Molecular Epidemiology of *Escherichia coli* isolates from Environmental, Food and Clinical Sources

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By

Divya P. S

Reg.No: 3664

Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682 016, India

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Author

Divya P. S
Research Scholar
Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

Supervising Guide

Dr. A. A. Mohamed Hatha
Associate Professor
Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

August 2014

Department of Marine Biology, Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682 016

Dr. A. A. Mohamed Hatha
Associate Professor

Certificate

This is to certify that the thesis entitled **"Molecular Epidemiology of *Escherichia coli* isolates from Environmental, Food and Clinical Sources"** is a bonafide record of original research work carried out by **Ms. Divya P. S**, under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee have been incorporated in this thesis and no part thereof has been presented before for the award of any other degree, diploma or associateship in any other University or institution.

Kochi - 682 016
August 2014

Dr. A. A. Mohamed Hatha
(Supervising Guide)

Declaration

I hereby declare that the thesis entitled **"Molecular Epidemiology of *Escherichia coli* isolates from Environmental, Food and Clinical Sources"** is an authentic record of the original research work done by me under the supervision and guidance of **Dr. A. A. Mohamed Hatha**, Associate Professor, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology and no part thereof has been presented before for the award of any other degree, diploma or associateship or any other similar title in any other University or Institution.

Kochi - 682 016
August 2014

Divya P. S

Dedicated to

Achan, Amma and Danu

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Abbreviations

A/E	-	Attaching and effacing
AA	-	Aggregative adherence
AAC	-	Aminoglycoside acetyltransferases
AMEs	-	Aminoglycoside-modifying enzymes
Amp	-	Ampicillin
ANOVA	-	One-Way Analysis of Variance
ANT	-	Aminoglycoside nucleotidyltransferases
APEC	-	Avian pathogenic *E. coli*
APH	-	Aminoglycoside phosphotransferases
ARGs	-	Antibiotic resistance genes
BFP	-	Bundle forming pilus
BLAST	-	Basic Local Aligment Search Tool
bp	-	Base pair
BSI	-	Bloodstream infections
C	-	Chloramphenicol
CAT	-	Chloramphenicol acetyltransferases
Caz	-	Ceftazidime
cDNA	-	Complementary deoxyribonucleic acid
CFs	-	Colonization factors
Cip	-	Ciprofloxacin
Co	-	Co-Trimoxazole
Cpd	-	Cefpodoxime
CT	-	Cholera toxin
C_t	-	Cycle threshold
Ctr	-	Ceftriaxone
Ctx	-	Cefotaxime
Cx	-	Cefoxitin
Cxm	-	Cefuroxime
DAEC	-	Diffusely adhering *E. coli*
DA-EPEC	-	Adhering enteropathogenic *E. coli*
DAF	-	Decay accelerating factor
DEC	-	Diarrheagenic *E. coli*
DEPC	-	Diethylpyrocarbonate
DHFR	-	Dihydrofolate reductase

DHPS	-	Dihydropteroate synthase
DNA	-	Deoxyribonucleic acid
DNase	-	Deoxyribonuclease
dNTP	-	Deoxy ribonucleotide
DTT	-	Dithiothreitol
EAEC	-	Enteroaggregative *E. coli*
EAF	-	Adherence factor
EAHEC	-	Enteroaggregative hemorrhagic *E. coli*
EDTA	-	Ethylene diamine tetra acetic acid
EHEC	-	Enterohemorrhagic *E. coli*
EIEC	-	Enteroinvasive *E. coli*
EMB	-	Eosin methylene blue
EMBL	-	European Molecular Biology Laboratory
EPEC	-	Enteropathogenic *E. coli*
ERIC	-	Enterobacterial repetitive intergenic consensus
ESBL	-	Extended-spectrum beta-lactamase
ETEC	-	Enterotoxigenic *E. coli*
ExPEC	-	Extraintestinal pathogenic *E. coli*
Gen	-	Gentamicin
HC	-	Hemorrhagic colit
HCl	-	Hydrochloric acid
HUS	-	Hemolytic uraemic syndrome
IMIs	-	Intramammary infection
IRT	-	Inhibitor-resistant TEM
kb	-	Kilo base pair
KCl	-	Potassium chloride
kD	-	Kilodalton
LEE	-	Locus of enterocyte effacement
LPS	-	Lipopolysaccharide
LT	-	Heat-labile enterotoxins
MAR	-	Multiple antibiotic resistance
MDR	-	Multidrug-resistant
MFS	-	Major facilitator superfamily
mg	-	Milligram
$MgCl_2$	-	Magnesium chloride
Min	-	Minutes
Ml	-	Millilitre

mM	-	Millimolar
MPN	-	Most probable number
MR	-	Methyl Red
Na	-	Nalidixic acid
NaCl	-	Sodium chloride
NAG	-	*N*-acetylglucosamine
NAM	-	*N*-acetylmuramic acid
NCBI	-	National Center for Biotechnology Information
NEMEC	-	Neonatal meningitis-associated *E. coli*
ng	-	Nanogram
OD	-	Optical density
PBPs	-	Penicillin-binding proteins
PCR	-	Polymerase Chain Reaction
pmol	-	Picomole
PMQR	-	Plasmid-mediated quinolone resistance
qPCR	-	Quantitative Polymerase Chain Reaction
QRDR	-	Quinolone resistance-determining region
RAPD	-	Randomly Amplified Polymorphic DNA
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
rpm	-	Revolutions per minute
rRNA	-	Ribosomal ribonucleic acid
R	-	Rough
RT	-	Respiratory tract
S	-	Streptomycin
SDS	-	Sodium dodecyl sulphate
sec	-	Seconds
SEPEC	-	Sepsis associated *E. coli*
ShET1	-	*Shigella* enterotoxin 1
SMART	-	The Study for Monitoring Antimicrobial Resistance Trends
SNPs	-	Single-nucleotide polymorphisms
SPSS	-	Statistical Package for the Social Science
ST	-	Heat-stable peptide toxins
STEC	-	Shiga toxin-producing *E. coli*
Te	-	Tetracycline
Tr	-	Trimethoprim

Tris	-	Tris(hydroxymethyl)aminomethane
UPEC	-	Uropathogenic *E. coli*
UPGMA	-	Unweighted pairgroup method arithmetic mean
USFDA	-	The Food and Drug Administration
UTI	-	Urinary tract infection
UT	-	Untypable
U	-	Units
V	-	Volt
VP	-	Voges Proskauer
VTEC	-	Verotoxin-producing *E. coli*
WHO	-	World Health Organization
%	-	Percentage
× g	-	Gravity (relative centrifugal Force)
°C	-	Degree celsius
µg	-	Microgram
µl	-	Microlitre

Chapter 1

General Introduction

1.1 Introduction

1.1.1 *Escherichia coli*

E. coli is familiar to biologists as a classical model system. *E. coli* strains are ubiquitous in molecular biology laboratories around the world, and the study of this organism has led to many of the landmark achievements in biology (Neidhardt, 1996). Most of this work has been carried out using derivatives of a single *E. coli* strain, K-12 (Bachmann, 1972). Outside of the laboratory, *E. coli* strains exist as an almost universal component of the lower-gut flora of humans and animals. Although usually a commensal, *E. coli* has an alter ego as a pathogen, associated with diarrheal disease and extra-intestinal infections (Kaper *et al*., 2004; Russo and Johnson, 2000; Nataro and Kaper, 1998), and it is in this role that *E. coli* is most likely to be recognised by the general public (Dixon, 1998; Thomas and Bettelheim, 1998).

E. coli was initially described (as *Bacterium coli commune*) by the German physician Theodor Escherich (1885). A related organism, *Shigella dysenteriae*

(initially *Bacillus dysentericus*) was identified as a causative agent of dysentery by the Japanese bacteriologist Kiyoshi Shiga (1897). The two genera were subsequently renamed as *Escherichia* and *Shigella* after their respective discoverers (Castellani and Chalmers, 1919). *E. coli* and *Shigella* were distinguished on the basis of motility, metabolic profile and clinical manifestation: *Shigella* spp. are nonmotile, obligate pathogens that typically cannot ferment lactose and are lysine decarboxylase and indole-negative, whereas *E. coli* are usually motile, lactose, lysine decarboxylase and indole-positive, and are suggested to be mostly commensal organisms (Edwards and Ewing, 1972).

E. coli are common inhabitants of the terminal small intestine and large intestine of mammals. They are often the most abundant facultative anaerobes in this environment. They can occasionally be isolated in association with the intestinal tract of non-mammalian animals and insects. The presence of *E. coli* in the environment is usually considered to reflect faecal contamination and not the ability to replicate freely outside the intestine. There is evidence however to suggest that *E. coli* may freely replicate in tropical fresh water (Bermudez and Hazen, 1988). *E. coli* is easily cultured in the clinical laboratory, but the identification of the different pathogenic genotypes requires virulence gene detection methods not typically available in most clinical laboratories. *E. coli* can be found secondarily in soil and water as the result of faecal contamination. Classically, its detection has been used as an indicator of poor water quality.

E. coli are Gram-negative, non-spore forming bacilli. They are approximately 0.5 µm in diameter and 1.0–3.0 µm in length. Within the

periplasm is a single layer of peptidoglycan. The peptidoglycan has a typical subunit structure where the *N*-acetylmuramic acid is linked by an amide bond to a peptide consisting of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and finally D-alanine.

E. coli is a facultative anaerobe. It is capable of reducing nitrates to nitrites. When growing fermentatively on glucose or other carbohydrates, it produces acid and gas. By traditional clinical laboratory biochemical tests, *E. coli* is positive for indole production and the methyl red test. Most strains are oxidase, citrate, urease and hydrogen sulphide negative. The classic differential test to primarily separate *E. coli* from *Shigella* and *Salmonella* is the ability of *E. coli* to ferment lactose, which the latter two genera fail to do. Aside from lactose, most *E. coli* strains can also ferment D-mannitol, D-sorbitol, L-arabinose, maltose, D-xylose, trehalose and D-mannose.

There are limited instances where pathogenic strains differ from the commensals in their metabolic abilities. For example, commensal *E. coli* strains generally use sorbitol, but *E. coli* O157:H7 does not. Most diarrheagenic strains cannot utilize D-serine as a carbon and nitrogen source, but uropathogenic and commensal faecal strains can use this enantiomer of serine (Roesch *et al*., 2003). Most *E. coli* strains are capable of growing over a wide range of temperature (approximately 15 - 48 °C). The growth rate is maximal in the narrow range of 37 - 42 °C (Ingraham and Marr, 1987). *E. coli* can grow within a pH range of approximately 5.5 - 8.0 with best growth occurring at neutrality. Some diarrheagenic *E. coli* strains have the ability to tolerate exposure to pH 2.0. Such an acid shock mimics transit through the stomach

and induces expression of sets of genes involved in survival and pathogenesis (Waterman and Small, 1996).

1.1.1.1 *E. coli* as an emerging pathogen

A recent food-borne outbreak involving an EAHEC (enteroaggregative hemorrhagic *E. coli*) strain (*E. coli* O104:H4) originating from sprouts in Germany and France (Buchholz *et al*., 2011) highlights the importance of newly emerging diarrheagenic *E. coli* pathotypes. In addition, the *E. coli* O104:H4 strain implicated in this outbreak was an EAEC that acquired the *stx* genes (Grad *et al*., 2013; Muniesa *et al*., 2012); this illustrates how the mobile *stx* genes can be transferred to different classes of pathogenic *E. coli* making them hyper virulent pathogens and meaning they have the potential to emerge as new shiga toxin-producing *E. coli* strains (Grad *et al*., 2013; Muniesa *et al*., 2012).

1.1.1.2 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) strains are leading causes of diarrhea in children living in developing countries and the most common cause of traveler's diarrhea (Kosek *et al*., 2003). ETEC bacteria are known to adhere to the small intestinal epithelium without inducing significant morphological changes, and to secrete proteins (enterotoxins) that alter the functions of enterocytes by increasing secretion and reducing absorption. Thus the main virulence attributes of ETEC are adhesins (primarily hair-like appendages called fimbriae or pili) and enterotoxins (proteins or peptides). In addition to adhesive and enterotoxic virulence factors, pathogenesis also involves host factors, the most important of which are receptors for adhesins and enterotoxins. Species specificity, which is a general characteristic of ETEC

infections, is largely due to the presence of specific receptors in only one or in a limited spectrum of animal species (Nagy and Fekete, 2005).

Strains of ETEC have two major virulence determinants: the enterotoxins (the heat-labile toxin/LT and heat-stable toxin/ST) and colonization factors (CFs). Enterotoxins are plasmid-regulated secreted proteins or peptides of ETEC bacteria acting on the intestinal epithelium, representing the following enterotoxin categories (O'Brien and Holmes, 1996): (1) Large-molecular-weight (88kDa) heat-labile enterotoxins (LT), and different variants; (2) small-molecular weight (11-48 amino acid containing) heat-stable peptide toxins (ST). The LT enterotoxins are produced predominantly by human and porcine ETEC, and are known to be derivatives of the cholera toxin (CT) of *Vibrio cholera* (Hirst *et al*., 1998; O'Brien and Holmes, 1996). The ST enterotoxins are produced by ETEC of human, porcine and bovine origin. The LT toxins have good antigenicity while ST toxins do not. LT toxins can be divided into two antigenically and biologically distinct but structurally similar groups: LTI and LTII. LTII toxins are rare and occur (in humans and in calves) in two antigenic variants (LTIIa and LTIIb) (O'Brien and Holmes, 1996). ST toxins have two classes: STa and STb (also referred to as STI and STII, respectively).

More than 25 CFs have been described but of these, only seven of these factors, i.e. CFA/I, CS1, CS2, CS3, CS4, CS5, and CS6 account for 50-70% of all clinical ETEC isolates. Furthermore, although ETEC clinical isolates may express more than one CF, native strains only express CFs in certain combinations. Thus, for example, CS1 or CS2 are generally expressed together with CS3, whereas some strains express CS3 alone. Similarly, CS4 or CS5 are generally expressed together with CS6, whereas there are many strains that express only

CS6. CFA/I has not been found to be naturally co-expressed with CS1, CS2, CS3, CS4, CS5, or CS6 (Qadri *et al*., 2005; Gaastra and Svennerholm, 1996).

1.1.1.3 Shiga toxin-producing *E. coli* (STEC)

Production of cytotoxins called shiga toxins is the leading trait of a group of *E. coli* for which three terms have been used concomitantly: shiga toxin-producing *E. coli* (STEC), vero cytotoxin-producing *E. coli* (VTEC), and enterohemorrhagic *E. coli* (EHEC). Some authors prefer to reserve the term EHEC for those *E. coli* that cause more severe illnesses characterised by bloody diarrhea or the hemolytic uraemic syndrome (HUS), in contrast to milder, non-bloody diarrhea caused by VTEC or STEC (Sharma, 2002; Bellin *et al*., 2001; Donnenberg and Whittam, 2001). However, this differentiation based on clinical severity is not paralleled by the presence of phenotypic or genotypic traits that might be used for assessing the potential risk to humans of isolates from animals, food, or the environment (Bell, 2002). Many STEC strains are part of the intestinal flora of domestic and wildlife animals, which excrete the bacteria with their faeces into the environment (European Centre for Disease Prevention and Control and European Food Safety Authority, 2011). Food produced from these animals can be contaminated with STEC strains derived from the faecal microbial flora of the producer animal (European Centre for Disease Prevention and Control and European Food Safety Authority, 2011; Martin and Beutin, 2011). Some, but not all STEC strains are known to have the capacity to cause life-threatening diseases in humans, such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Melton-Celsa *et al*., 2012). These STEC strains, which are also called enterohemorrhagic *E. coli* (EHEC), belong to a few *E. coli* serotypes, and

share similarities in their *stx*-types, virulence plasmids and chromosomally encoded effectors involved in colonization of the human intestine (Wong *et al*., 2011; Abu-Ali *et al*., 2010).

Shiga toxin-producing *E. coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), is the most important recently emerged group of foodborne pathogens. It is a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic uremic syndrome (HUS), which is the main cause of acute renal failure in children (Beutin *et al*., 2004; Blanco *et al*., 2004; Paton and Paton, 1998; Karmali, 1989). Since its identification as a pathogen in 1982, STEC O157:H7 has been the cause of a series of outbreaks that happened especially in Canada, Japan, Germany, U.K and U.S (Chattaway *et al*., 2011; Mora *et al*., 2004; Karmali, 1989). Human and bovine STEC elaborate two potent phage encoded cytotoxins called shiga toxins (*stx1* and *stx2*) or verotoxins (*VT1* and *VT2*) (Paton and Paton, 1998; Karmali, 1989). In addition to toxin production, another virulence associated factor expressed by STEC is a protein called intimin which is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa (Jerse *et al*., 1990). Intimin is encoded by the chromosomal gene *eae*, which is part of a pathogenicity island termed the locus for enterocyte effacement (LEE) (Kaper *et al*., 1998). Severe diarrhea (especially HC) and HUS were closely associated with STEC types carrying the *eae* gene for intimin (Paton and Paton, 1998; Karmali, 1989). Differentiation of intimin alleles represents an important tool for STEC typing in routine diagnostics as well as in epidemiological and clonal studies.

1.1.1.4 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) strains are causal agents of persistent diarrhea in developing countries (Savarino, 1993; Cravioto *et al*., 1991). EAEC group includes *E. coli* strains carrying the *aggR* regulon that comprises virulence genes defining typical, pathogenic EAEC and *aggR*-negative, atypical strains considered as non-pathogenic (Dudley *et al*., 2006; Nataro *et al*, 1998). EAEC pathotype most prominently is associated with persistent diarrhea and prolonged carriage of invasive EAEC strains may disturb the delicate balance between the host innate immune response and the gut microbiota. Moreover, invasive capability of EAEC may not only protect the bacteria from the host immune response and bacteriocins produced by normal intestinal microflora, but may also facilitate horizontal gene transfer and acquisition of a new virulence factors.

This category of diarrheagenic *E. coli* displays a typical aggregative adherence (AA) pattern when associated with cultured epithelial cells and inert surfaces, in which bacteria aggregate to each other forming a stacked brick-like configuration (Nataro *et al*., 1987). Biofilm formation is an intrinsic property of EAEC strains. The mechanisms of pathogenesis of EAEC infection are not fully clear mainly because the strains comprising this pathogroup are very heterogeneous (Czeczulin *et al*., 1999; Elias *et al*., 1999; Monteiro-Neto *et al*., 1997). A variety of virulence traits have been described in these bacteria, which include: fimbrial and afimbrial adhesins, enterotoxins, such as a heat-stable enterotoxin (EAST-1) (Savarino *et al*., 1996), the plasmid-encoded toxin (Pet) (Navarro-Garcia *et al*., 1998), the *Shigella* enterotoxin 1 (ShET1) (Fasano *et al*., 1995; Noriega *et al*., 1995), *Pic*, a

mucinase involved in intestinal colonization (Henderson *et al*., 1999), and a secreted protein termed dispersin (Sheikh *et al*., 2002). Adherence to the intestinal mucosa, increased mucus production, and an inflammatory response are characteristics of EAEC infections (Steiner *et al*., 1998; Hicks *et al*., 1996). The thick mucus-containing biofilm is encrusted with EAEC on the surface of cells and may be one of the reasons why most of the individuals develop a chronic EAEC infection. Several fimbrial adhesins have been described to be important in the establishment of aggregative adherence pattern by different EAEC strains (Kaper *et al*., 2004; Nataro *et al*., 1998). Three distinct plasmid-encoded fimbrial adherence factors have been identified in EAEC. Two of them are bundle-forming fimbrial structures designated as aggregative adherence fimbriae I and II (AAF/I and AAF/II).

Nucleotide sequences analyses of AAF/I (agg) and AAF/II (aaf) operons have indicated that these fimbriae belong to the Dr family of adhesins found in other extra-intestinal *E. coli* strains (Czeczulin *et al*., 1997; Nataro *et al*., 1992). A third fimbrial structure (AAF/III), which is closely related to the *agg* and *aaf* operons, was identified in EAEC strain 55989 (Bernier *et al*., 2002). Hence, the three AAFs are phylogenetically related. There are reports in favour and against a role of type 1 pili (T1P) in adherence and biofilm formation of EAEC strains (Moreira *et al*., 2003; Sheikh *et al*., 2001). Analyses of the prevalence of AAFs among clinical strains of different geographic origins indicate that only a minority (~ 15%) of them possess any of the described 3 AAF operons (Bernier *et al*., 2002; Okeke *et al*., 2000; Elias *et al*., 1999; Nataro and Kaper, 1998; Czeczulin *et al*., 1997), suggesting that adherence is mediated by other fimbriae yet to be identified in these AAF-negative strains.

1.1.1.5 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. There are a few biochemical traits that can be used to distinguish enteroinvasive *E. coli* (EIEC) from *Shigella,* but the principal virulence genes are shared. The diagnostic confusion between *Shigella* and EIEC is evident in that EIEC isolates are nonmotile and 70% are nonlactose fermenters (Silva *et al*., 1980). In addition, EIEC share with *Shigella* the inability to decarboxylate lysine, a trait common to other *E. coli*. The traits that EIEC share with *E. coli* but not *Shigella* are the ability to produce gas from glucose and fermentation of xylose.

Recent phylogenetic studies have suggested that *Shigella* and EIEC form a single pathovar of *E. coli*. EIEC strains are regarded as precursors of full-blown *Shigella* evolved from *E. coli* (Lan *et al*., 2004). All the *Shigella* may be derived from the EIEC sequence type 270 or the ST 280 (Wirth *et al*., 2006). EIEC penetrate and multiply within epithelial cells of the colon, causing widespread cell destruction. Clinical manifestations include a dysentery-like diarrhea with fever (Nataro and Kaper, 1998). Much of EIEC pathogenesis is the result of the multiple effects of its plasmid-borne, type III secretion system secreting multiple proteins such as IpaA, IpaB, IpaC and IpgD (Sansonetti *et al*., 2000). The *IpAH* gene encoding the invasive plasmid antigen H is located on both the chromosome and the invasion plasmid (Dutta *et al*., 2001).

1.1.1.6 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) strains are defined as intimin-containing diarrheagenic *E. coli* that possess the ability to form A/E lesions on

intestinal cells and that do not possess shiga toxin genes (Kaper, 1996). EPEC are further classified as typical, when possessing the EAF (for EPEC adherence factor) plasmid that encodes localized adherence (LA) on cultured epithelial cells mediated by the Bundle Forming Pilus (BFP); whereas atypical EPEC strains do not possess the EAF plasmid (Trabulsi *et al*., 2002). Typical EPEC, a major cause of infant diarrhea in developing countries, are rare in industrialized countries, where atypical EPEC seem to be a more important cause of diarrhea (Trabulsi *et al*., 2002). Typical EPEC strains are isolated mainly from humans, whereas atypical EPEC strains have been isolated from different animal species, including cattle, sheep and goats (Blanco *et al*., 2005; Cortes *et al*., 2005; Orden *et al*., 2003; Trabulsi *et al*., 2002).

The attaching and effacing (A/E) lesion is the key feature of EPEC pathogenesis and is characterised by intimate attachment of bacteria to the apical enterocyte membrane and localised destruction of the brush border microvilli with specific aggregation of actin beneath the site of adherence. The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island on the *E. coli* chromosome that contains the genes encoding intimin (*eae*), a type III secretion system, a number of secreted proteins (*espA, espB*) and the translocated intimin receptor Tir (tir) (Levine *et al*., 1988; Nataro and Kaper, 1998). Typical EPEC strains also carry the large EPEC adherence factor (EAF) plasmid, which presents a cluster of genes that encodes a type IV pili (bundle forming pilus-BFP), which interconnects bacteria within microcolonies and thus promotes their stabilisation (Nataro and Kaper, 1998; Jerse and Kaper, 1991).

Intimin, an outer membrane protein, is responsible for the intimate adherence between bacteria and enterocyte membranes. The ESP proteins are involved in the formation of a translocon that delivers effector molecules to the host cell and disrupts the cytoskeleton, subverting the host cell functions (Frankel *et al*., 1998). Several studies have identified variants (a, h and g) within the *eae*, *tir*, *espA* and *espB* genes of EPEC (Beutin *et al*., 2003; Adu-Bobie *et al*., 1998). Typical and atypical EPEC both carry the LEE region and are distinguished by phenotypic and genotypic characteristics, virulence properties and reservoirs. Typical EPEC, who's only known reservoir is human beings, expresses the LA phenotype, carries the virulence plasmid pEAF and is a leading cause of infantile diarrhea in developing countries. Atypical EPEC has both animals and humans as reservoirs, can express LA, AA and DA phenotypes in adherence assays, does not harbour the EAF plasmid or *stx* (shiga toxin) genes and seems to be an important cause of diarrhea in industrialised countries. Atypical EPEC appear to be an emerging pathogen (Trabulsi *et al*., 2002).

1.1.1.7 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) are types of EPEC that contain a characteristic, diffuse pattern of adherence to HEp-2 cell monolayers (Bilge *et al*., 1989). On the basis of this, two subclasses of DAEC strains have been proposed: diffusely adhering enteropathogenic *E. coli* (DA-EPEC) harboring an LEE island (Beinke *et al*., 1998) and those DAECs expressing adhesins (*draA-E* and *draP*) of the *Afa/Dr* family (Berger *et al*., 2004; Nowicki *et al*., 2001). DAEC may cause diarrhea in very young children (Scaletsky *et al*., 2002). They are differentiated from the other diarrheagenic *E. coli* by a

distinct adhesion phenotype, again on HEp-2 cells. The adhesion is brought about by F1845 fimbriae, which belong to the Dr family of adhesins (also found in some UPEC strains). The Dr adhesins recognize and bind to host cell surface decay accelerating factor (DAF). DAEC bound to cultured cells elicit a cytopathic phenotype and activation of signal transduction pathways.

1.1.1.8 Extraintestinal pathogenic *E. coli* (ExPEC)

E. coli from infections of the urinary tract, blood stream, central nervous system, respiratory tract, and peritoneum are quite distinct from commensal and intestinal pathogenic *E. coli*. It recently has been proposed that these strains of *E. coli* be collectively termed ExPEC, rather than split up as uropathogenic *E. coli* (UPEC), sepsis associated *E. coli* (SEPEC), and neonatal meningitis-associated *E. coli* (NEMEC). UPEC are a heterogeneous group of clones (Donnenberg and Welch, 1996). Within the UPEC grouping are cystitis, pyelonephritis and urosepsis isolates. These strains are the principal causes of morbidity and mortality from either community or hospital-acquired *E. coli* infections. As much as 90% of all community-acquired urinary tract infections and greater than 30% of the hospital-acquired UTIs are caused by *E. coli* (Haley *et al*., 1985). There have been reports of community-wide outbreaks of UTIs by multidrug-resistant UPEC clones (Manges *et al*., 2001). Characteristic virulence traits that are present in most ExPEC include various adhesins (e.g. P and type I fimbriae), factors to avoid or subvert host defense systems (e.g. capsule, lipopolysaccharide), mechanisms for nutrient acquisition (e.g. siderophores), and toxins (e.g. hemolysin, cytotoxic necrotizing factor 1).

1.1.2 Serotyping

The species *E. coli* is serologically divided in serogroups and serotypes on the basis of its antigenic composition (somatic or O antigens for serogroups and flagelar or H antigens for serotypes). Many strains express a third class of antigens (capsular or K antigens) that although important in pathogenesis only occasionally are used in serotyping. The species comprise intestinal and extraintestinal pathogens. Systematic O serotyping of *E. coli* began in the early 1930s (Nataro and Kaper, 1998), and many studies showed that the O serotype of *E. coli* are generally associated with pathogenesis (Yayue *et al*., 2006; Wang *et al*., 1998). O serotyping became important tools to classify *E. coli* in clinical settings. It has been shown repeatedly that antigenic typing of *E. coli* is extremely useful in epidemiological studies (Vu-Khac *et al*., 2007; Blanco *et al*., 2006b; Machado *et al*., 2000; Orskov and Orskov, 1992).

1.1.3 Phylogenetic analysis

Currently, there are four well-recognized phylogroups and these have been designated A, B1, B2 and D. Clermont *et al*., (2000) developed a multiplex PCR-based method that enables strains of *E. coli* to be assigned to a phylogroup using a dichotomous key approach based on the presence or absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2). Strains belong to phylogenetic group B2 carry the greatest number of virulence factors and followed by those in group D, whereas most commensal strains belong to groups A and B1 (Moreno *et al*., 2008; Johnson *et al*., 2005b; Moreno *et al*., 2005; Sannes *et al*., 2004; Johnson *et al*., 2002a; Johnson and Stell, 2000). Virulence factors are generally carried on plasmids,

pathogenicity islands, or phages and are supposed to be highly interchangeable among bacterial strains through horizontal gene transfer (Hacker and Kaper, 2000).

1.1.4 Antibiotic resistance

E. coli, a member of the *Enterobacteriaceae* family, is a common inhabitant of the human and animal gut. It is the most common cause of Gram-negative nosocomial and community-acquired infections. Resistance to at least two classes of antibiotic agents in *E. coli* is nowadays an ordinary finding in human and veterinary medicine and has an increasing impact on available therapeutic options. The clinical use of antibiotics, and therefore the effective treatment of bacterial infections, is under considerable threat due to the emergence of bacteria that are resistant to many classes of commonly used antibiotics. Antibiotic agents can be found in sewage effluents, particularly in places where these drugs are extensively used, such as hospitals, pharmaceutical production plants and around farms where animal feed contains these agents. Antibiotics present in these sources, found to play a significant role in the natural selection and survival of resistant strains. The wild dissemination of antibiotic resistance among bacterial populations is an increasing problem worldwide. Multidrug-resistant bacterial strains are prevalent in human and animal isolates all over the world.

1.1.5 Antibiotic resistance genes

The escalating problem of emergence of antibiotic resistant bacteria and their resistant genes is becoming a major global health issue (Levy, 2002; Chee-Sanford *et al*., 2001). Antibiotic resistance genes may be spread on

mobile genetic elements such as plasmids, transposons and integrons. Bacteria carrying integrons might transfer antibiotic resistance genes from animals to animals as well as to humans (Aarestrup *et al*., 1999; Recchia and Hall, 1995). Many gene cassettes of integrons contain antibiotic resistance genes and thus play an important role in the dissemination of antibiotic resistance genes and the development of multidrug resistance. The spread of antibiotic resistance genes present in gene-transfer units and dissemination of multiresistant bacteria in nature may have consequences for human health and the evolution of environmental microbiota (Martinez, 2009). Antibiotic resistance may develop through mutations in chromosomal DNA or horizontal gene transfer of mobile elements.

1.1.6 Genotyping

Traditional phenotyping methods such as biotyping, serotyping, and phage typing of isolates, provide insufficient information for epidemiological purposes. Molecular genetic methods have revolutionized the fingerprinting of microbial strains. Genotyping methods such as ERIC – PCR, RAPD and RFLP are examples of the methods exploited successfully in many laboratories to diagnose, discriminate and survey several important bacterial pathogens. Enterobacterial repetitive intergenic consensus (ERIC) PCR relies on amplification of genomic DNA fragment using sets of primers complimentary to the short repetitive sequences. On the other hand, in Randomly Amplified Polymorphic DNA (RAPD) the primer of 8-10 bp binds somewhere in the sequence, but it is not certain exactly where (Nath *et al*., 2010). RAPD analysis is a technique based on a simple PCR amplification of genomic (DNA) with single primers of arbitrary nucleotide sequence. Because RAPD

reveals considerable polymorphism in genomic DNA, it has been extensively used as a genetic marker for estimating genetic, taxonomic and phylogenetic relationship in plants and animals. In RFLP, a known sequence is amplified, cut with a restriction enzyme and the restriction fragment profiles are compared between different strains.

1.2 Broad objectives

1) To isolate, characterize and analyse the serological diversity of *E. coli* isolates from environmental, seafood and clinical sources.

2) To find out the phylogenetic groups (clonal relationship) among *E. coli* isolates from environmental, seafood and clinical sources.

3) To monitor the prevalence of drug resistance among the *E. coli* isolates from environmental, seafood and clinical sources.

4) To find out the prevalence of antibiotic resistance genes and integrons associated with antibiotic resistance in *E. coli* isolates from environmental, seafood and clinical sources.

5) To analyze the prevalence of various virulence factor genes in *E. coli* isolates from environmental, seafood and clinical sources.

6) To investigate the genetic relatedness among *E. coli* isolates from environmental, seafood and clinical sources using ERIC PCR, RAPD and RFLP.

7) To characterize the strain CUSMBES11 by Real - Time PCR

Chapter 2

Isolation, identification and serotyping of *Escherichia coli* isolates from estuarine, seafood and clinical sources

2.1 Introduction

The species *E. coli* is serologically divided in serogroups and serotypes on the basis of its antigenic composition (somatic or O antigens for serogroups and flagellar or H antigens for serotypes). Many strains express a third class of antigens (capsular or K antigens) that although important in pathogenesis only occasionally are used in serotyping. The species comprise intestinal and extraintestinal pathogens. The intestinal pathogens are also known as diarrheagenic *E. coli* (DEC) of which six categories have been characterized: enteropathogenic *E. coli* (EPEC), shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely

adhering *E. coli* (DAEC) (Hunt, 2010; Smith *et al*., 2007; Kaper *et al*., 2004; Nataro and Kaper, 1998). The extraintestinal pathogens (EXPEC) are more prevalent strains include those associated with urinary tract infections (UPEC), neonatal meningitis (MAEC), and bacteremia.

Systematic O serotyping of *E. coli* began in the early 1930s (Nataro and Kaper, 1998), and many studies showed that the O serotype of *E. coli* are generally associated with pathogenesis (Yayue *et al*., 2006; Wang *et al*., 1998). O serotyping became important tools to classify *E. coli* in clinical settings. It has been shown repeatedly that antigenic typing of *E. coli* is extremely useful in epidemiological studies (Vu-Khac *et al*., 2007; Blanco *et al*., 2006b; Machado *et al*., 2000; Orskov and Orskov, 1992).

2.2 Review of Literature

2.2.1 Serotyping

Lipopolysaccharide (LPS) is a key component of the outer membrane of Gram-negative bacteria. It commonly comprises three distinct regions: the proximal hydrophobic lipid A region, the distal hydrophilic O antigen, and the interconnecting core oligosaccharide. The O antigen, which consists of many repeats of an oligosaccharide (O unit), is one of the most variable cell constituents due to the variations in the types of sugars present, the arrangement of the sugars within O unit, and the linkages between O units (Reeves and Wang, 2002; Reeves, 1994). The highly variable nature of the O antigen provides the basis for serotyping, and 186 O antigen forms (serotypes) have been recognized in *E. coli* (including *Shigella*) (Feng *et al*., 2004; Samuel and Reeves, 2003). In general, most genes required for O-antigen biosynthesis are clustered on the *E. coli* chromosome between the

colanic acid biosynthesis gene cluster and the histidine biosynthesis (his) operon (Reeves *et al*., 1996). The organization of O-antigen biosynthesis gene clusters is O-antigen-specific. Sequence comparisons in many bacterial species and strains have shown that the variation between O-antigens has been generated by horizontal transfer of a part or the entire O-antigen gene clusters (Cunneen and Reeves, 2007; Fegan *et al*., 2006; Wang *et al*., 2002; Curd *et al*., 1998; Sugiyama *et al*., 1998; Xiang *et al*., 1994). Thus, the serotype of a strain correlates to some extent with its phylogeny, and often provides valuable information on its pathotype.

The O-antigen is subject to intense selection by the host immune system, which may be the major factor for the maintenance of many different O-antigen forms (Reeves and Wang, 2002; Reeves, 1994). There are three main classes of genes involved in O-antigen synthesis: (1) genes for synthesis of nucleotide sugar precursors; (2) genes encoding glycosyl transferases for sequential transfer of sugars from their respective nucleotide precursors to the carrier lipid, undecaprenolphosphate to form O units; and (3) O unit processing genes encoding O unit flippase (*wzx*) and O-antigen polymerase (*wzy*) (Reeves and Wang, 2002). The role of *wzx* is to translocate or flip the O units formed at the cytoplasmic face of the membrane to the periplasmic face. The O units are then polymerised to form a long chain O-antigen at the periplasmic face of the membrane by *wzy*. Both *wzx* and *wzy* are specific to individual O-antigens (Liu *et al*., 1996). The genes specifically involved in O-antigen synthesis are generally grouped together on the chromosome as a gene cluster. In *E. coli* and *Salmonella enterica*, the O-antigen gene clusters lie between the *galF* and *gnd* genes (Reeves, 1994). The O-antigen genes are found generally very close to each other, often overlapping in their reading frames, and thought to be

transcribed as a unit (Samuel and Reeves, 2003). The sugar residues in lipid A and the core region are decorated to a varying extent with phosphate groups or phosphodiester-linked derivatives, which ensure micro heterogeneity in each strain. The lipid A part is highly conserved in *E. coli*. The core, however, contains five different basic structures, denoted R1 to R4 and K12. The O-polysaccharide is linked to a sugar in the outer core. The O-antigen usually consists of 10-25 repeating units containing two to seven sugar residues. Thus, the molecular mass of the LPS present in smooth strains will be up to 25 kDa.

The structural variation of O-antigens is almost entirely dependent on the variation of the O-antigen gene clusters. Typically, O-antigen gene clusters have a GC content lower than the average level of the genome, indicating the gene clusters have been arisen from other species by lateral transfer (Lan and Reeves, 1996; Aoyama *et al*., 1994). Inter and intra species lateral transfers of O-antigen genes appear to play important roles for expanding O-antigen polymorphism. Both transfer of O-antigen genes for assembly of new O-antigen gene clusters and transfers of entire O-antigen gene clusters between clones of a species by homologous recombination were observed (Wang *et al*., 2002; Lan and Reeves, 2000, 1996).

Since its introduction by Kauffmann in the 1940s (Kauffmann, 1944, 1943), serotyping has been the most widely used method of identifying strains of *E. coli* for epidemiological and other purposes. The potential of serological techniques for strain discrimination is considerable. Some 170 O (lipopolysaccharide), 71 K (capsular), and 56 H (flagellar) antigens have been distinguished, and several thousand combinations of these antigens have been identified among isolates of *E. coli* from natural sources (Orskov and Orskov,

1984; Orskov *et al*., 1984, 1977). Three epidemiologically important observations have been made concerning this extensive antigenic diversity. First, the relative frequencies of many of the antigens vary with the source of the isolates; for example, some O and K antigens are more common among strains associated with extraintestinal infections than among those recovered from the normal intestinal flora (Lidin-Janson *et al*., 1977; Robbins *et al*., 1974). Second, the O, K, and H antigens are non-randomly associated with one another, and specific O:K:H serotypes are, in turn, non-randomly associated with certain other phenotypic traits, such as fermentation markers and virulence determinants (Orskov and Orskov, 1977). Third, some multiple antigen serotypes associated with pathogenicity are worldwide in distribution and temporally stable (Orskov *et al*., 1976; Orskov and Orskov, 1977). Together, these observations were the basis for formulation of the "clone concept," a hypothesis postulating that the genetic structure of natural populations of *E. coli* is that of an array of stable cell lineages (clones), among which there is very little recombination of chromosomal genes (Orskov *et al*., 1976).

2.2.2 EPEC

EPEC produce potentially fatal infant diarrhea, noticeably in developing countries (Nataro and Kaper, 1998; Trabulsi *et al*., 2002), and have been isolated from different animal species (Blanco *et al*., 2005; Cortes *et al*., 2005; Krause *et al*., 2005; Nataro and Kaper, 1998) and from a variety of foods, including chicken products (Omaye, 2004; Nataro and Kaper, 1998). EPEC strains have been isolated from a variety of animal species, such as cattle, goats, sheep, chickens, pigeons and gulls (Cortes *et al*., 2005). Outbreaks of infantile gastroenteritis caused by enteropathogenic *E. coli* (EPEC) were common in

Britain from the 1940s to the 1960s and continued until the early 1970s. Serotypes isolated from infants included O114:H2, O119:H6, O127: H6, O128:H2, and O142:H6 (Smith *et al*., 1996). There were usually high attack rates in these outbreaks, often with high mortality. Since the 1970s, there has been a marked change in the epidemiology of EPEC in developed countries such as Britain (Smith *et al*., 1996). In developing countries, EPEC continues to be a leading cause of infant diarrhea (Ratchtrachenchai *et al*., 2004; Mani *et al*., 2003). Several reports (Blanco *et al*., 2006a; Nguyen *et al*., 2006; Beutin *et al*., 2003; Zhang *et al*., 2002b), have found that humans can be infected with a large spectrum of serologically different atypical EPEC strains.

Historically, EPEC strains have been identified by serotype, i.e., the distinct combination of O (somatic) and H (flagellar) antigens, which have been linked epidemiologically to infantile diarrhea. In 1987, the World Health Organization (World Health Organization, 1987) recognized EPEC serotypes of 12 different O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158). According to Germani, (1995) enteropathogenic *E. coli* (EPEC), traditionally represented by many serogroups, the most important ones being O26, O55, O86, O111, O114, O119, O125, O127, O128 and O142. The most prevalent serogroups within this group of *E. coli* are: O18, O20, O25, O26, O44, O55, O86, O91, O111, O114, O119, O125ac, O126, O127, O128, O142 and O158 (Nataro and Kaper, 1998).

2.2.3 EIEC

Enteroinvasive *E. coli* (EIEC) closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon, causing widespread cell destruction.

Clinical manifestations include a dysentery-like diarrhea with fever (Nataro and Kaper, 1998). Humans are a major reservoir for EIEC (Kaper *et al*., 2004). According to several reports (Stenutz *et al*., 2006; Germani, 1995; Echeverria *et al*., 1991) enteroinvasive *E. coli* (EIEC), often nonmotile and belonging to serogroups O28, O29, O112, O124, O136, O143, O144, O152, O164, O167 and O173.

2.2.4 EAEC

Enteroaggregative *E. coli* (EAEC) has emerged as an important pathogen causing diarrheal disease in multiple epidemiologic and clinical settings (Harrington *et al*., 2006). This pathogen has been associated with diarrheic syndrome in developing and industrialized countries which has been isolated from cases of acute and persistent diarrhea in children, adults, travelers and immune-compromised individuals (Pereira *et al*., 2008; Savarino, 1993; Cravioto *et al*., 1991). EAEC strains have been isolated from humans and from animal species, such as calves, piglets and horses. However, the most significant strains have been derived from humans (Uber *et al*., 2006). *E. coli* belonging to this category cause watery diarrhea, which is often persistent and can be inflammatory. The serogroups that have been identified within the EAEC group are O3, O7, O15, O44, O77, O86, O111, O126 and O127 (Stenutz *et al*., 2006).

2.2.5 ETEC

Enterotoxigenic *E. coli* (ETEC) strains are leading causes of diarrhea in children living in developing countries and the most common cause of traveler's diarrhea (Kosek *et al*., 2003). ETEC cause diarrhea without fever and are distinct from other *E. coli* pathotypes by their production of enterotoxins. ETEC has been

isolated from pigs, cattle (Nagy and Fekete, 1999) and humans (Kaper *et al*., 2004). However, the most significant strains have been derived from humans (Nataro and Kaper, 1998). Antigen heterogeneity is a striking feature among ETEC strains, as demonstrated in a survey that evaluated the diversity, distribution, and association of ETEC phenotypes in epidemiological studies carried out in different parts of the world (Wolf, 1997).

The most common O serogroups reported in ETEC are O6, O8, O11, O15, O20, O25, O27, O68, O77, O78, O85, O114, O115, O126, O128, O139, O148, O149, O153, O159, O166, O167, and O173 (Stenutz *et al*., 2006; Nataro and Kaper, 1998; Wolf, 1997; Germani, 1995; Penaranda *et al*., 1983). The predominant non-β-hemolytic ETEC serogroups identified in porcine neonatal diarrhea are: O8, O9, O20, O64 or O101 (Chapman *et al*., 2006; Fairbrother *et al*., 2005).

2.2.6 STEC

Shiga toxin-producing *E. coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), is the most important recently emerged group of foodborne pathogens. *E. coli* strains belonging to the STEC group are phenotypically, genetically and serologically highly diverse. More than 400 serotypes of STEC have been isolated from human patients and even more STEC types were isolated from food, animals and the environment (Baranzoni *et al*., 2014; Hussein, 2007; Scheutz and Strockbine, 2005; Blanco *et al*., 2001). It is a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic uremic syndrome (HUS), which is the main cause of acute renal failure in children (Beutin *et al*., 2004; Blanco *et al*., 2004; Paton and Paton, 1998; Karmali, 1989). Most outbreaks of HC and HUS have been

attributed to strains of enterohemorrhagic serotype O157:H7 (Chapman *et al*., 2006; Mora *et al*., 2004; Banatvala *et al*., 2001; Blanco *et al*., 2001; Karmali, 1989). However, as non-O157 STEC strains are more prevalent in animals and as contaminants in foods, humans are probably more exposed to these strains (Tahmasby *et al*., 2014; Beutin *et al*., 2004; Blanco *et al*., 2004). Infections with some non-O157 STEC types, such as O26:H11 or H−, O91:H21 or H−, O103:H2, O111:H−, O113:H21, O117:H7, O118:H16, O121:H19, O128:H2 or H−, O145:H28 or H− and O146:H21 are frequently associated with severe illnesses in humans, but the role of other STEC non-O157 types in human disease needs further examination (Beutin *et al*., 2004; Blanco *et al*., 2004; Pradel *et al*., 2000; Karmali, 1989). Non-O157 STEC strains are mostly commensal bacteria in animals, with a high potential for food-borne transmission to humans (Caprioli *et al*., 2005). However, the majority of non-O157 STEC strains have low virulence. Ruminants, primarily cattle, are the predominant reservoir of STEC (Caprioli *et al*., 2005). Most documented outbreaks caused by STEC are attributed to only a few serotypes, mainly O157:H7, but also O groups O26 and O111 (Nataro and Kaper, 1998).

Shiga toxin- producing *E. coli* (STEC) or verotoxigenic *E. coli* (VTEC), which were found to be associated with many serogroups and most frequently with O26, O55, O103, O111, O128 and O157 (the most epidemic serogroup) (Germani, 1995). According to and Stenutz *et al.,* (2006) the most common EHEC serogroups are: O4, O5, O16, O26, O46, O48, O55, O91, O98, O111ab, O113, O117, O118, O119, O125, O126, O128, O145, O157 and O172. Several EHEC serogroups have been described: O176, O177, O178, O179, O180 and O181 (Scheutz *et al*., 2004).

2.2.7 UPEC and/or ExPEC

The subset of *E. coli* that causes uncomplicated cystitis and acute pyelonephritis is distinct from the commensal *E. coli* strains that make up most of the *E. coli* populating the lower colon of humans. It has been traditionally described that certain serotypes of *E. coli* were consistently associated with uropathogenicity and were designated as uropathogenic *E. coli* (UPEC). *E. coli* from a small number of O serogroups - O4, O6, O14, O22, O75 and O83 – cause 75% of these urinary tract infections (Stenutz *et al*., 2006). The serogroups most commonly associated with uropathogenicity are O1, O2, O4, O6, O7, O18 and O75 (Coimbra *et al*., 2000). Strains belonging to this group possess a unique set of virulence determinants that include specialized fimbriae. A specific subset of *E. coli* strains, most often belonging to serogroups O1, O2, O4, O6, O7, O16, O18 and O75, expressing α-hemolysin and other virulence determinants, are implicated in most cases of *E. coli* septicemia. Moreover, extraintestinal *E. coli* is the most common cause of neonatal meningitis. The serogroups most commonly linked to meningitis are O1, O7, O6, and O18 (Orskov and Orskov, 1985). P-fimbriae are known to occur in association with a limited number of *E. coli* serotypes (Blanco *et al*., 1997a; Johnson *et al*., 1997; Vrn, 1996; Blanco *et al*., 1992; Johnson, 1991; Pere *et al*., 1988). The most frequent serogroups of the P-fimbriated strains belonged to one of the six serogroups O1, O2, O4, O6, O7, O18 (Vrn, 1996). Fathollahi *et al*., (2009) also observed a correlation between the *pap* operon and the O-serogroups of the strains. There are also a few reports of an association of STEC O17:H18, O103:H2, O145:H28, and OX3:H2 strains with urinary tract infections (Scheutz *et al*., 2000; Tarr *et al*., 1996). *E. coli* strains from a relatively small number of O serogroups, mainly O1, O2, O4,

O6, O7, O18 and O75 have been reported to account for a major part of O-groupable UTI strains from different parts of the world (Blanco *et al*., 1997a).

2.3 Objectives

1) To isolate and characterize *E. coli* from Cochin estuary, seafood and clinical sources.
2) To find out the prevalence of various serotypes of *E. coli* isolates at different stations in Cochin estuary.
3) To find out the occurrence of various serotypes of *E. coli* isolates in seafood and clinical sources.

2.4 Materials and Methods

2.4.1 Isolation of *E. coli* from Cochin estuary

2.4.1.1 Description of the study area

The water samples were collected from five different stations along Cochin estuary (Figure 2.1). The stations were selected based on their closeness to satellite townships and waste inputs. Two of the stations such as Chitoor (station 1) and Thevara (station 4) were fixed upstream, two in the central part of the estuary namely Bolgatty (station 2) and Marine Science Jetty (station 3), and one at the Barmouth (station 5). The sampling stations (Figure 2.2) were fixed in and around Cochin estuary as they were suspected to receive high levels of sewage inputs.

2.4.1.2 Collection of samples

The water samples were collected monthly from five different stations along Cochin estuary for a period of three years from January 2010- December 2012. The water samples were collected in 1 litre sterile plastic bottles

(Tarson, India) one foot below the surface to get a better representation of the water column. Water samples were transported to the laboratory in an ice box and subjected to bacteriological examination within four hours of collection.

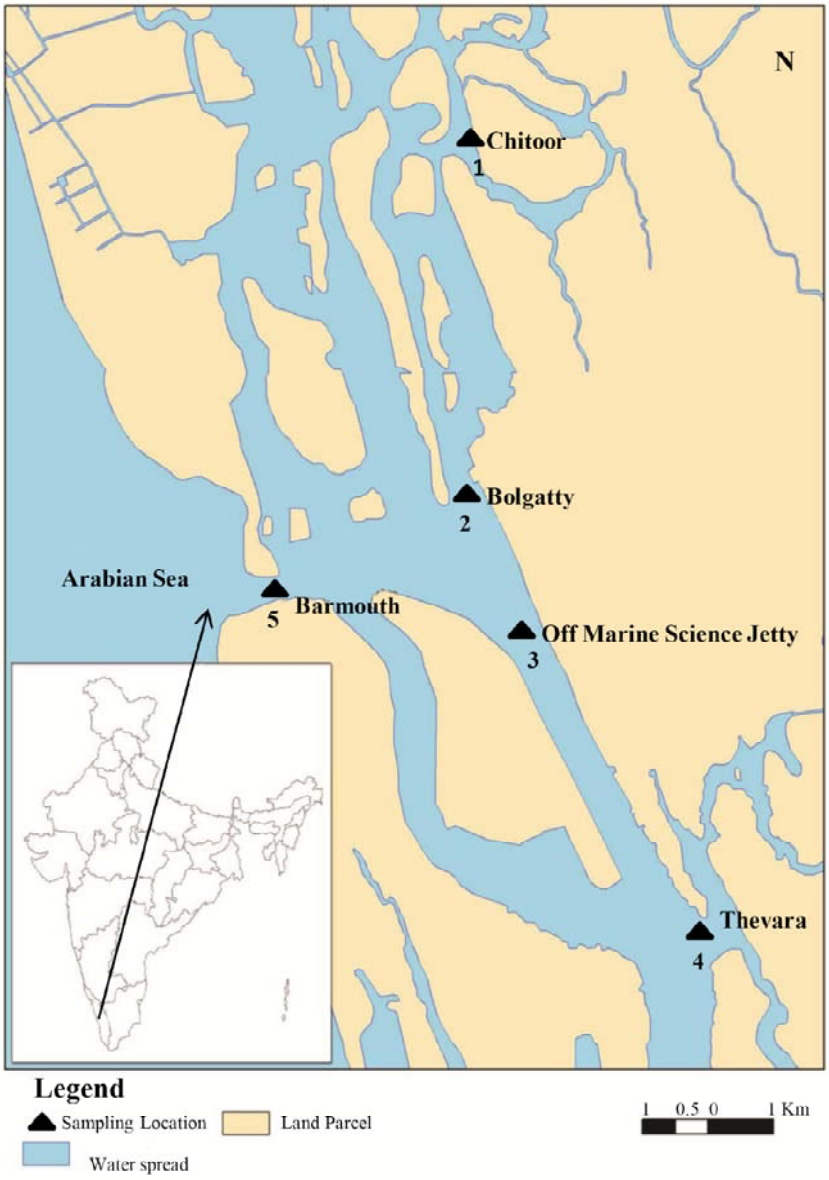


Figure 2.1 Cochin estuary map showing sampling locations

Station 1 (Chitoor)

Station 2 (Bolgatty)

Station 3 (Off Marine Science Jetty)

Station 4 (Thevara)

Station 5 (Barmouth)

Figure 2.2 Figure showing the five sampling stations set at Cochin estuary

2.4.1.3 Isolation of *E. coli* from water samples

Samples were analysed for faecal coliforms by most probable number method. The most probable number (MPN) load of faecal coliform bacteria was determined by three-tube dilution method using MacConkey broth (Hi-Media, India) as medium. Ten ml, 1 ml and 0.1 ml of water samples were inoculated into respective dilution tubes containing inverted Durham's tubes. Inoculated tubes were incubated at 37 ^{o}C for 24 hours and observed for growth and gas production. For isolation of *E. coli*, one loopfuls from positive MacConkey broth tube showing growth and gas production were streaked onto Eosin methylene blue (EMB) (Hi-Media, India) plates and incubated at 37 °C for 24 hours. After incubation, plates were examined, typical *E. coli* like colonies were selected, restreaked to ensure purity and inoculated on sterile nutrient agar (Hi-Media, India) vial for further characterization.

2.4.2 Isolation of *E. coli* from seafood samples

Two groups of seafood samples such as fish and shellfish collected from the Cochin estuary were used for isolation of *E. coli*. The samples for isolation were collected from various parts of fish such as body surface, gill and intestine. Sterile cotton swabs were used to collect samples. In order to collect samples from body surface fish was rinsed with sterile distilled water thoroughly. Using a sterile cotton swab the content of the body surface were transferred to the sterile diluents and analysed by MPN method. Gill and intestine samples were also collected using sterile cotton swab. In order to facilitate collection of the samples from intestine an incision was made near the anal region and the swab samples were processed for isolation of *E. coli* as in the case of samples from body surface and gill.

Shellfish (*Villorita cyprinoides*) collected from the shell fish beds in the Cochin estuary by professional collectors were brought to the laboratory in pre-sterilised polythene bags in an ice chest and proceeded within 4 hours from collection. The samples were processed for isolation of *E. coli* as per USFDA (Feng *et al*., 2002). Briefly, the shellfish were opened aseptically by a sterile scalpel and the meat along with valvular fluid is transferred to sterile polythene bags. Ten grams of samples were then homogenised with 90 ml of sterile distilled water in a stomacher (IUL instruments, Spain) for 90 seconds. Samples were processed for isolation of *E. coli* as in the case of samples from fish.

2.4.3 Clinical Isolates of *E. coli*

One hundred clinical *E. coli* isolates were collected from one public health centre, one hospital and five medical labs in and around Cochin City, Kerala.

2.4.4 Biochemical Identification of *E. coli*

Typical colonies, which were isolated from EMB agar plates and maintained on nutrient agar vials were subjected to IMViC test. i.e., indole production in tryptone broth, the ability to produce various organic acids from mixed acid fermentation of dextrose in Methyl red test, ability to produce non-acidic byproducts in Voges-Proskauer test and ability to utilise sodium citrate as the sole carbon source in citrate test. The cultures giving + + - - reaction were confirmed as *E. coli*.

2.4.4.1 Indole test

Indole test is used to check the release of indole ring from the breakdown of tryptophan by *E. coli*. Presumptive *E. coli* cultures were inoculated into tryptone broth and incubated at 37 °C for 48 hours. After incubation, 1 ml of

Kovac's reagent was added to the medium. Development of a red ring in the reagent layer is considered as indole positive.

2.4.4.2 Methyl Red (MR) test

In this test the ability of *E. coli* to produce various organic acids and the subsequent reduction of pH to 4.6 or below resulting from mixed acid fermentation of dextrose was checked. Cultures suspected as *E. coli* were inoculated into MR-VP broth (Hi-Media, India) and incubated at 37 °C for 48 hours. After incubation a few drops of methyl red reagent was added. A persistent red colour on addition of methyl red indicated positive for methyl red test. Yellow or orange colour is considered as negative.

2.4.4.3 Voges Proskauer (VP) test

Voges Proskauer (VP) test was done to determine the ability of *E. coli* to produce non-acidic by-products such as acetyl methyl carbinol from dextrose. Suspected colonies were inoculated into MR-VP broth and inoculated at 37 °C for 48 hours. After incubation a few drops of Barrits reagent 'A' was added to the medium. An equal volume of Barrits reagent 'B' was also added. The tubes were allowed to stand for 15 minutes before reading the result. Development of cherry red colour was considered as positive for Voges-Proskauer test. *E. coli* is unable to produce acetyl methyl carbinol and hence gives negative result.

2.4.4.4 Citrate test

In the citrate test the ability of *E. coli* to utilise sodium citrate as the sole carbon source has been assessed. Presumptive *E. coli* cultures were inoculated into Simmons citrate agar and incubated at 37 °C for 48 hours. Development

of deep prussion blue colour was recorded as positive test for citrate utilization. *E. coli* is unable to utilize sodium citrate as the sole carbon source and hence gives negative result.

2.4.5 Serotyping of *E. coli*

Confirmed *E. coli* cultures were serotyped at National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli, Himachal Pradesh, India.

2.4.6 Molecular characterization of *E. coli*

2.4.6.1 Isolation of DNA from *E. coli*

DNA from the bacterial genome was extracted as per standard Proteinase-K digestion method (Sambrook *et al*., 1989). Bacterial cultures were suspended in Luria Bertani broth (Hi-Media, India) and incubated in an orbital incubator (Orbitek, India) at 37 °C, 110 rpm for 12 hours. The 12 hour old bacterial cells were pelleted at 15000 × g for 10 minutes and then suspended in TEN (Tris-HCl (pH 7.2), 10 mM EDTA, 250 mM NaCl) buffer having 1% sodium dodecyl sulphate (Hi-Media, India). Proteinase-K (GeNei^{TM}, India) was then added to a final concentration of 100 µg/ml and mixed gently. The suspension was incubated at 37 °C for 60 min. DNA obtained by sequential phenol-chloroform and chloroform-isoamyl alcohol extractions was precipitated by adding 2.5 volumes of absolute ethanol, and DNA was suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA-pH 7.5). DNA was checked for purity by agarose gel electrophoresis.

2.4.6.2 Polymerase Chain Reaction (PCR) for detection of *uid A* gene in *E. coli*

The polymerase chain reaction (PCR) was used to detect the presence of the *uid* gene, which codes for the β-D-glucuronidase enzyme. A 147 bp

coding region of the *E. coli uid* gene was amplified by PCR, using the 20 and 21-mer primers UAL-754 (5'-AAAACGGCAAGA AAAAGCAG-3') and UAR-900 (5'-ACGCGTGGTTACAGTCTTGCG-3') (Bej *et al*., 1991). The optimized protocol was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 2.5 µl of Taq buffer (Tris (pH 9.0) at 25°C, KCl and Triton X-100), 2.5 mM each of dNTP mixture, 1 pmol/µl of each of the primers, 1 U of Taq polymerase (GeNei^{TM}, India) and 1 µl of the DNA template. Amplification was performed with a thermal cycler programmed for 1 cycle of 2 min at 94 °C; 25 cycles of 1 min at 94 °C, 1.5 min at 58 °C, 2 min at 72 °C; 1 cycle of 5 min at 72 °C. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei^{TM}, India), and visualized by Gel Documentation System (BioRad Gel Doc^{TM} EZ Imager, USA).

2.5 Results

2.5.1. Diversity of *E. coli* serotypes in Cochin estuary

2.5.1.1 Overall diversity of *E. coli* serotypes in Cochin estuary

Serotyping of the 223 *E. coli* isolates were from Cochin estuary revealed that they belonged to 58 different serotypes (Table 2.1). About 18% of isolates were untypable (UT) and 15% were rough (R). *E. coli* pathogenic serotypes such as O25, O60, O75 were isolated with high frequency, where as O157, O153, O148 were encountered very rarely. All isolates were confirmed by molecular level identification by polymerase chain reaction with primers UAL-754 and UAR-900 to amplify the amino coding region of *uid A* produced amplified DNA bands of 147 bp for all *E. coli* isolates (Figure 2.3).

Stations close to Cochin City, such as Bolgatty and Off Marine science jetty yielded more diverse pathogenic serotypes. Spatial distribution of various pathogenic serotypes of *E. coli* in Cochin estuary is given in Table 2.2.

In station 1, 2 and 5, more diverse serotypes were obtained during pre-monsoon period, while post-monsoon period yielded diverse serotypes in station 3 and 4. Temporal distribution of various pathogenic serotypes of *E. coli* in Cochin estuary is given in Table 2.3.

Table 2.1 Percentage incidence of different *E. coli* serotypes in Cochin estuary

Serotype	% incidence	Serotype	% incidence	Serotype	% incidence	Serotype	% incidence
UT	17.93	O56	1.79	O20	0.89	O157	0.44
R	15.24	O76	1.79	O55	0.89	O163	0.44
O25	7.62	O103	1.34	O64	0.89	O18	0.44
O60	3.58	O114	1.34	O69	0.89	O2	0.44
O75	3.58	O149	1.34	O90	0.89	O34	0.44
O4	3.13	O21	1.34	O91	0.89	O39	0.44
O8	2.69	O22	1.34	O100	0.44	O41	0.44
O1	2.24	O29	1.34	O105	0.44	O47	0.44
O147	2.24	O59	1.34	O112	0.44	O52	0.44
O37	2.24	O10	0.89	O113	0.44	O58	0.44
O28	1.79	O102	0.89	O13	0.44	O6	0.44
O30	1.79	O11	0.89	O135	0.44	O61	0.44
O35	1.79	O116	0.89	O148	0.44	O62	0.44
O44	1.79	O166	0.89	O153	0.44	O87	0.44
O5	1.79	O170	0.89	O154	0.44	O9	0.44

Classic serotypes associated with shiga toxin-producing and enterotoxigenic *E. coli* were detected highly.

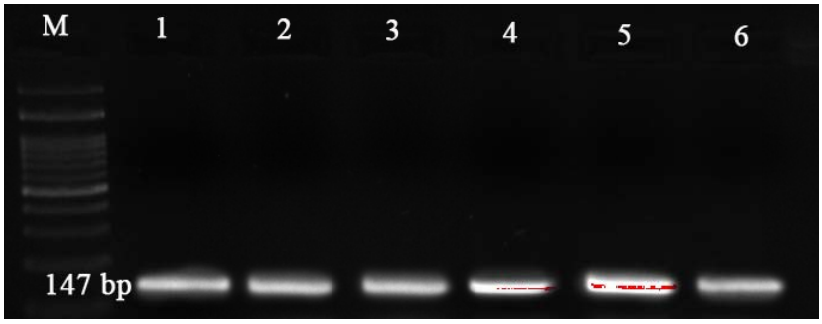


Figure 2.3 Gel image showing detection of *uidA* gene in *E. coli*.
Lanes, M: 100 bp Marker; 1-6: *E. coli* isolates

Table 2.2 Spatial distribution of various pathogenic serotypes of *E. coli* in Cochin estuary

Station No*	Pathogenic Serotypes
1.	O1, O18, O21, O22, O25, O28, O44, O55, O60, O91, O105, O112, O114, O163.
2.	O1, O2, O4, O5, O10, O13, O21, O20, O25, O29, O34, O37, O41, O44, O52, O59, O60, O75, O87, O90, O116, O147, O154, O157.
3.	O6, O8, O20, O22, O25, O29, O35, O37, O39, O60, O75, O147, O148, O149, O153, O166.
4.	O8, O9, O11, O25, O28, O44, O60, O75, O76, O113, O116, O147, O166.
5.	O1, O4, O5, O25, O28, O29, O64, O75, O102, O103, O114, O149.

* 1, Chitoor; 2, Bolgatty; 3, Off Marine Science Jetty; 4, Thevara; 5, Barmouth

Table 2.3 Temporal distribution of various pathogenic serotypes of *E. coli* in Cochin estuary

Station No*	Pre-monsoon	Monsoon	Post-Monsoon
1.	O25, O114, O28, R, UT, O25, O112, O58, O163, O44	UT, O18, O114, O105, O55, O22	R, UT, O25, O60, O21, O1, R, O91
2.	O59, R, O60, O2, O116, O4, O41, UT, O52, O30, O147, O30, O60, O25, O5, O44, O61	UT, O30, O20, O4, O59, O157, O44, O154, O25, O10, O34	UT, O90, O25, R, O75, O29, O1, O69, O37, O13, O87, O21, O147
3.	R, O147, O37, UT, O135, O20, , O75, O60, O39, O8	O166, O30, O8, UT, O149, O29, O6	O25, UT, O35, O22, O60, O153, O147, R, O149, O148, O37, O8
4.	UT, R, O100, O75, O9, O25, O8	O11, UT, O90, R, O116, O76, O60, O62, O44	O11, R, O60, O75, O147, O28, UT, O113, O166
5.	O4, O64, UT, O75, O29, O64, O103, O114, O5, R	O5, R, O28, O170, O75, O1, O102, O170	R, O149, UT, O25, O28, O56,

* 1, Chitoor; 2, Bolgatty; 3, Off Marine Science Jetty; 4, Thevara; 5, Barmouth

2.5.1.2 Distribution of different classes of *E. coli* serotypes in Cochin estuary

Enteroaggregative *E. coli* serotype was represented by only one serotype O44. The 6 strains in EIEC belonged to 2 serotypes. About 4% of isolates were with classic EIEC serotypes such as O29, and O28. The classical EPEC comprised 15 strains belonging to serotypes O13, O21, O34, O37, O64, O10, and O9. The isolated serotypes included in classic enterotoxigenic *E. coli* were O11, O112, O114, O147, O148, O149, O153, O166, O20, O25, O41, O6, and O8. More than 30% of isolates belonged to ETEC serotype. About 29% of isolates were represented by classic STEC serotypes. The STEC belonged to 19 serotypes. The serotypes were O102, O103, O105, O113, O116, O154,

O157, O163, O22, O35, O39, O4, O5, O52, O55, O60, O76, O87, and O91. The best-known serotypes that conform to the features of UPEC were O1, O18, O2, O59, and O75. More than 11% of isolates were non pathogenic and belonged to serotypes such as, O47, O56, O58, O100, O135, O170, O30, O61, O62, O69, O90 (Figure 2.4).

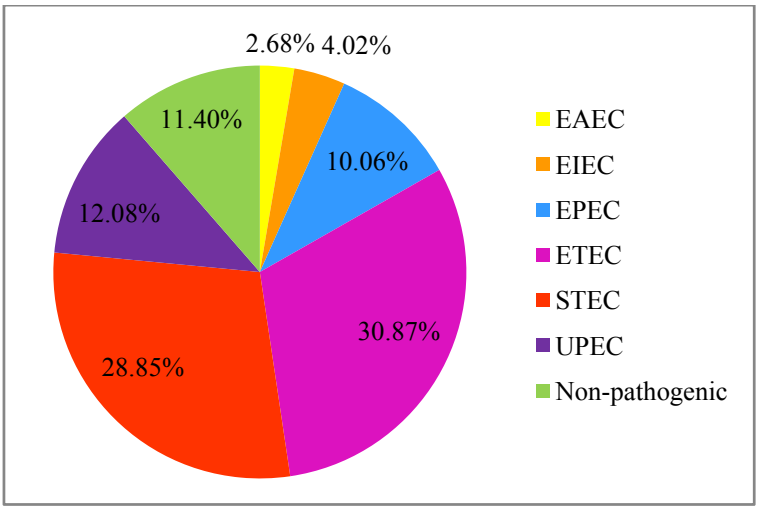


Figure 2.4 Distribution of different classes of *E. coli* serotypes in Cochin estuary.

2.5.1.3 Diversity of *E. coli* serotypes in station 1 (Chitoor)

Out of 47 *E. coli* strains isolated from station 1 (Chitoor), 36 isolates were serotyped. About 14% of isolates were untypable and 14% were rough. Fifteen diverse serotypes were obtained from this station (Table 2.4). Classic serotypes associated with shiga toxin-producing and enterotoxigenic *E. coli* were detected frequently. The percentage of incidence of O25 was very high, which is a traditional ETEC serotype.

Table 2.4 Percentage incidence of different *E. coli* serotypes in station 1 (Chitoor)

Serotype	% incidence	Serotype	% incidence
O25	16.66	O112	2.77
R	13.88	O163	2.77
UT	13.88	O18	2.77
O1	5.55	O28	2.77
O114	5.55	O44	2.77
O21	5.55	O58	2.77
O22	5.55	O60	2.77
O55	5.55	O91	2.77
O105	2.77		

2.5.1.3.1 Distribution of different classes of *E. coli* serotypes in station 1 (Chitoor)

About 34% of isolates were represented by classic STEC serotypes belonging to 6 serotypes such as O105, O163, O22, O55, O60, and O91 (Figure 2.5). Enterotoxigenic *E. coli* also contributed nearly 34% which included serotypes such as O112, O114, and O25. Enteroaggregative *E. coli* serotype was represented by only one serotype O44. The traditional EIEC serotype comprised only one strain belonging to serotype O28. The classical EPEC serotype comprised 2 strains belonging to serotype O21. The best-known UPEC serotypes were O1, and O18. Only one isolates was non pathogenic and belonged to serotype O58.

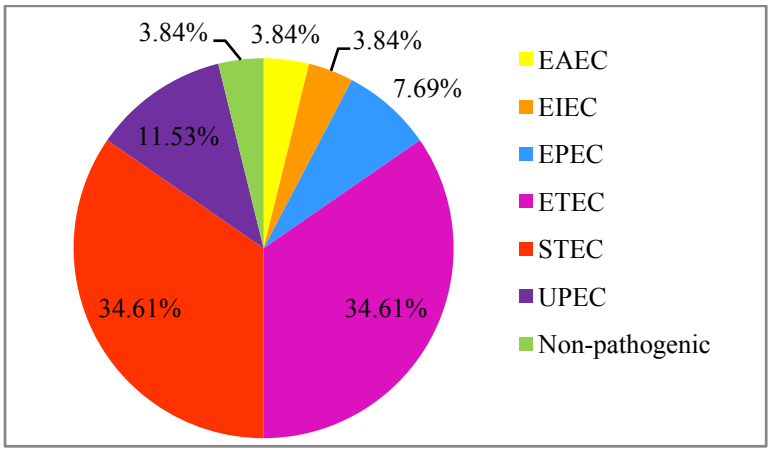


Figure 2.5 Distribution of different classes of *E. coli* serotypes in station 1 (Chitoor)

2.5.1.4 Diversity of *E. coli* serotypes in station 2 (Bolgatty)

Out of 75 *E. coli* strains isolated from station 2 (Bolgatty), 64 isolates were serotyped. About 12% of isolates were untypable and 14% were rough. Twenty eight diverse serotypes were obtained from this station (Table 2.5). Classic serotypes associated with shiga toxin-producing *E. coli* were dominant. The percentage incidence of O4 serotype was very high, which is a traditional STEC serotype.

Table 2.5 Percentage incidence of different *E. coli* serotypes in station 2 (Bolgatty)

Serotype	% incidence	Serotype	% incidence	Serotype	% incidence
R	14.06	O41	3.12	O29	1.56
UT	12.5	O60	3.12	O34	1.56
O4	9.37	O69	3.12	O44	1.56
O25	6.25	O116	1.56	O47	1.56
O30	4.68	O13	1.56	O5	1.56
O59	4.68	O154	1.56	O52	1.56
O1	3.12	O157	1.56	O61	1.56
O10	3.12	O2	1.56	O75	1.56
O147	3.12	O20	1.56	O87	1.56
O37	3.12	O21	1.56	O90	1.56

2.5.1.4.1 Distribution of different classes of *E. coli* serotypes in station 2 (Bolgatty)

The isolated serotypes included in classic enterotoxigenic *E. coli* were O147, O20, O25, and O41 which contributed 14% (Figure 2.6). About 34% of isolates were represented by classic STEC serotypes belonging to 9 serotypes such as O116, O154, O157, O4, O5, O52, O60, O87, and O90. The two strains belonged to EAEC represented by one serotype O44. The traditional EIEC serotype comprised only one strain belonging to serotype O29. The classical EPEC comprised 7 strains belonging to serotypes O10, O13, O34, and O37. The best-known UPEC serotypes were O1, O2, O59, and O75. About 15% of isolates were non pathogenic and belonged to serotypes such as, O30, O47, O61, and O69.

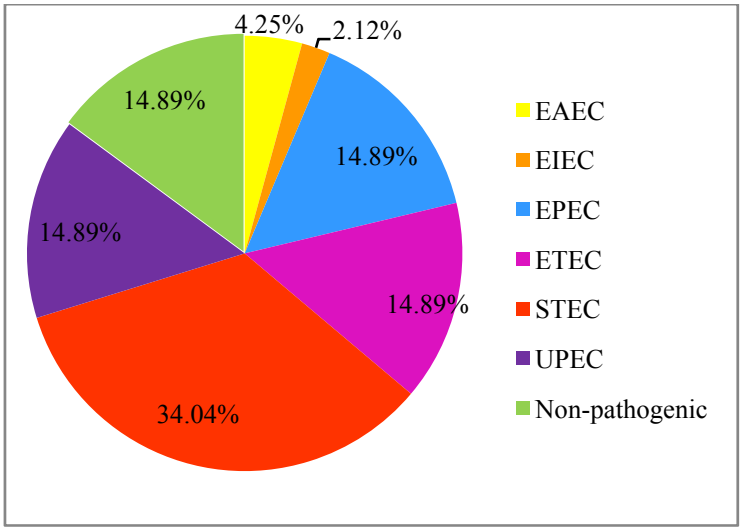


Figure 2.6 Distribution of different classes of *E. coli* serotypes in station 2 (Bolgatty)

2.5.1.5 Diversity of *E. coli* serotypes in station 3 (Off Marine Science Jetty)

Twenty diverse serotypes were obtained from this station (Table 2.6). About 27% of isolates were untypable and 7.5% were rough. Classic serotypes associated with enterotoxigenic *E. coli* were detected highly. The percentage of incidence of O8 was very high, which is a traditional ETEC serotype.

Table 2.6 Percentage incidence of different *E. coli* serotypes in station 3 (Off Marine Science Jetty)

Serotype	% incidence	Serotype	% incidence	Serotype	% incidence
UT	27.5	O135	2.5	O20	2.5
O8	10	O148	2.5	O22	2.5
O37	7.5	O153	2.5	O25	2.5
R	7.5	O39	2.5	O29	2.5
O147	5	O6	2.5	O30	2.5
O149	5	O75	2.5	O35	2.5
O60	5	O166	2.5		

2.5.1.5.1 Distribution of different classes of *E. coli* serotypes in station 3 (Off Marine Science Jetty)

The isolated serotypes included in classic enterotoxigenic *E. coli* were represented by O147, O148, O149, O153, O166, O20, O25, O6, and O8 which together contributed 53% of isolates (Figure 2.7). The traditional EIEC serotype comprised only one strain belonging to serotype O29. About 11% of isolates were with classic EPEC serotypes represented by O37. About 19% of isolates were represented by classic STEC serotypes belonging to 4 serotypes such as O22, O35, O39, and O60. The best-known UPEC serotype was O75. More than 7% of isolates were non pathogenic and belonged to serotypes such as O135, and O30.

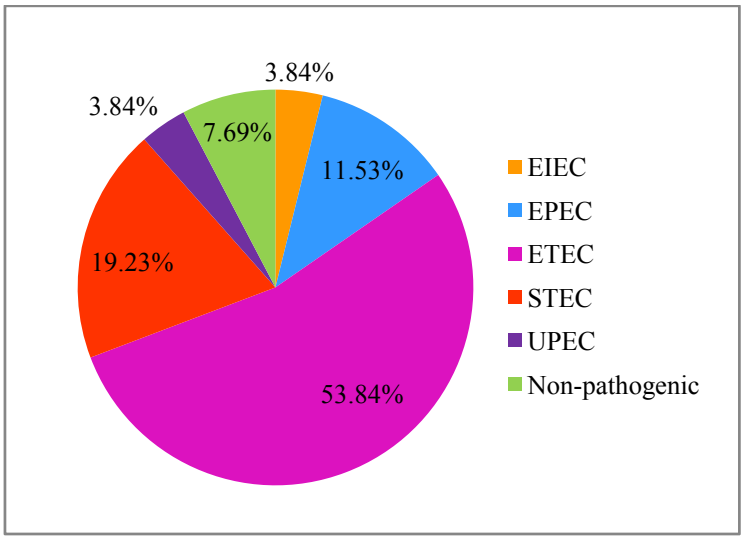


Figure 2.7 Distribution of different classes of *E. coli* serotypes in station 3 (Off marine Science Jetty)

2.5.1.6 Diversity of *E. coli* serotypes in station 4 (Thevara)

Classic serotypes associated with enterotoxigenic *E. coli* were dominant at this station. The percentage of incidence of O75 was very high, which is a traditional UPEC serotype. About 24% of isolates were untypable and 19% were rough. Eighteen diverse serotypes were obtained from this station (Table 2.7).

Table 2.7 Percentage incidence of different *E. coli* serotypes in station 4 (Thevara)

Serotype	% incidence	Serotype	% incidence	Serotype	% incidence
UT	24.39	O100	2.43	O28	2.43
R	19.51	O113	2.43	O44	2.43
O75	9.75	O116	2.43	O62	2.43
O60	7.31	O147	2.43	O76	2.43
O11	4.87	O166	2.43	O9	2.43
O8	4.87	O25	2.43	O90	2.43

2.5.1.6.1 Distribution of different classes of *E. coli* serotypes in station 4 (Thevara)

About 26% of isolates were represented by classic STEC serotypes. The STEC belonged to 4 serotypes such as O113, O116, O60, and O76. The isolated serotypes included in classic enterotoxigenic *E. coli* were O11, O147, O166, O25, and O8. More than 30% of isolates belonged to ETEC serotype (Figure 2.8). EAEC serotype was represented by O44 only. The traditional EIEC serotype comprised only one strain belonging to serotype O28. The classic EPEC serotype comprised only one strain belonging to serotype O9. The best-known UPEC serotype was O75. About 13% of isolates were non pathogenic and belonged to serotypes such as O100, O63, and O90.

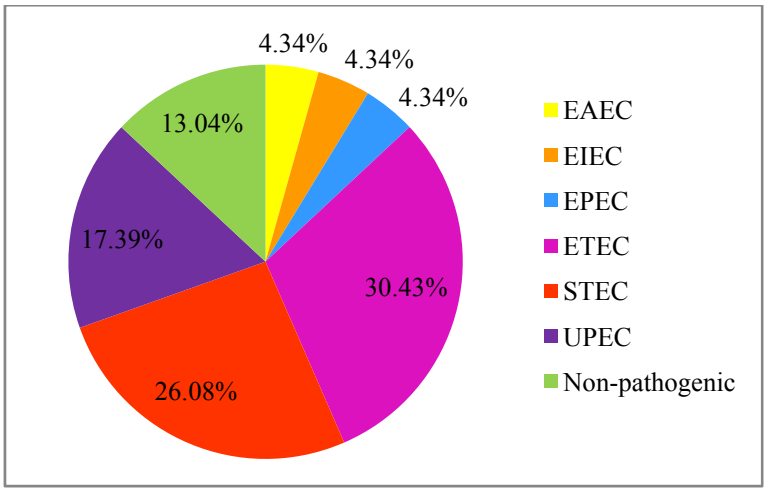


Figure 2.8 Distribution of different classes of *E. coli* serotypes in station 4 (Thevara)

2.5.1.7 Diversity of *E. coli* serotypes in station 5 (Barmouth)

Out of 60 *E. coli* strains isolated from station 5 (Barmouth), 42 isolates were serotyped. About 14% of isolates were untypable and 21% were rough.

Sixteen diverse serotypes were obtained from this station (Table 2.8). Classic serotypes associated with shiga toxin-producing *E. coli* were frequently encountered at this station. The percentage of incidence of O25 was very high, which is a traditional ETEC serotype.

Table 2.8 Percentage incidence of different *E. coli* serotypes in station 5 (Barmouth)

Serotype	% incidence	Serotype	% incidence
R	21.42	O64	4.76
UT	14.28	O75	4.76
O25	11.9	O1	2.38
O103	7.14	O114	2.38
O5	7.14	O149	2.38
O102	4.76	O29	2.38
O170	4.76	O4	2.38
O28	4.76	O56	2.38

2.5.1.7.1 Distribution of different classes of *E. coli* serotypes in station 5 (Barmouth)

The isolated serotypes included in classic enterotoxigenic *E. coli* were O114, O149, and O25, which together contributed more than 25% of isolates (Figure 2.9). About 33% of isolates were represented by classic STEC serotypes. The serotypes were O102, O103, O4, and O5. The classical EIEC comprised 3 strains belonging to serotypes O28, and O29. The 2 strains in classical EPEC serotype belonged to O64. The best-known UPEC serotypes were O1, and O75. About 11% of isolates were non pathogenic and belonged to O170.

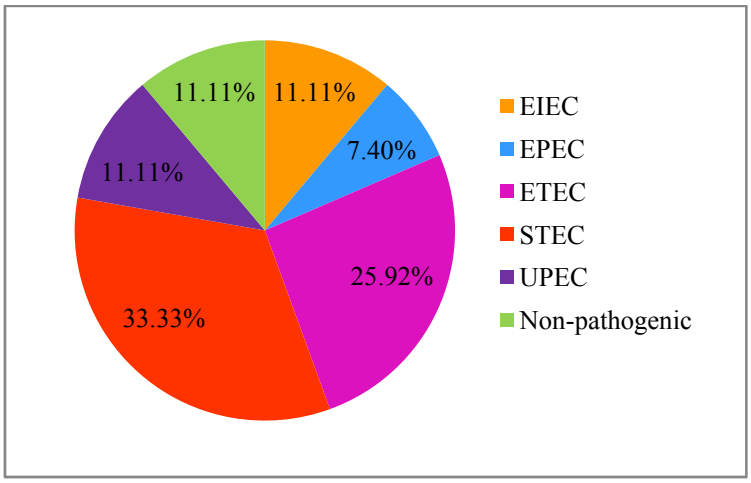


Figure 2.9 Distribution of different classes of *E. coli* serotypes in station 5 (Barmouth)

2.5.2 Diversity of *E. coli* serotypes in seafood sources from Cochin estuary

Out of 100 *E. coli* strains isolated from seafood, sixteen diverse serotypes were obtained (Table 2.9). About 21% of isolates were untypable and 16% were rough. Diversity of *E. coli* serotypes was relatively low in seafood samples. Classic serotypes associated with enteropathogenic *E. coli* were frequently encountered. The percentage of incidence of O14 was very high, which is a traditional UPEC serotype.

Table 2.9 Percentage incidence of different *E. coli* serotypes in seafood sources

Serotype	% incidence	Serotype	% incidence
UT	21.05	O132	2.63
O14	15.78	O159	2.63
R	15.78	O3	2.63
O16	5.26	O56	2.63
O23	5.26	O60	2.63
O41	5.26	O69	2.63
O106	2.63	O7	2.63
O119	2.63	O82	2.63
O13	2.63	O88	2.63

2.5.2.1 Distribution of different classes of *E. coli* serotypes from seafood sources

More than 12% of isolates belonged to ETEC serotype (Figure 2.10) and 12% of isolates were represented by classic STEC serotypes such as O106, O119, O60. The traditional EAEC serotype comprised only one strain belonging to serotype O3. The classical EPEC comprised 6 strains belonging to serotypes O13, O132, O23, O82, and O88. The isolated serotypes included in classic enterotoxigenic *E. coli* were O159, and O41. The best-known UPEC serotypes were O14, O16, and O7. More than 8% of isolates were non pathogenic and belonged to serotypes such as O56, and O69.

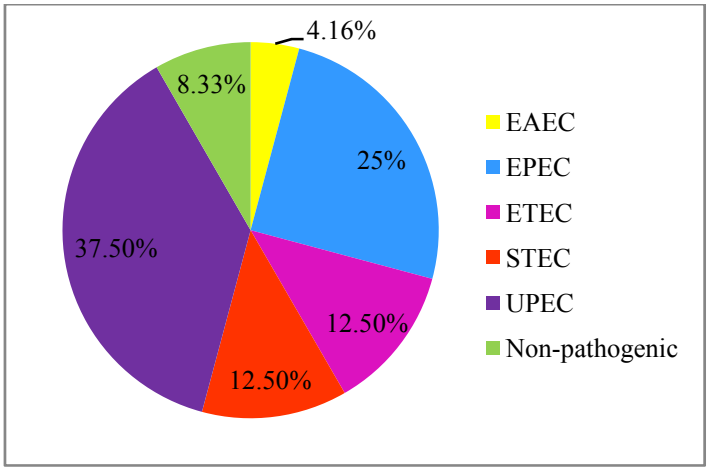


Figure 2.10 Distribution of different classes of *E. coli* serotypes in seafood sources

2.5.3 Diversity of *E. coli* serotypes in clinical sources from Cochin City

Diversity of *E. coli* serotypes was much lower in clinical sources when compared to estuarine and seafood sources. Except for nearly 50% of *E. coli*, which were untypable, remaining was represented by two pathogenic classes such as EPEC and UPEC. Classic serotypes associated with enteropathogenic

E. coli were dominated. The percentage of incidence of O82 was very high, which is a traditional EPEC serotype. About 42% of isolates were untypable and 8% were rough (Table 2.10).

Table 2.10 Percentage incidence of different *E. coli* serotypes in clinical sources

Serotype	% incidence
UT	41.66
O82	25
O2	16.66
O172	8.33
R	8.33

2.5.3.1 Distribution of different classes of *E. coli* serotypes in clinical sources

The classical EPEC comprised 4 strains belonging to serotypes O172, and O82. More than 66% of isolates belonged to EPEC serotype. About 33% of isolates were represented by classic UPEC serotypes. The best-known UPEC serotype was O2 (Figure 2.11).

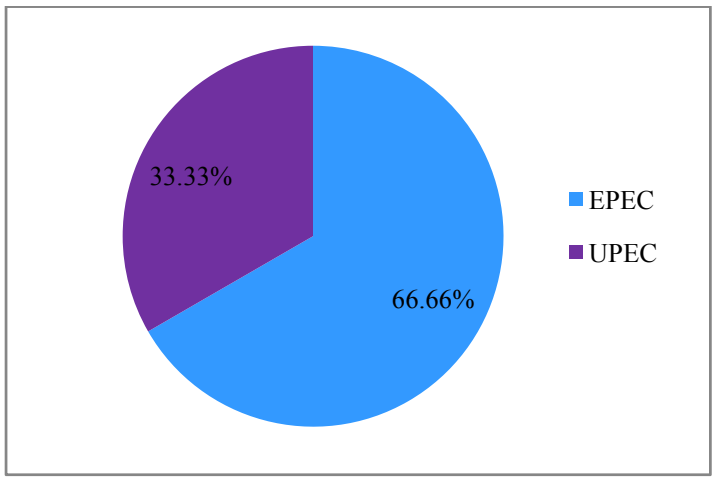


Figure 2.11 Distribution of different classes of *E. coli* serotypes in clinical sources

2.6 Discussion

2.6.1 Serotyping of *E. coli* isolates

Though *E. coli* considered as a commensal, causes a wide variety of intestinal diseases. The objective of this observation was to look at the diversity of *E. coli* serotypes in various sources such as seafood, clinical and estuarine water to determine the prevalence of pathogenic class of serotypes. All the biochemically confirmed *E. coli* were reconfirmed by polymerase chain reaction of *uidA* gene. Bej *et al*., (1991) suggested that a PCR-based method for *uidA* gene is more sensitive in detecting *E. coli* isolates from water samples. The *uid* gene was detected in all *E. coli* isolates.

Conventional serotyping methods for *E. coli* somatic and flagellar antigens are still important technique in many laboratories for diagnosis and surveillance (Perez *et al*., 2010). Furthermore, serologic antigens are not directly involved in virulence, but can provide important information about the circulating serotypes in the communities and in outbreaks (Nataro and Kaper, 1998).

Serotyping is a common method used for the characterization of clinical isolates of *E. coli* and has a broad use in epidemiology and also in medical diagnosis. The existing association between serotype and pathotype makes this method a valuable tool for typing *E. coli* and other bacterial species. Although, serotyping plays an important role in the characterization of pathogenic *E. coli*, there are many limitations in conventional serogrouping for it to be used as a regular diagnostic or epidemiological tool (Gordon, 2010). The serotyping of clinical isolates of *E. coli* is under constant development and usually it is possible to identify the isolated strains. In some cases, however, it is not possible to

properly characterize the strain with available monospecific polyclonal antisera, either due to auto agglutination or because the isolated *E. coli* strain is novel and appropriate antisera have not been raised (Sumathi *et al*., 2008).

The transition of *E. coli* from smooth (S) to rough (R) form is another challenging phenomenon in serotyping of *E. coli*. The R forms do not synthesise O-antigen due to specific mutations in genes within the *rfb* gene cluster which encodes the O-antigen. As a result, the R strains cannot be typed using conventional serological methods (Orskov and Orskov, 1978). Our results revealed good number of rough strains in the estuarine environment. Though detailed studies are required, our results indirectly indicate the conducive nature offered by the estuarine environment for mutation leading to the emergence of rough strains. In an earlier study conducted in Cochin estuary by Chandran *et al*., (2008) recovered 81 strains belonged to 43 different serotypes. Serological test classified 4.93% were untypable in their study.

2.6.2 Diversity of *E. coli* serotypes in Cochin estuary

In our study only one O157 isolate (0.44%) was obtained and it was from station 2 (Bolgatty). When an analysis of the geographic distribution of *E. coli* O157 was done by Sehgal *et al*., (2008), it was observed to be widely distributed in all parts of India showing wide prevalence of this strain in almost all regions of the country and 1.1% of O157 isolates were from Kerala. The newly emerged pathogen O157:H7 is an example of a strong association of specific serotype with a pathotype (Day *et al*., 1983). It could be diagnosed before any virulence properties of this strain were known. Bonnet *et al*., (1998) shown that *E. coli* serotypes other than O157:H7 can also cause serious

disease. Perelle *et al*., (2007) reported that contamination by the pathogenic *E. coli* serotypes, including O103, O157, and O145 representing a major public health concern. In our study 1.34% of *E. coli* isolates were with O103 serotype and those were obtained from station 5 (Barmouth). Two recent outbreaks due to *E. coli* have occurred with serotypes not previously known to cause large scale outbreaks O103:H25 (Sekse *et al*., 2009; Schimmer *et al*., 2008) and O104:H4 (Bielaszewska *et al*., 2011; Frank *et al*., 2011; Scheutz *et al*., 2011). These two outbreaks resulted in severe burden on the public health system, and the O104:H4 outbreak was one of the world's largest *E. coli* food-borne outbreaks in history. As the number of STEC serotypes is high, there is always the possible outbreak of any *E. coli* of any serovar or pathovar.

In our study only one O113 isolate (0.44%) was obtained and it was from station 4 (Thevara). STEC strains belonging to serotype O113:H21 have been shown responsible for cases of HUS (Nataro and Kaper, 1998; Paton and Paton, 1998). Infections with some non-O157 STEC types, such as O26:H11 or H2, O91:H21 or H2, O103:H2, O111:H2, O113:H21, O117:H7, O118:H16, O121:H19, O128:H2 or H2, O145:H28 or H2 and O146:H21, have been associated with severe illness in humans (Coombes *et al*., 2008; Bettelheim, 2007) and with a number of outbreaks (Werber *et al*., 2007; Hiruta *et al*., 2001; McMaster *et al*., 2001). Contreras *et al*., (2011) isolated shiga toxin-producing *E. coli* from children and reported that the most common serogroups were O26, O111 and O145. Beutin *et al*., (2004) reported that certain STEC strains belonging to serogroups O26, O103, O111, O145, and O157 were more frequently isolated from humans with severe diseases such as hemorrhagic colitis and HUS. *E. coli* strains belonging to serotypes O157:[H7], O145:[H28],

O111:[H8], O103:H2, and O26:[H11] are recognized classical EHEC types which occur in different countries worldwide.

Shiga toxin-producing (STEC) and enteropathogenic *E. coli* (EPEC) are serologically diverse, emerging foodborne pathogens and leading cause for a spectrum of human illness ranging from hemorrhagic diarrhea to even fatal consequences such as hemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpurea (TTP) and hemorrhagic colitis (HC) (Croxen and Finlay, 2010; Gyles and Fairbrother, 2010). Aidar-Ugrinovich *et al*., (2007) reported that the most frequent serotypes among STEC strains were O7:H10, O22:H16, O111:H−, O119:H− and O174:H21, whereas O26:H11, O123:H11 and O177:H11 were the most prevalent among EPEC strains. In their study, serotypes not previously reported were also found among STEC strains: O7:H7, O7:H10, O48:H7, O111:H19, O123:H2, O132:H51, O173:H−, and O175:H4. Whereas in our result, all the above serotypes were absent.

Numerous outbreaks have been attributed to STEC strains of serotype O157:H7, but it has been recognized that STEC strains causing gastrointestinal diseases in humans may belong to a wide range of serogroups. Non-O157 serogroups, most commonly O26, O55, O103, O111 and O145, have been shown to cause diarrhea in humans (Tozzi *et al*., 2003; Scotland *et al*., 1993). The percentage of incidence for O55 was 0.89% in our study and this serotype was isolated from station 1 (Chitoor) only. Infections with some non-O157 STEC types, such as O26:H11 or O26:H, O91:H21 or O91:H, O103:H2, O111:H, O113:H21, O117:H7, O118:H16, O121: H19, O128:H2 or O128:H, O145:H28 or O145:H, and O146:H21, are frequently associated with severe illness in humans, but the roles of other non-O157 STEC types in human

disease require further examination (Mora, 2002; Blanco *et al*., 2001; Beutin, 1999; Boerlin *et al*., 1999).

In our result, 0.89% of isolates from estuary was O91 serotype and obtained from station 1 (Chitoor). STEC strains belonging to serogroup O91 were associated with HUS cases in adult patients in central France (Bonnet *et al*., 1998). Pradel *et al*., (2000) reported a case of HUS associated with an O91:H10 isolate that could be differentiated from O91:H21 by using ribotyping. Food vehicles have been identified as the only risk factors for adults with sporadic STEC O91 infection in Germany (Werber *et al*., 2007). Serotype O91 is the second most frequently isolated STEC serogroup in routine food samples (Mellmann *et al*., 2009).

Serotypes such as O148, O149 were obtained from Cochin estuary with a percentage of incidences of 0.44% and 1.34%, respectively. Serotype O149 was obtained from Off Marine Science Jetty and Barmouth. Whereas serotype O148, was obtained from station 3 only. Scott *et al*., (2009) reported that the isolates identified in their study contained many virulence factors that are associated with human disease and serotypes O2:H27, O149:H1, ONT: H25, O148:H8 and O174:H21 have been each been identified as infrequent human pathogens from several countries in Europe and North America. In our study, serotype O2 was obtained from station 2 (Bolgatty) only. Ramteke and Tewari (2007) analysed the serogroups of *E. coli* in drinking water from India and found that 78.9% were typable of which 26.3% were pathogenic serotypes. Pathogenic serogroup detected in their study were O4, O25, O86, O103, O157, O8, and 0113. In our study, we also isolated the above serotypes from Cochin estuary except O86.

E. coli expressing O4 lipopolysaccharide (LPS) and/or H5 flagellin frequently causes extraintestinal infections in humans and domestic animals (Blanco *et al*., 1994a; Orskov and Orskov, 1992; Gransden *et al*., 1991). *E. coli* O4 organisms are common etiologic agents of human, canine, and feline genitourinary tract infections, manifested as pyelonephritis, cystitis, prostatitis, urosepsis, and asymptomatic bacteriuria (Yuri *et al*., 1999; Terai *et al*., 1997; Blanco *et al*., 1996; Donnenberg and Welch, 1996; Johnson, 1991). *E. coli* O4 and/or H5 antigens are also associated with enteric disease. In our study, 3.13% of isolates from estuary were of O4 serotype and obtained from station 2 (Bolgatty), and 5 (Barmouth). Shiga toxin-producing *E. coli* (STEC) O4:H-negative (Tzipori *et al*., 1988), O4:H5 (Gunzburg *et al*., 1998), O4:H10 (Willshaw *et al*., 1992), O2:H5 (Bockemuhl *et al*., 1992, von Wulffen *et al*., 1989), and O75:H5 (Bockemuhl *et al*., 1992) strains have caused hemorrhagic colitis and hemolytic uremic syndrome cases, while non-STEC O4 and enteroaggregative O4 have caused pediatric diarrhea outbreaks (Cobeljic *et al*., 1996; Colonna *et al*., 1992). STEC O4:H-negative (Wieler *et al*., 1996), O4:H4 (Blanco *et al*., 1994b), O4:H21 (Suthienkul *et al*., 1990), O4:H25 (Wilson *et al*., 1996), and O2:H5 (Sandhu *et al*., 1996) have been isolated from healthy cattle, while STEC O4 and non-STEC O4 have been associated with calves (Orden *et al*., 1998), pigs (Garabal *et al*., 1996), and lambs (Blanco *et al*., 1996) with diarrhea. While the *E. coli* O4 and H5 antigens are both markers of strain pathogenic potential, the O4 antigen moiety may itself function as an urovirulence factor (Russo *et al*., 1996). In the United States, O4:HNM, O45:H2, O111:HNM, and O145:HNM strains were isolate from sporadic cases of hemorrhagic colitis in the early 1980s (Tzipori *et al*., 1988).

In our results, classical EPEC comprised 15 strains belonging to serotypes O13, O21, O34, O37, O64, O10, and O9. Carneiro *et al*., (2006) isolated fifty six *E. coli* strains from pasteurised milk and serogrouped as EPEC. The strains were distributed among 7 EPEC serogroups (O26, O55, O111, O114, O125, O127, O128, O158). In a study by Saridakis *et al*., (1997) reported that 19 EPEC isolates belonged to 12 serotypes with isolates of O26:H, O119:H25 and O114:H- being the most prevalent. Jenkins *et al*., (2006) reported that the EPEC strains showed a wide range of O:H types, with O70:H11 and O111:H– being the most frequently detected. Certain O serogroups, such as O2, O33, O71, O103, O111, O114, and O129, were associated with more than one H type. In a study by Blanco *et al*., (2006a), atypical EPEC strains belonged to 65 O:H serotypes, including 43 new serotypes not previously described among human EPEC. Aslani and Alikhani, (2009) analysed the enteropathogenic *E. coli* serotypes isolated from children and reported that O142:H48, O86:H48, O111:H21 and O127:H21 were the most prevalent serotypes.

Rodriguez-Siek *et al*., (2005) reported that both UPEC and APEC isolates identified as O1, O2, O4, O6, O8, O11, O15, O18, O19, O21, O23, O25, O36, O75, O77, O82, O83, O86, O109, O112 and O117. More than 2% and 1% of *E. coli* isolates belonged to serotype O1 and O59 respectively. Serotypes such as O2 and O18 were represented by 0.44% of estuarine isolates. Fathollahi *et al*., (2009) studied the UPEC serotypes and reported that 66.14% were O-serogroupable and belonging to O1, O6, O15, O18 and O20 serogroups, while 33.86% were O-non typeable. About 4% of *E. coli* isolates from estuary belonged to serotype O75. Serogroup O75 strains are among the most common cause of extraintestinal infections (Devine *et al*., 1989; Nimmich *et al*., 1988; Kaijser and Jodal, 1984; Orskov and Orskov, 1975).

2.6.2.1 Spatial distribution of *E. coli* serotypes in Cochin estuary

Spatial variation in the distribution of *E. coli* serotypes revealed, presence of more pathogenic *E. coli* serotypes at stations 2 and 3, which are close to Cochin City. Twenty four diverse serotypes were obtained from Bolgatty and 16 serotypes from Off marine Science Jetty. The population explosion and industrialization in and around Cochin contributed to the outflow of enormous amount of sewage effluents in to the estuary and which might have given a selection pressure for diversity of serotypes and mutation in genes within *rfb* gene cluster. The pollution in the Cochin estuary, which is considered as the nursery ground for many species of marine and estuarine fin fishes and shell fishes, thus posing serious threat to public health.

2.6.2.2 Temporal distribution of *E. coli* serotypes in Cochin estuary

The prevalence of *E. coli* serotypes showed a variation during different seasons. It was found that all the stations showed high prevalence either in pre-monsoon or post-monsoon period, but showed comparatively low diversity in monsoon season. Station 2, Bolgatty yielded more diverse serotypes in pre-monsoon period, whereas station 3 (Off marine Science Jetty) showed more serotypes in post-monsoon period. The relative frequency of bacteria from human sources increased after cumulative rainfall and extreme run-off events, which might cause competition within different classes of bacteria.

2.6.3 Diversity of *E. coli* serotypes in clinical sources

Serotypes such as O172, O2, and O82 were recovered from *E. coli* of clinical origin. Vaishnavi *et al*., (2010) reported that among 100 clinical

isolates from India, only 25 were typable and they belonged to 14 different O-serogroups comprising O153, O102, O25, O130, O169, O1, O8, O15, O37, O86, O101, O127, O143, and O160.

2.6.4 Diversity of *E. coli* serotypes in seafood sources

In our study, we isolated serotypes such as O106, O119, O13, O132, O14, O159, O16, O23, O3, O41, O56, O60, O69, O7, O82, and O88 from seafood sources. Cortes *et al*., (2005) analysed the serotypes of VTEC strains from healthy goats and found that those belonged to 25 different serotypes. They also reported that the most frequent serotypes among VTEC from goats were O5:H, O76:H19, O126:H8, O146:H21. Parma *et al*., (2000) showed that VTEC strains were distributed among 31 serotypes, some of which (O20:H19, O91:H21, O113:H21, O116:H21, O117:H7, O171:H2, OX3:H21) were shared between bovine and food strains. Vu-Khac *et al*., (2007) reported that seventy seven isolates originating from diarrheic piglets were of O149, none of isolates from healthy piglets belonged to this serogroup. An O39:H- atypical EPEC strain was responsible for a foodborne diarrheal outbreak in 1991, involving 100 adults in Minnesota (Hedberg *et al*., 1997). Whereas in our study, O39 was absent in seafood isolates, while 0.44% of estuarine isolates belonged to this serotype.

A recent food-borne outbreak involving an EAHEC (enteroaggregative hemorrhagic *E. coli*) strain (*E. coli* O104:H4) originating from sprouts in Germany and France (Buchholz *et al*., 2011) highlights the importance of newly emerging diarrheagenic *E. coli* pathotypes. In addition, the *E. coli* O104:H4 strain implicated in this outbreak was an EAEC that acquired the *stx* genes (Grad *et al*., 2013; Muniesa *et al*., 2012); this illustrates how the mobile *stx* genes can be transferred to different classes of pathogenic *E. coli* making them

hyper virulent pathogens and meaning they have the potential to emerge as new shiga toxin-producing *E. coli* strains (Grad *et al*., 2013; Muniesa *et al*., 2012).

Pradel *et al*., (2000) reported the most common serotypes from healthy cattle in France as OX3:H2, O113:H21, O113:H4, OX3:H21, O6:H10, OX178:H19, O171:H2, O46:H38, O172:H21, O22:H16, O91: H10, and O91:H21. Porcine pathogenic *E. coli* involved in postweaning diarrhea typically belong to serogroups O8, O138, O139, O141, O147, O149 and O157, of which O149 seems to be the predominant serogroup in most countries (Noamani *et al*., 2003; Frydendahl *et al*., 2002; Blanco *et al*., 1997b; Sojka, 1965). Montenegro *et al*., (1990) reported that nearly 40% of the *E. coli* strains from cattle belonged to serogroups known to be pathogenic for humans, i.e., O22, O39, O82, O91, O113, O116, O126, and O136. In a study by Djordjevic *et al*., (2004) the most prevalent serotypes isolated from sheep were O91:H (46 isolates; 42.2%), O75:H8 (15 isolates; 13.8%), O5:H (9 isolates; 8.3%), O123:H (7 isolates; 6.4%), and O128:H2 (7 isolates;6.4%). About 3% of seafood isolates in our study belonged to serotype O82.

In conclusion, we would like to highlight that the diversity of *E. coli* serotypes in the Cochin estuary has increased considerably when compared to our previous studies (Chandran *et al*., 2008) which might be due to large scale influx of organic wastes into the estuary from the satellite townships along the banks of Cochin estuary. It may also be worthwhile for the clinical laboratories of this locality to further characterise the *E. coli* serotypes isolated from diarrheal patients so as to get a clear picture of the emergence of pathogenic strains of *E. coli* in the study area.

Chapter 3

Phylogenetic analysis of *Escherichia coli* isolates from estuarine, seafood and clinical sources

3.1 Introduction

E. coli is widespread in the environment and is a component of the intestinal microbiota of most warm blooded animals, including humans (Bettelheim, 1997a). *E. coli* is also a major cause of extraintestinal infections in humans, being the major agent of urinary tract infections and one of the most common agents of bacteraemia (Russo and Johnson, 2000; Bettelheim, 1997b; Eykyn *et al*., 1990). The *E. coli* strains are usually referred to as commensal, intestinal pathogenic or extraintestinal pathogenic (Russo and Johnson, 2000). The pathogenic strains have been associated with several diseases including diarrhea, urinary tract infections and meningitis (Russo and Johnson, 2003). In developing countries, diarrheal diseases are often associated with infant and child deaths (Sobel *et al*., 2004; Murray and Lopez, 1996).

Among the *E. coli* pathotypes responsible for extraintestinal infections are UPEC (uropathogenic *E. coli*), and MNEC (meningitis-associated *E. coli*) (Kaper *et al*., 2004). *E. coli* from these pathotypes can cause hemolytic uremic syndrome, urinary tract infection, newborn meningitis, sepsis, and others (Kaper *et al*., 2004; Dobrindt *et al*., 2003). The intestinal pathogenic *E. coli* belong to the pathotypes: ETEC (enterotoxigenic *E. coli*), EPEC (enteropathogenic *E. coli*), EIEC (enteroinvasive *E. coli*), EHEC (enterohemorrhagic *E. coli*), EAEC (enteroaggregative *E. coli*) and DAEC (diffusely adherent *E. coli*). These pathotypes have been associated with cases of mild and severe diarrhea in adults and children, mostly in developing countries (Kaper *et al*., 2004).

The existence of distinct phylogroups or 'subspecies' within *E. coli* has long been acknowledged (Wirth *et al*., 2006; Desjardins *et al*., 1995; Herzer *et al*., 1990; Selander *et al*., 1987; Ochman and Selander, 1984).

3.2 Review of Literature

3.2.1 Phylogenetic analysis

Clermont *et al*., (2000) developed a multiplex PCR-based method that enables strains of *E. coli* to be assigned to a phylogroup using a dichotomous key approach based on the presence or absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2). *chuA*, a gene required for heme transport in enterohemorrhagic O157:H7 *E. coli* (Bonacorsi *et al*., 2000; Torres and Payne, 1997; Swenson *et al*., 1996; Whittam, 1996; Mills and Payne, 1995), *yjaA*, a gene initially identified in the recent complete genome sequence of *E. coli* K-12, the function of which is unknown (Blattner *et al*., 1997), and an anonymous DNA fragment designated TSPE4.C2 from

subtractive library (Bonacorsi *et al*., 2000). To date, the method has been used in over 150 population-level studies of *E. coli*. The utility of the Clermont method was validated as part of the original study. However, the validation process was based on relatively few strains, largely collected from humans or human-associated animals. This methodology has been used, with different purposes, by authors interested in the assignment of *E. coli* strains to the phylogenetic groups (Aslam *et al*., 2014; Bagheri *et al*., 2014; Biswal *et al*., 2014; Henriques *et al*., 2014; Liu *et al*., 2014; Brisse *et al*., 2012; Giufre *et al*., 2012; Contreras *et al*., 2011; Suojala *et al*., 2011; Bukh *et al*., 2009; Martinez-Medina *et al*., 2009a, b; Petersen *et al*., 2009; Unno *et al*., 2009; Clermont *et al*., 2008; Jaureguy *et al*., 2008; Johnson *et al*., 2008c; Piatti *et al*., 2008; Erjavec *et al*., 2007; Houdouin *et al*., 2007; Martinez *et al*., 2006; Moreno *et al*., 2006; Rodriguez-Siek *et al*., 2005; Skurnik *et al*., 2005; Dixit *et al*., 2004; Marynard *et al*., 2004; Sannes *et al*., 2004; Bonacorsi *et al*., 2003; Gordon and Cowling, 2003; Bingen-Bidosis *et al*., 2002; Zhang *et al*., 2002a; Duriez *et al*., 2001). Since the method appeared in the literature, only one study has commented on the frequency with which strain are correctly assigned using the Clermont phylo-grouping method (Walk *et al*., 2007).

To increase the discrimination power of *E. coli* population analyses, it has been proposed the use of subgroups A_0, A_1, B_1, B_2, B_3, D_1 and D_2, that are determined by the combination of the genetic markers (Escobar-Paramo *et al*., 2006). Some authors analyzed the distribution of the main phylogenetic groups among *E. coli* strains isolated from human and animal faeces. Recently, Doumith *et al*., (2012) validated an updated version of the previously described (Clermont *et al*., 2000) multiplex method for phylogrouping *E. coli*. The assay used new primers designed to accommodate sequence variations in

the three targeted markers, thereby increasing the sensitivity and reliability of the method and achieving better congruence with MLST data.

3.2.2 *E. coli* phylogenetic groups

E. coli strains can be separated into four main phylogenetic groups: A, B1, B2 and D (Herzer *et al*., 1990; Selander *et al*., 1987). Groups A and B1 often include commensal strains (Johnson *et al*., 2001a) and group B2, and to a lesser extent group D, usually allocate extraintestinal pathogenic strains (Johnson and Stell, 2000; Picard *et al*., 1999). Groups A and B1 are considered to be sister groups and group B2 is considered by some to represent the 'ancestral lineage' of *E. coli* (Lecointre *et al*., 1998). Genome size varies among the four phylogroups with A and B1 strains having smaller genomes than B2 or D strains (Bergthorsson and Ochman, 1998). The intestinal pathogenic strains are usually assigned to groups A, B1 and D (Pupo *et al*., 1997). Strains of the four groups differ in their phenotypic characteristics, including their ability to exploit different sugars, their antibiotic resistance profiles and their growth rate-temperature relationships (Gordon, 2004). Strains of the four phylogroups also appear to differ in their ecological niches, life-history characteristics and propensity to cause disease. Moreover, based on comparisons of the distribution of *E. coli* phylogenetic groups among humans of different sexes and ages, it has been suggested that *E. coli* genotypes are likely influenced by morphological, physiological, and dietary differences (Gordon *et al*., 2005). In addition, climate has also been proposed to influence the distribution of strains within *E. coli* phylogenetic groups (Escobar-Paramo *et al*., 2004b). Previous research by Gordon and Cowling, (2003) revealed the prevalence of phylogroup B2 strains among herbivorous

and omnivorous mammals and a prevalence of phylogroup B1 among birds and carnivorous mammals, which supports their hypothesis of geographic effects in the *E. coli* population structure among hosts.

There are several reports indicating that there is a potential relationship between *E. coli* phylogenetic groups, age, and disease. Atypical EPEC (Law, 2000) and EAEC isolates were distributed among the various groups (Escobar-Paramo *et al*., 2004a; Girardeau *et al*., 2003; Donnenberg and Whittam, 2001). The pathotypes linked to chronic and mild diarrhea (EPEC, EAEC, and diffusely adherent *E. coli*) are distributed across all the four phylogenetic groups (Escobar-Paramo *et al*., 2004a; Johnson and Russo, 2002; Johnson, 2002). The distribution (presence/absence) of a variety of genes thought to enable a strain to cause extra-intestinal disease also varies among strains of the four phylogroups (Johnson *et al*., 2001a). In addition, a variety of putative virulence factors associated with extraintestinal infections are nonrandomly distributed among strains of the 4 phylogenetic groups, with strains from groups B2 and D harbouring a greater frequency and diversity of virulence traits compared with strains of groups A and B1 (Johnson *et al*., 2001a; Bingen *et al*., 1998; Boyd and Hartl, 1998).

Gordon and Cowling, (2003) reported, after analyzing non-domesticated vertebrates in Australia, that climate, host diet and body mass can influence the distribution of *E. coli* into the phylogenetic groups A, B1 B2 and D, in mammals. Skurnik *et al*., (2005) studied the phylogenetic group distribution of intestinal *E. coli* from healthy individuals, who recently migrated from a temperate to a tropical area and suggested that the composition of the commensal intestinal flora of humans is not static but changes dynamically in response to new environmental conditions.

Bailey *et al*., (2010) re-examined published data on the distribution of phylogenetic groups among human commensal *E. coli* isolates (Moreno *et al*., 2009, 2008; Johnson *et al*., 2007; Nowrouzian *et al*., 2006; Sabate *et al*., 2006; Gordon *et al*., 2005; Nowrouzian *et al*., 2005; Machado *et al*., 2005; Escobar-Paramo *et al*., 2004b; Sannes *et al*., 2004; Watt *et al*., 2003; Mereghetti *et al*., 2002; Obata-Yasuoka *et al*., 2002; Zhang *et al*., 2002a; Duriez *et al*., 2001; Clermont *et al*., 2000). The combined data from those 1,889 strains thus reveal that groups A and B2 are both somewhat more abundant (32.0% and 29.4% of the total, respectively) than B1 or D (17.9% and 20.7% of the total, respectively) in human faeces, and a similar conclusion has recently been drawn by others (Tenaillon *et al*., 2010). While both geographic and temporal variation as well as specific features of the populations used may have influenced the outcome of the various studies, the differences could also simply be a reflection of the enormous overall diversity in the *E. coli* species pool.

3.2.2.1 Phylogenetic group B2 and D (Pathogenic groups)

E. coli group B2 strains are an excellent model with which to investigate the evolution of virulence in a facultative pathogen. On the one hand, group B2 strains are responsible for many extraintestinal infections and are therefore a major public health concern (Donnenberg, 2002; Duriez *et al*., 2001), whereas on the other hand, group B2 strains can be the strains most frequently isolated from the faeces of asymptomatic humans (Escobar-Paramo *et al*., 2006, 2004b; Zhang *et al*., 2002a). Moreover, it seems that in industrialized countries, the prevalence of B2 strains isolated in human faeces has substantially increased over the last 2 decades (Nowrouzian *et al*., 2006; Escobar-Paramo *et al*., 2004b). Other studies have demonstrated that B2 and D

strains are usually more pathogenic than A and B1 strains (Le Gall *et al*., 2007; Picard *et al*., 1999; Bingen *et al*., 1998; Boyd and Hartl, 1998). Thus, a great deal can be learnt concerning the characteristics of an unknown strain by determining its phylogroup membership. Nowrouzian *et al*., (2005) isolated *E. coli* strains from the commensal intestinal flora of 70 Swedish infants and suggested that strains from the phylogenetic group B2 have evolved to survive in the human intestine. The contamination of surface water by faecal pollution is a serious problem since it represents a risk to both animal and human health. Faecal pollution can be introduced from multiple sources. Surface runoff and field drainage water from fields containing grazing animals, slurry spreading, farmyard runoff, direct faecal inputs and others can contribute to riverine faecal coliform loads (Vinten *et al*., 2004).

Boyd and Hartl, (1998) reported that among the *E. coli* strains in the *E. coli* reference and the diarrheagenic *E. coli* collections, strains in phylogenetic group B2 carry the greatest number of virulence factors, followed by those in group D. Virulence factors carried by group B2 strains are thought to contribute to their strong colonizing capacity; a greater number of virulence genes have been detected in resident strains than in transient ones (Lim *et al*., 2007). Moreover, a mouse model of extraintestinal virulence showed that phylogenetic group B2 strains killed mice at greater frequency and possessed more virulence determinants than strains in other phylogenetic groups, suggesting a link between phylogeny and virulence genes in *E. coli* extraintestinal infection (Picard *et al*., 1999). In contrast, Johnson and Kuskowski, (2000) suggested that a group B2 ancestral strain might have simply acquired virulence genes by chance and that these genes were vertically inherited by group members during clonal expansion. However,

numerous studies published to date suggest that there is a relationship between the genomic background of phylogenetic group B2 and its association with virulence factors (Le Gall *et al*., 2007; Nowrouzian *et al*., 2005; Escobar-Paramo *et al*., 2004a; Johnson and Stell, 2000; Picard *et al*., 1999).

Groups B2 and D strains are less frequently isolated from the environment (Walk *et al*., 2007) or fish, frogs and reptiles than A or B1 strains (Gordon and Cowling, 2003). In mammals, B2 strains are more frequently isolated from hosts possessing hindgut modifications for microbial fermentation than strains of the other phylogroups (Gordon and Cowling, 2003). B2 strains have been shown to persist for longer periods in infants than other strains of *E. coli* (Nowrouzian *et al*., 2006). *E. coli* isolates belonging to phylogenetic group B2 have been shown to predominate in infants with neonatal bacterial meningitis (Johnson *et al*., 2002c) and among urinary tract and rectal isolates (Zhang *et al*., 2002a). Also, Nowrouzian *et al*., (2005) and Moreno *et al*., (2005) reported that strains belonging to phylogenetic group B2 persisted among the intestinal microflora of infants and were more likely to cause clinical symptoms. *E. coli* isolates recovered from extraintestinal body sites are more likely to be B2 or D strains than to be A or B1 strains (Gordon, 2004). The majority of strains isolated from extraintestinal body sites are members of group B2 and to a lesser extent group D (Picard *et al*., 1999; Cherifi *et al*., 1991).

Verocytotoxin-producing *E. coli*, like O157:H7, belongs to group D (Bidet *et al*., 2005) and cattle are the main reservoirs of this pathogen. The prevalence of groups B2 and D and of the *chuA* and *yjaA* genes in humans and pigs might suggest that faecal contamination by these animals can present a high risk of extra-intestinal pathogenic *E. coli*. Thus, the correct identification

of this kind of faecal contamination can also be useful to the appropriate management of environmental pollution.

3.2.2.2 Phylogenetic group A and B1 (Non-Pathogenic groups)

Among human-source *E. coli* isolates phylogroups A and B1 occasionally cause extraintestinal infection, but typically contain fewer virulence genes than either B2 or D strains and have low capacity for virulence (Picard *et al*., 1999). Escobar-paramo *et al*., (2006) observed the prevalence of group B1 in birds, A and B1 in non-human mammals, and A and B2 in humans. Numerous studies published to date reported that the majority of the shiga toxin-producing *E. coli* strains belonged to phylogenetic group B1 (Ishii *et al*., 2007; Girardeau *et al*., 2005; Escobar-Paramo *et al*., 2004a). Baldy-Chudzik *et al*., (2008) analyzed faeces from zoo animals and found a prevalence of group B1 in herbivorous animals and a prevalence of group A in carnivorous and omnivorous animals. Dixit *et al*., (2004) observed that *E. coli* strains isolated from different regions of the gut of pigs belonged to the phylogenetic groups A and B1. Phylogenetic group A was also the predominant group in pig *E. coli* isolates in some previous studies (Holzel *et al*., 2012; Wu *et al*., 2007; Dixit *et al*., 2004), whereas group B1 was the predominant in others (Lay *et al*., 2012; Tenaillon *et al*., 2010; Bibbal *et al*., 2009). Carlos *et al*., (2010) reported that omnivorous mammals presented a prevalence of phylogroup A, while the herbivorous mammals presented a prevalence of phylogroup B1.

3.2.3 Phylogenetic group distribution among *E. coli* isolates from environmental sources

Sabate *et al*., (2008) found that in both human and animal wastewater, *E. coli* A, B1 and D were prevalent, and strains from both origins showed a

similar virulence profile in each phylogroup. Walk *et al*., (2007) demonstrated that the majority of the *E. coli* strains that are able to persist in the environment belong to the B1 phylogenetic group. Hamelin *et al*., (2007) studied the phylogenetic group distribution in *E. coli* isolates from different aquatic system and reported that the percentages of *E. coli* isolates in each phylogenetic group differed according to the location. Orsi *et al*., (2007) observed a significant association between the phylogenetic groups and spring water where strains from groups B2 and D were more prevalent than strains from groups A and B1. Orsi *et al*., (2007) also reported the prevalence of groups B2 and D, in the drinking water. Orsi *et al*., (2008) studied the phylogenetic group distribution of *E. coli* strains isolated from the Sorocaba and Jaguari Rivers located in the State of Sao Paulo, Brazil and reported that *E. coli* strains from group D were found in both rivers while one strain from group B2 was isolated from the Sorocaba River. Walk *et al*., (2007) studied the phylogenetic group distribution of *E. coli* isolates from fresh water beaches and reported that the distribution of groups was similar across the sites, and the frequency of phylogroup isolation was statistically independent of beach sites.

3.2.4 Phylogenetic group distribution among *E. coli* isolates from food sources

Jakobsen *et al*., (2010) studied the phylogenetic group distribution among *E. coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs and reported that chicken dominated by A and B1 isolates. Among the animal isolates, isolates of phylogenetic groups A and B1 were mostly porcine isolates; most pet isolates belonged to phylogenetic group B2, whereas 63% of avian isolates were in group D (Marynard *et al*., 2004). Unno *et al*., (2009) also reported that phylogenetic group distribution pattern seen among *E. coli*

isolates from migrating wild geese was significantly different from that seen among isolates from domesticated chicken and duck, although the chicken and duck isolates showed similar phylogenetic distribution patterns. The results of that study also indicated that *E. coli* isolates belonging to phylogenetic group A were more frequently found in chickens, ducks, and swine, whereas those belonging to phylogenetic group B1 were predominantly found in isolates obtained from beef and dairy cattle.

Wu *et al*., (2007) observed a high prevalence of *E. coli* group A isolates from weaned pigs. Unno *et al*., (2009) reported that *E. coli* strains in phylogenetic group B2 were rarely found in humans and domesticated animals in South Korea, and that the majority of strains containing virulence genes belonged to phylogenetic group B1 and were isolated from beef cattle. They also suggested that the relationship between the presence and types of virulence genes and phylogenetic groupings may differ among geographically distinct *E. coli* populations.

3.2.5 Phylogenetic group distribution among *E. coli* isolates from clinical sources

Bingen *et al*., (1998) analyzed phylogenetic groups of the *E. coli* strains causing neonatal meningitis and reported that all strains of the phylogenetic A group were isolated from high-risk patients, while the majority of B2 group strains were isolated from normal-risk neonates and one B1 group and 2 D group strains were isolated from normal-risk neonates. Bonacorsi *et al*., (2003) analyzed the virulence of French and North American NMEC isolates and reported that the distribution of phylogenetic groups B2 and D differed significantly between France and North America, whereas the distribution of

phylogenetic groups A and B1 was similar. Few previous studies have focused on the distribution of the 4 phylogenetic groups of extraintestinal pathogenic *E. coli* (Bingen-Bidois *et al*., 2002; Johnson *et al*., 2002a; Johnson and Stell, 2000; Bingen *et al*., 1998). Bonacorsi *et al*., (2003) also confirmed the predominance of groups B2 and D and the minor contribution of groups B1/A. Johnson *et al*., (2002a) found that group B1 was the second largest group after group B2, whereas group B1 was the smallest group in a study by Bonacorsi *et al*., (2003) in ECNM collection in both France and North America.

Bukh *et al*., (2009) reported that phylogroups A and B1 were associated with sites of infection other than the urinary tract, and resistance to multiple antibiotics was most prevalent for groups A and D. Bukh *et al*., (2009) also reported that phylogenetic group B2 was predominant in *E. coli* community-acquired bacteraemia and B2 was the least resistant of the four groups. Sannes *et al*., (2004) studied blood culture isolates from a US veterans hospital and found a predominance of phylogroup B2 isolates, with phylogroup D as second. A similar distribution was reported in a French study including 161 isolates from two university hospitals (Jaureguy *et al*., 2007). A Spanish cohort study including 185 adult patients from one university hospital found a predominance of phylogroup D, followed by A, B1 and B2 (Martinez *et al*., 2006). Culham and Wood, (2000) reported that the neonatal meningitis isolates were concentrated in phylogenetic group B2. Giufre *et al*., (2012) found out human and avian isolates strongly differed in phylogenetic group assignment, in which B2 and A predominated among human and avian isolates, respectively.

Houdouin *et al*., (2007) reported that *E. coli* isolates caused acute pyelonephritis, mainly belonged to group B2 and D. In a previous work,

differences were found among phylogenetic groups with respect to the primary source of bacteremia (Jaureguy *et al*., 2007). B2 was significantly associated with urosepsis and immunocompetent hosts, whereas non-B2 isolates were associated with non-urinary tract origins and immunecompromised hosts. Jaureguy *et al*., (2008) also reported that bacteremic *E. coli* isolates mainly belonged to group B2.

Rodriguez-Siek *et al*., (2005) compared the *E. coli* isolates implicated in human urinary tract infection and avian colibacillosis and reported that the majority of avian pathogenic *E. coli* (APEC) fell into group A, whereas the majority of UPEC were found in group B2. Suojala *et al*., (2011) studied the phylogeny of *E. coli* isolates, from both persistent and non-persistent intramammary infection (IMIs), and reported that majority of isolates belonged to phylogenetic group A. Group B2 accounted for most isolates in both populations (Veterans with Bacteremia and Uninfected Control Subjects), although for a somewhat greater proportion in the bacteremia isolates. The only statistically significant difference in prevalence involved group B1, which was the least prevalent group among the bacteremia isolates (Sannes *et al*., 2004). Group B2 strains were the most common and group B1 strains were the least common in both the UTI and rectal specimen collections (Zhang *et al*., 2002a). Martinez-Medina *et al*., (2009a) reported that phylogroup B2 was more prevalent in Adherent-Invasive *E. coli* (AIEC) subtypes than in non-AIEC.

3.2.6 Distribution of virulence genes among various phylogenetic groups of *E. coli*

Girardeau *et al*., (2005) reported that phylogenetic group A exhibited a significant higher prevalence of various virulence factors analyzed (specifically,

astA, HPI, *stx*1c, and *stx*2-$_{NV206}$). Girardeau *et al*., (2005) also reported that difference in the prevalence of *eae* between phylogenetic group D and group B1 and group A was highly significant, favouring isolates in phylogenetic group D. Bingen *et al*., (1998) found that most of the isolates belonged to phylogenetic groups B2 and D and harbored *papG* allele II and the *aer* and *fyuA*/*irp-2* genes. Bonacorsi *et al*., (2003) found out, the two isolates representative of group A, one from North America and one from France, were avirulent in our animal model, which suggests that, although capsular K1, HPI, and aerobactin may be a prerequisite for extraintestinal virulence, they are not sufficient to transform a commensal into a meningitis-causing strain. Phylogenetic studies have indicated that STEC/EHEC strains fall principally into phylogenetic groups A, B1 and D (Ziebell *et al*., 2008; Bidet *et al*., 2005; Girardeau *et al*., 2005; Escobar-Paramo *et al*., 2004a; Donnenberg and Whittam, 2001). Obligatory pathogens responsible for acute and severe diarrhea (EHEC, ETEC, and enteroinvasive *E. coli*) also group within the A and B1 groups. Contreras *et al*., (2011) reported that most of the STEC strains belonged to phylogenetic group B1, followed by D, A, and B2. Contreras *et al*., (2011) also reported that among diarrheal strains, isolates belonged to group B1 group D.

Culham and Wood, (2000) reported that virulence markers linked to neonatal meningitis (including *sfa* or *foc* and *ibe-10*) were also present at the highest frequency in group B2. In contrast, Culham and Wood, (2000) also reported that the UTI-associated marker *pap* was present at the highest frequencies in non-B2 neonatal meningitis isolates and in group B2 ECOR strains. Less virulent strains belonging to phylogenetic group A were also revealed by Houdouin *et al*., (2007) to be more frequent in patients with clinically

significant urinary tract abnormalities. Sannes *et al*., (2004) observed a stepwise continuum of VF scores from group B2 (highest), to group D (intermediate), and to groups A and B1 (lowest), with bacteremia isolates accounting for the highest and rectal isolates accounting for the lowest VF scores within groups B2 and D. They also noticed that the group B2 rectal isolates had VF scores that were similar to those of the group D bacteremia isolates, whereas the group D rectal isolates had VF scores that were similar to those of the group A and group B1 bacteremia and rectal isolates. Zhang *et al*., (2002a) observed, for group B2 isolates from Michigan, a higher prevalence of VFs among urine isolates from women with acute cystitis than among faecal isolates from healthy women.

Virulence factors, such as P pili (*pff*), S fimbrial adhesin (*sfa*), adhesions of the Dr family (*drb*), aerobactin (*aer*), group II capsule (*kpsMT*), outer membrane T (*ompT*), cytotoxic necrotizing factor 1 (*cnf1*), and hemolysin (*hly*) were strongly associated with group B2 and D strains (Zhang *et al*., 2002a). Most animal isolates belonging to group B2 contained the virulence genes *sfaDE*, *papC*, and *hlyA* (Marynard *et al*., 2004). Picard *et al*., (1999) studied the link between phylogeny and virulence in *E. coli* and reported that *pap* operon was found in strains of phylogenetic groups D, B1, and B2 but was significantly more frequent in the B2 group, whereas the *sfa/foc* operon is strictly restricted to strains of the B2 phylogenetic group. Piatti *et al*., (2008) reported that within group B2, fluoroquinolone-resistant strains showed lower prevalences of *papC*, *hlyA*, and *cnf1* than their susceptible counterparts and in contrast, the incidence of *iutA* appeared higher for refractory isolates, including group B2, than for susceptible isolates. Unno *et al*., (2009) reported

that among the strains examined, virulence genes were mainly found in phylogenetic group B1 strains isolated from beef cattle.

3.2.7 Phylogenetic group distribution and antibiotic resistance

Some authors (Holzel *et al*., 2012; Walk *et al*., 2007) have described associations between phylogenetic groups and integron presence; nevertheless, these associations were not detected in other studies (Koczura *et al*., 2012; Skurnik *et al*., 2005). Boczek *et al*., (2007) studied the occurrence of UPEC in waste water and reported that sulphonamide resistant isolates were approximately evenly distributed over the four phylogenetic groups.

Sabate *et al*., (2008) reported that among group A strains, quinolone and fluoroquinolone resistance was frequent in strains from chicken wastewater and pig wastewater. In contrast, among group B1 *E. coli* isolates, quinolone and fluoroquinolone resistance was similar in strains from chicken and human wastewater. Sabate *et al*., (2008) also reported that majority of strains with ESBL (Extended spectrum beta-lactamase) production came from phylogenetic groups A and B1 and were of chicken origin. Houdouin *et al*., (2006) analysed the phylogenetic background and carriage of pathogenicity island-like domains in relation to antibiotic resistance profiles among *E. coli* urosepsis isolates and found associations between nalidixic acid susceptibility, phylogenetic group B2 and carriage of pathogenicity-island-like domains.

Bukh *et al*., (2009) reported that antibiotic resistance to one and more than three antibiotics, respectively, was most frequent in group D, followed by A, B1 and B2. Bukh *et al*., (2009) also reported that groups A and D were associated with resistance to antibiotics including ampicillin, sulphonamide,

trimethoprim, gentamicin and quinolones. Several workers reported that phylogenetic group D had the highest prevalence of antibiotic resistance (Corvec *et al*., 2007; Jaureguy *et al*., 2007; Sannes *et al*., 2004). Erjavec *et al*., (2007) found out chloramphenicol-resistant and nalidixic acid-resistant isolates were statistically associated with the A phylogenetic group, tetracycline-resistant isolates associated with phylogenetic groups A and D, streptomycin-resistant isolates associated with phylogenetic group D, whereas all susceptible strains were statistically associated with the B2 group. Giufre *et al*., (2012) observed an association with ciprofloxacin resistance and group A isolates among human isolates.

Lee *et al*., (2010) reported that the distribution of *E. coli* isolates into phylogenetic groups was not significantly different between the two populations (CTX-M-producing isolates and non-ESBL-producing isolates) of *E. coli*. Both CTX-M-producing and non-ESBL-producing populations were polyclonal and were distributed across major phylogenetic groups A, B1, B2, and D in proportions (Brisse *et al*., 2012; Lee *et al*., 2010; Jaureguy *et al*., 2008). Valat *et al*., (2012) reported that most of the ESBL-producing isolates belonged to group A, followed by D, B1, and B2. Corvec *et al*., (2007) also found an association between group A and expression of chromosomal β-lactamase. Clermont *et al*., (2008) reported that CTX-M-15 isolates belonged to phylogenetic group B2. Strains of the D2 genotype produce only CTX-M-type enzymes, and tend to have fewer virulence factors and to be more resistant to fluoroquinolones than B2 strains (Branger *et al*., 2005).

A previous study of hospital-acquired *E. coli* isolates producing various types of ESBLs from different parts of France showed that whereas the

preponderance of the SHV- and TEM-producing strains were from group B2, the greatest proportion of CTX-M producers was from group D (Branger *et al*., 2005), whereas Sallem *et al*., (2012) reported that most ESBL-producing *E. coli* isolates belonged to phylogroups B1 and A. Most ESBL-producing *E. coli* isolates belonged to the virulent phylogenetic groups B2 and D (Baudry *et al*., 2009, Carattoli *et al*., 2008; Coque *et al*., 2008b; Lavigne *et al*., 2007; Pitout *et al*., 2005; Leflon-Guibout *et al*., 2004). In contrast, Pallecchi *et al*., (2007) reported that CTX-M-producing isolates belonged to all phylogenetic groups and no significant correlation was observed between the nature of the CTX-M determinant and the phylogenetic group. Machado *et al*., (2005) reported that clinical-ESBL *E. coli* isolates mainly corresponded to group D, whereas most clinical non ESBL isolates belonged to the group B2, and most commensal non-ESBL belonged to the group A.

3.3 Objectives

To find out the phylogenetic groups of *E. coli* isolates from Cochin estuary, seafood and clinical sources

3.4 Materials and Methods

In the present study a total of 300 *E. coli* isolates of estuarine origin, 100 *E. coli* of clinical and 100 *E. coli* of seafood origin were grouped into various phylogenetic groups by triplex PCR.

3.4.1 Isolation of DNA from *E. coli*

DNA from the bacterial genome was extracted as described in section 2.4.6.1

3.4.2 Phylogenetic analysis

The phylogenetic group was determined by a triplex PCR method (Clermont *et al.*, 2000). The primers used were ChuA.1 (5′-GACGAACCAACGGTCAGGAT-3′) and ChuA.2 (5′-TGCCGCCAGTACC AAAGACA-3′), YjaA.1 (5′-TGAAGTGTCAGGAGACGCTG-3′) and YjaA.2 (5′-ATGGAGAATGCGTTCCTCAAC-3′), and TspE4C2.1 (5′-GAGTAATGTC GGGGCATTCA-3′) and TspE4C2.2 (5′-CGCGCCAACAAAGTATTACG-3′). This PCR is based on the amplification of two genes (*chuA* and *yjaA*) and one genomic fragment (TSPE4.C2). The optimized protocol was carried out with a PCR mix of 20 µl contained 1.5 mM $MgCl_2$, 2.5 µl of Taq buffer (Tris (pH 9.0) at 25 °C, KCl and Triton X-100), 2 mM each of dNTP mixture, 20 pmol each of the primers, 2.5 U of Taq polymerase (GeNei^{TM}, India) and 1 µl of the DNA template. The amplification consisted of following steps: initial denaturation at 94 °C for 5min, followed by 30 cycles of denaturation (30 sec at 94 °C), annealing (30 sec at 55 °C) and extension (30 sec at 72 °C), and a final extention step of 7 min at 72 °C. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei^{TM}, India), and visualized by Gel Documentation System (BioRad Gel Doc^{TM} EZ Imager, USA). The data of the three amplicons resulted in assignment of strains to phylogenetic groups as follows: $ChuA^+$, $yjaA^+/ChuA^+$,$YjaA^+$, $TspE4.C2^+$, group B2; $ChuA^+$, $yjaA^-/ChuA^+$, $TspE4.C2^+$, group D; $ChuA^-$, $TspE4.C2^+/YjaA^+$, $TspE4.C2^+$, group B1; $ChuA^-$, $TspE4.C2^-/ ChuA^-$, $YjaA^+$, $TspE4.C2^-$, group A (Figure 3.1).

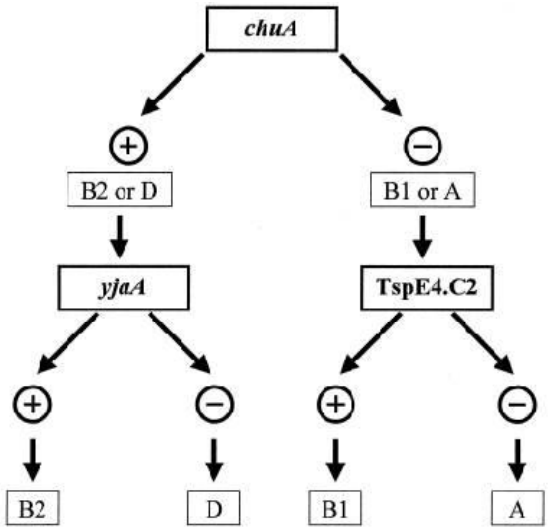


Figure 3.1 Dichotomous decision tree proposed by Clermont *et al*., (2000)

3.4.3 Statistical analysis

Statistical analysis of the results of this study was carried out with SPSS (Statistical Package for the Social Science). One-Way analysis of Variance (ANOVA, Games-Howell) was applied to test difference in phylogenetic group distribution among different stations and different sources. Significance level was set at $\alpha = 0.05$.

3.5 Results

3.5.1 Phylogenetic group distribution of *E. coli* isolates from Cochin estuary

A total of 300 *E. coli* strains were isolated from Cochin estuary. The combination of PCR products obtained (279, 211 and 152 bp) allowed the allocation of the *E. coli* isolates to one of the four major phylogenetic groups such as A, B1, B2 and D (Figure 3.2). Triplex PCR analysis of *E. coli* isolates

(n = 300) revealed 35.66% of the isolates belonged to phylogenetic group A, followed by group B2 (25.66%), B1 (23%), and D (15.66%) (Figure 3.3).

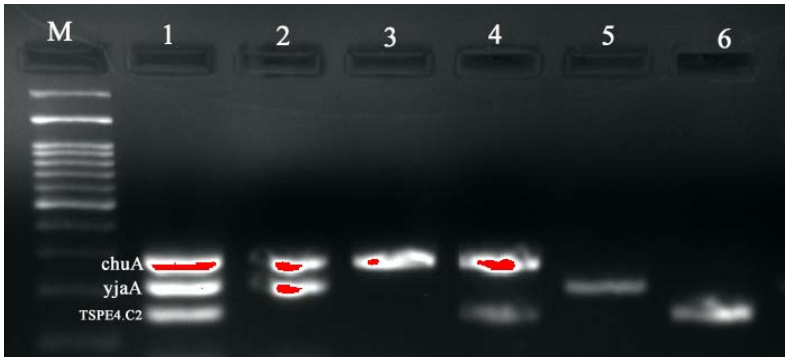


Figure 3.2 Gel image of triplex PCR end products showing detection of *E. coli* phylogenetic groups

Lane M, 100 bp Marker; lanes 1 and 2, group B2; lane 3 and 4, group D; lane 5, group A; lane 6, group B1

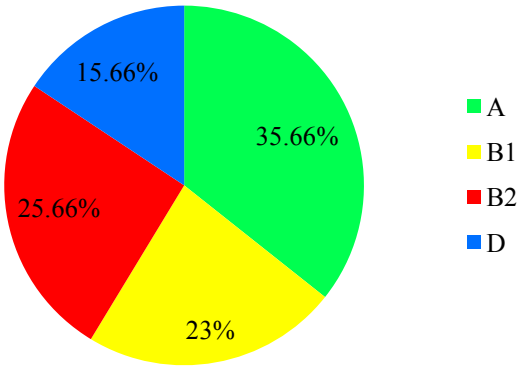


Figure 3.3 Overall distribution of various phylogenetic groups among *E. coli* isolates (n = 300) from Cochin estuary

3.5.1.1 Phylogenetic group distribution of *E. coli* isolates from different stations set at Cochin estuary

Figure 3.4 showed the percentage of phylogenetic group distribution among *E. coli* strains from different stations along Cochin estuary. Out of the 47

isolates from station 1 (Chitoor), 19 (40.43%) were found to belong to group B1, 17 (36.17%) to group A, 8 (17.02%) to group D and 3 (6.38%) to group B2.

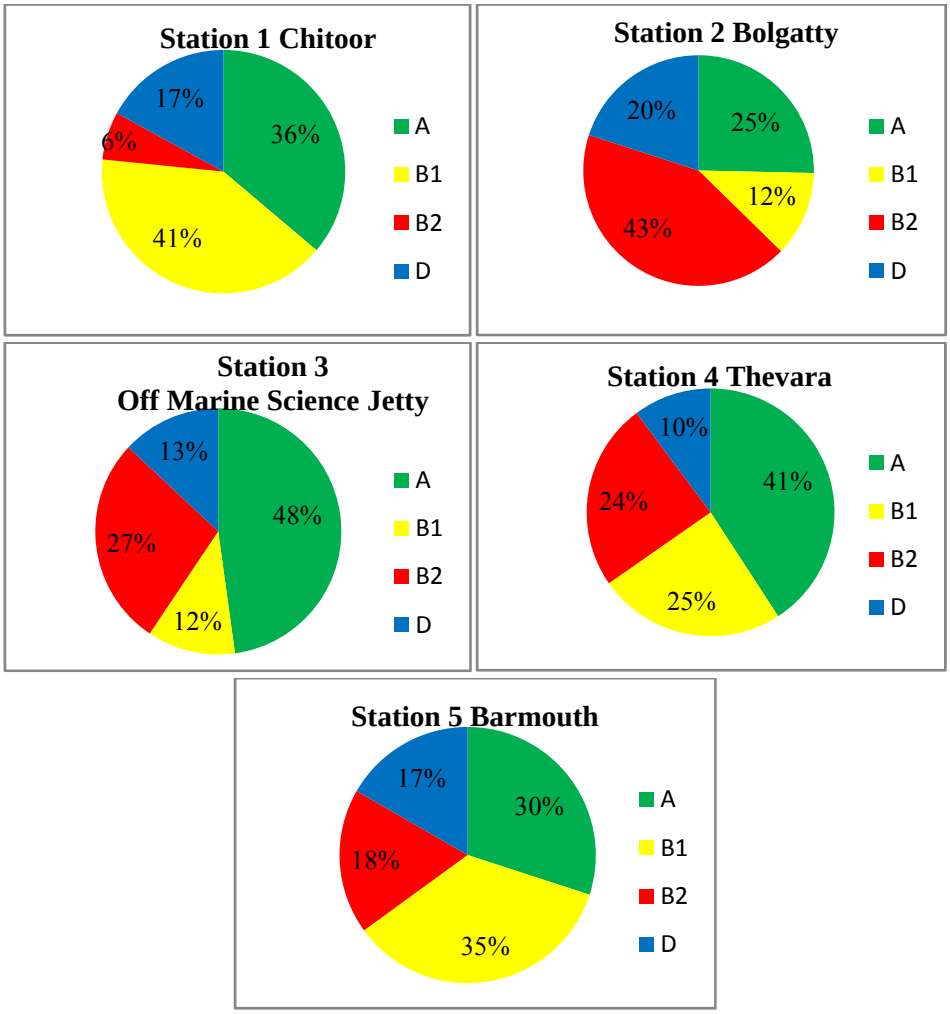


Figure 3.4 Phylogenetic distribution among *E. coli* isolates from different stations in Cochin estuary

Phylogenetic analysis revealed that group B2 *E. coli* isolates were predominant in station 2 (Bolgatty), with a prevalence of 42.66% followed by group A (25.33%), group D (20%) and group B1 (12%). In station 3 (Off

Marine Science jetty), 47.83% of isolates belonged to phylogenetic group A, followed by group B2 (27.54%), D (13.04%) and B1 (11.59%). Of the 49 strains isolated from station 4 (Thevara), majority of the *E. coli* isolates were belonged to phylogenetic group A (20 strains, 40.81%), followed by group B2, B1 (12 strains each, 24.48%) and D (5 strains, 10.2%). In station 5 (Barmouth), 35% of isolates belonged to phylogenetic group B1, followed by group A (30%), B2 (18.33%) and D (16.66%).

3.5.1.2 Relative distribution of various phylogenetic groups of *E. coli* isolates among different stations set at Cochin estuary

There was a significant difference in the distribution of different phylogenetic groups among different stations (ANOVA, p = 0.049). Gams-Howell analysis showed that the distribution of phylogenetic group A was significantly higher than phylogenetic group D among different stations (p = 0.015).

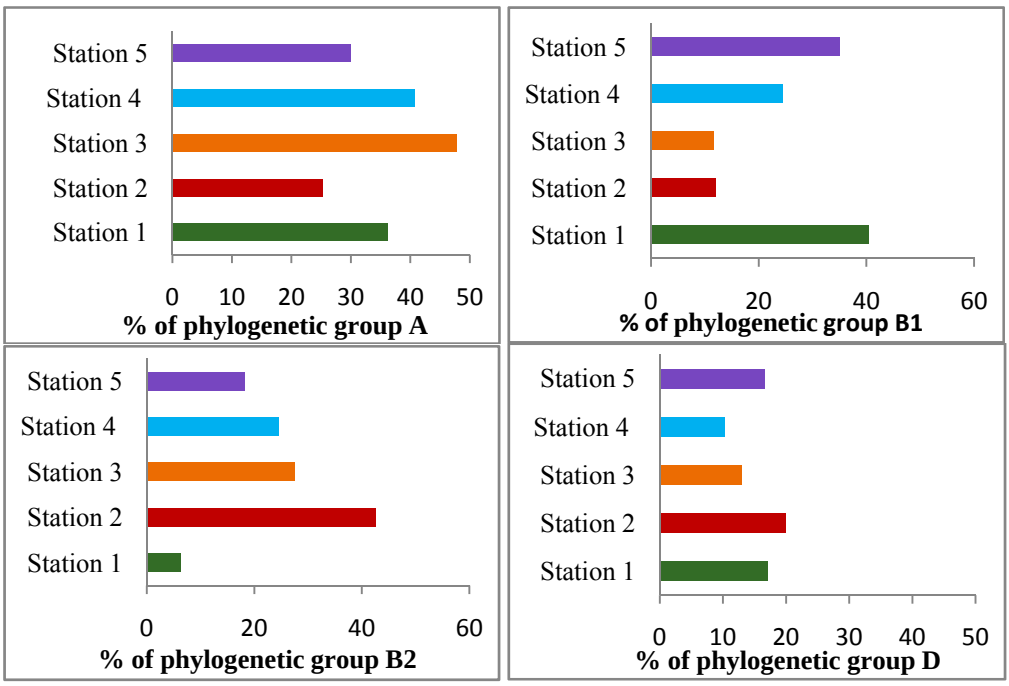


Figure 3.5 Percentage of each phylogenetic group (A, B1, B2, D) distribution in different stations at Cochin estuary

E. coli isolates belonged to phylogenetic group A were mainly isolated from station 3 (Off Marine Science Jetty), followed by station 4 (Thevara), station 1 (Chittor), 5 (Barmouth), and 2 (Bolgatty). *E. coli* isolates belonged to phylogenetic group B1 were mainly isolated from station 1 (Chittor), followed by station 5 (Barmouth), and station 4 (Thevara). Station 2 and 3 showed similar distribution for phylogenetic group B1 isolates. Station 2 (Bolgatty) yielded more phylogenetic group B2 isolates compared to other stations. While station 3 (Off Marine Science Jetty), 4 (Thevara), and 5 (Barmouth) showed a moderate distribution of group B2 isolates. Group B2 isolates were less frequently obtained from station 1 (Chitoor). Between 20% - 10% of phylogenetic group D were isolated from different stations. Station 2 (Bolgatty) yielded more group D isolates than station 4 (Thevara), 1 (Chitoor) and 3 (Off Marine Science Jetty). Group D isolates were less frequently obtained from Station 4 (Thevara) (Figure 3.5).

3.5.2 Phylogenetic group distribution among *E. coli* isolates from seafood sources

A total of one hundred *E. coli* isolates from seafood samples were subjected to phylogenetic analysis. Triplex PCR analysis of *E. coli* isolates of seafood origin revealed that 52% of the isolates belonged to phylogenetic group A, followed by group B1 (23%), B2 (16%), and D (9%) (Figure 3.6).

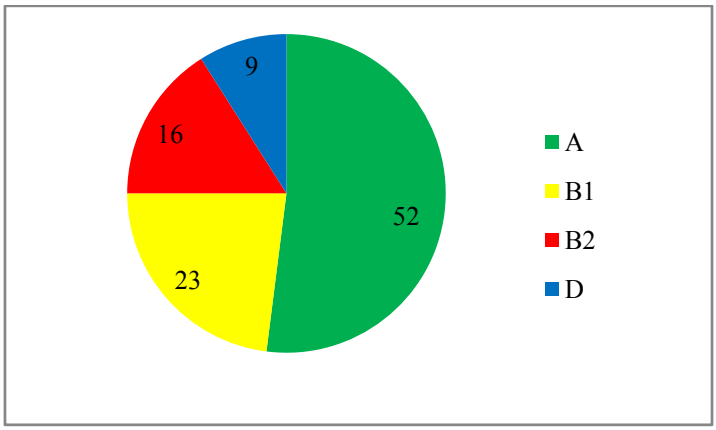


Figure 3.6 Distribution of various phylogenetic groups among *E. coli* isolates from seafood sources

3.5.3 Phylogenetic group distribution among *E. coli* isolates from clinical sources

A total of one hundred *E. coli* isolates from clinical samples were subjected to phylogenetic analysis. Figure 8 shows the triplex PCR results of *E. coli* of clinical origin. Phylogenetic analysis of *E. coli* isolates of clinical origin revealed that 80% of the isolates belonged to phylogenetic group B2, followed by group A (12%), D (6%), and B1 (2%) (Figure 3.7).

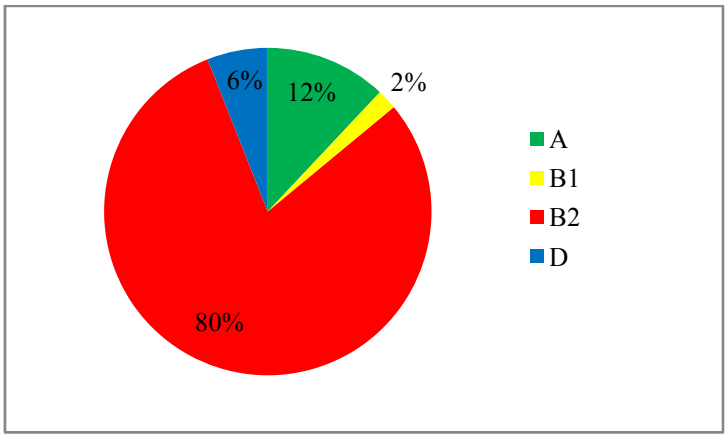


Figure 3.7 Distribution of various phylogenetic groups among *E. coli* isolates from clinical sources

3.5.4 Relative distribution of phylogenetic groups among *E. coli* isolates from three different sources

Figure 3.8 shows relative distribution of different phylogenetic groups of *E. coli* such as group A, B1, B2, and D, from different sources. *E. coli* phylogenetic group A isolates were frquently encountered in seafood sources when compared to esturine and clinical sources. Phylogenetic group B1 was less frequently isolated from clinical origin, whereas seafood and estuarine sources showed equal prevalence for group B1 isolates. *E. coli* belonging to phylogenetic group B2 isolates was prevalent in clinical sources. Phylogenetic group D showed moderate prevalence in different sources. Group D isolates of *E. coli* were relatively frequent in estuarine sources, followed by seafood and clinical sources. There was no significant difference in the distribution of different phylogenetic groups among different sources (ANOVA, $p > 0.05$).

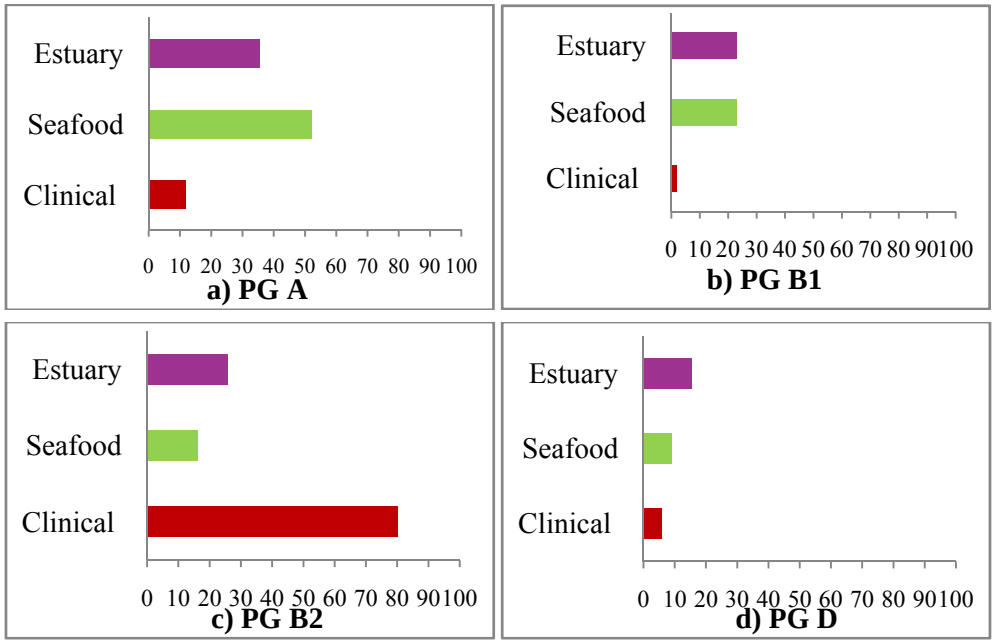


Figure 3.8 a-d Relative distribution of each phylogenetic group (A, B1, B2, and D) among *E. coli* isolates from different sources

3.6 Discussion

3.6.1 Distribution of various phylogenetic groups of *E. coli* isolates from Cochin estuary

E. coli causes a wide variety of diseases, including diarrhea, hemolytic-uremic syndrome and hemorrhagic colitis. The presence of *E. coli* in water is an indication of faecal contamination and represents a risk of disease (Leclerc *et al*., 2001).

When the *E. coli* strains isolated from all the stations were considered, A and B2 group strains were the most common (35.66% and 25.66% respectively), followed by B1 and D strains (23% and 15.66% respectively). Distribution of phylogenetic group A *E. coli* isolates in our study showed a good agreement with results of those who studied the distribution in well water (Orsi *et al*., 2007). In contrast to our study, higher prevalence of group A was reported in two rivers in Brazil (Orsi *et al*., 2008); polluted urban water (Higgins *et al*., 2007) and in mineral water (Orsi *et al*., 2007). While Higgins *et al*., (2007) and Hamelin *et al*., (2007) reported a low frequency of group A isolates in fresh water bodies compared to our results. Walk *et al*., (2007) also reported a low prevalence of group A isolates from fresh water beaches, which was also lower than our results.

Fifteen percentage of the *E. coli* isolates from the Cochin estuary belong to phylogenetic group D. Similar levels of occurrence of phylogenetic groups was reported from different environments (Brisse *et al*., 2012; Orsi *et al*., 2008; Higgins *et al*., 2007; Orsi *et al*., 2007; Walk *et al*., 2007; Bonacorsi *et al*., 2003). Distribution of group B1 of estuarine *E. coli* isolates for our study showed a good

agreement with those for other studies in spring and mineral water (Orsi *et al*., 2007), river (Orsi *et al*., 2008), and fresh water bodies (Higgins *et al*., 2007).

Of the five stations set at Cochin estuary, two of them (station 2 and 3) were much close to Cochin estuary. It was interesting to note that highest percentages of *E. coli* isolates (32 strains, 42.66%) belonged to group B2 were isolated from the station 2 (Bolgatty). The results suggest the possible release of these organisms through hospital waste from many of the hospitals in and around Cochin City. Hospital wastewater is often contaminated by antibiotic agents, which even in sub inhibitory concentrations may promote selection and survival of resistant strains (Kim *et al*., 2007a).

The percentage of B2 strains isolated from the Cochin estuary (23%) was higher than encountered in the previous studies conducted in water samples (Orsi *et al*., 2008; Hamelin *et al*., 2007; Higgins *et al*., 2007; Orsi *et al*., 2007; Walk *et al*., 2007). The large number of strains belonging to group B2 pose a significant risk to people uses this natural water body for fishing and recreation. Concerning the phylogenetic distribution of B2 group among different stations, stations 3, 4 and 5 showed moderate frequency, with percentages of 27.54%, 24.48%, and 18.33%, respectively. Frequency of B2 isolates in station 1 (Chitoor) was lower (6.38%). *E. coli* strains belonging to group B2 are highly pathogenic and frequently responsible for extraintestinal infections in humans (Duriez *et al*., 2001; Lecointre *et al*., 1998). Therefore, the presence of these strains, especially in the water samples, deserves attention. The strains from group B2 are usually responsible for extraintestinal infections and exhibit several virulence factors such as adhesins and toxins (Johnson and Stell, 2000; Picard *et al*., 1999). These strains can cause

meningitis, intra-abdominal infections and pneumonia (Russo and Johnson, 2003). Sabate *et al*., (2008) reported that the *E. coli* phylogenetic group B2 is frequently found among human sewage samples, probably because this pathogroup is better adapted to human than animal intestine.

The distribution of phylogenetic groups of *E. coli* varied among five stations. *E. coli* belonged to group D, were moderately distributed among stations 1, 2 and 3, with percentages of 17.02%, 20%, and 13.04%, respectively. Similarly, frequency of D isolates at station 4 and 5 were lower, with percentages of 16.66% and 10.2%, respectively. The phylogenetic group D includes pathogenic strains such as O157:H7, which is highly virulent and can cause diarrhea, hemolytic uremic syndrome and hemorrhagic colitis (Parry and Palmer, 2000). Though we can't say with full confidence, the most probable source of phylogenetic group D isolates of *E. coli* may be chicken carcases from retail outlets of chicken and the effluents from nearby markets which often drained into the nearby water bodies.

It is interesting to note that highest percentages of *E. coli* isolates belonged to group A were isolated from the station 3 (Off Marine Science Jetty) and 4 (Thevara), whereas in stations 1, 2 and 5 moderate percentages of isolates were detected. The phylogenetic groups A and B1 usually include commensal *E. coli* strains (Duriez *et al*., 2001). Pathogenic intestinal *E. coli* strains have been found in groups A, B1 and D (Russo and Johnson, 2000; Picard *et al*., 1999).

Between 35-40% of *E. coli* isolated from stations 1 (Chitoor) and 5 (Barmouth) were belonged to phylogenetic group B1. The lowest percentages of B1 group were detected in stations 2 and 3, with percentages of 12% and

11.9%, respectively. Carlos *et al*., (2010) reported that *E. coli* strains from phylogenetic group B1 were present in all the hosts analyzed (human, chicken, cow, goat, pig and sheep) but were more prevalent in cow, goat and sheep samples. In a study by Walk *et al*., (2007), it was shown that *E. coli* isolated from fresh water beaches were predominated by phylogenetic group B1 (56%), followed by group A (23%), group D (15%) and B2 (6%). Girardeau *et al*., (2005) analysed the phylogenetic background of shiga toxin-producing *E. coli* isolates and reported that about 70% of strains belonged to group B1. In a study by Contreras *et al*., (2011), it was shown that shiga toxin-producing *E. coli* strains belonged to phylogenetic group B1 (52 %), D (28 %), A (17 %) and B2 (3 %).

Similar to our observations, spatial variation of different phylogenetic groups of *E. coli* were reported elsewhere. Hamelin *et al*., (2007) used a custom microarray to genotype *E. coli* isolated from the Detroit and St. Clair rivers; distributions of the genotypes varied among the six sites sampled. While the urban site 4 had all genotypes represented, for site 3, associated with agriculture, wildlife, and smaller municipalities, B1 and D predominated. For site 6, which was chosen because of its proximity to a sea gull colony, genotypes A and B1 predominated. Higgins *et al*., (2007) studied the phylogenetic distribution of *E. coli* isolated from fresh stream, polluted urban stream, and fresh water bodies; distributions of the genotype varied among the three sites sampled. For fresh water stream, group B1 and D were predominated; group A and B2 were equally distributed. For polluted urban stream, group A and B1 were dominated; B2 and D were equally distributed. For fresh water bodies, group D and B2 were dominated. Orsi *et al*., (2007) studied the phylogenetic group distribution of *E. coli* isolated from different sources such as well water,

spring water, mineral water and distribution system and reported high prevalence of group A isolates in mineral water, group B1 in well water, B2 in distribution system, and group D in spring water.

3.6.2 Distribution of various phylogenetic groups of *E. coli* isolates from seafood sources

When *E. coli* isolated from seafood sources were analysed by triplex PCR, A group was the most common (52%), followed by B1 (23%), B2 (16%), and D (9%). This shows clear deviation from the patterns of distribution of various phylogenetic groups of *E. coli* in estuarine water. Marchant *et al*., (2013) reported a similar prevalence for group B1 and D isolates.

Distribution of group A of seafood *E. coli* isolates for our study showed a good agreement with porcine isolates (Marynard *et al*., 2004) and chicken isolates (Unno *et al*., 2009). Distribution of group B1 of seafood *E. coli* isolates for our study showed a good agreement with duck isolates (Unno *et al*., 2009). *E. coli* from estuary and seafood sources were dominated by group A isolates. While *E. coli* from estuary and seafood sources showed moderate distribution for group B1, B2 and D, group B2 isolates were relatively more prevalent in estuarine water when compared to seafood.

Giufre *et al*., (2012) studied the phylogenetic distribution of *E. coli* in chicken and reported that group A (34%) and B1 (29%) were dominated, followed by group D (27%) and B2 (8.9%). These findings are much similar to the patterns we encountered in seafood. Specific reports on distribution of phylogenetic groups of *E. coli* in seafood are not available for comparison.

Unno *et al*., (2009) studied the phylogenetic group distribution in domesticated animals (chicken, duck, beef cattle, dairy cattle, and swine); distributions of the genotypes varied among the animals. In chickens *E. coli* were localized to phylogenetic group A (55%), followed by strains in groups B1 (31.7%) and D (13.3%). A similar pattern of distribution was also found among isolates from domesticated ducks; where about 63%, 24%, and 13% of strains were in phylogenetic groups A, B1, and D, respectively. In contrast, *E. coli* isolates from beef cattle had the greatest percentage of group B1 strains (79.2%) among all sources and fewer isolates belonging to groups A (15.1%) and D (5.7%). A similar trend was observed among *E. coli* isolates from dairy cattle, where 62% of the isolates belonged to group B1 and a smaller percentage belonged to groups A (32.0%) and D (5.7%).

Swine isolates showed a unique phylogenetic group distribution, with an extremely low percentage of group D (0.7%) strains, a relatively high percentage of group A (64.7%) strains, and a moderate percentage of group B1 (34.5%) strains. Unno *et al*., (2009) also studied the phylogenetic group distribution of isolates from migrating wild geese and reported that the majority of isolates (60.4%) were in phylogenetic group B1, and 16.7%, 14.6% and 8.3% of the remaining isolates were in phylogenetic groups B2, A, and D, respectively. In a study by Hatha *et al*., (2013) on diarrheagenic *E. coli* in migratory bird (*Branta leucopis*) reported that 31.7% of the isolates belonged to group B2, followed by B1, A and D.

3.6.3 Distribution of various phylogenetic groups of *E. coli* isolates from clinical sources

Phylogenetic analysis of *E. coli* isolates of clinical origin revealed that 80% of the isolates belonged to phylogenetic group B2, followed by group A (12%), D (6%), and B1 (2%). *E. coli* from clinical sources were mainly dominated by group B2 strains. Between 12-2% of isolates were belonged to group A, D and B1. *E. coli* isolated from seafood showed high prevalence for phylogenetic group A than from esturine and clinical sources. Phylogenetic group B1 was less frequently isolated from *E. coli* of clinical origin, whereas *E. coli* of seafood and estuarine origin showed equal prevalence for group B1. *E. coli* of clinical origin showed high prevalence for phylogenetic group B2 isolates than from estuary and seafood.

Distribution of group B2 of clinical *E. coli* isolates for our study showed a good agreement with those for other studies in neonatal meningitis (Johnson *et al*., 2008c; Bonacorsi *et al*., 2003), UPEC (Moreno *et al*., 2006), and inflammatory bowel disease (Petersen *et al*., 2009). Many other studies conducted in clinical samples, showed lower prevalence for B2 group than our study (Giufre *et al*., 2012; Martinez-Medina *et al*., 2009a; Jaureguy *et al*., 2008; Piatti *et al*., 2008; Houdouin *et al*., 2007; Rodriguez-Siek *et al*., 2005; Sannes *et al*., 2004; Zhang *et al*., 2002a). Group B1 isolates were less frequently distributed in our clinical isolates, which was consistent with many studies (Giufre *et al*., 2012; Jaureguy *et al*., 2008; Johnson *et al*., 2008c; Houdouin *et al*., 2007; Moreno *et al*., 2006; Rodriguez-Siek *et al*., 2005; Bonacorsi *et al*., 2003; Zhang *et al*., 2002a). Distribution of group A of clinical *E. coli* isolates for our study showed a good agreement with those for other studies in clinical isolates (Giufre *et al*., 2012; Johnson *et al*., 2008c; Houdouin *et al*., 2007; Moreno *et al*., 2006; Rodriguez-Siek *et al*., 2005; Sannes *et al*., 2004; Bonacorsi *et al*., 2003; Zhang *et al*., 2002a). In contrast to

our study, prevalence of group D was higher in many studies than our results (Giufre *et al*., 2012; Suojala *et al*., 2011; Jaureguy *et al*., 2008; Johnson *et al*., 2008c; Houdouin *et al*., 2007; Moreno *et al*., 2006; Rodriguez-Siek *et al*., 2005; Sannes *et al*., 2004; Bonacorsi *et al*., 2003; Zhang *et al*., 2002a).

Bingen *et al*., (1997) reported that *E. coli* isolated from neonatal meningitis patients were dominated by group B2 and D. Bonacorsi *et al*., (2003) and Bingen-Bidois *et al*., (2002) reported that *E. coli* urosepsis isolates were dominated by group B2 and D. Brisse *et al*., (2012) classified clinical *E. coli* into groups A, B1, B2, and D on the basis of gene sequences were 23%, 14%, 48%, and 15%, respectively. Bukh *et al*., (2009) reported that B2 was the most prevalent group for all sites of infection, ranging from 69.9% in cases with a urinary tract site of infection to 54.8% in cases with a hepatobiliary tract site of infection.

Sannes *et al*., (2004) studied 63 blood culture isolates from a US veteran's hospital and found a predominance of phylogenetic group B2 isolates, with phylogenetic group D as second (67% and 19%, respectively). A similar distribution was reported in a French study including 161 isolates from two university hospitals (Jaureguy *et al*., 2007). A Spanish study including 185 adult patients from one university hospital found that phylogenetic group D accounted for 52% of the incidences, whereas groups A, B1 and B2 accounted for 12%–18% each (Martinez *et al*., 2006). Bukh *et al*. (2009) studied 1553 *E. coli* isolates from community-acquired bacteraemia and found a predominance of B2 isolates (65.9%), followed by D (16.6%), A (13.1%) and B1 (4.4%). Giufre *et al*., (2012) studied the phylogenetic distribution of *E. coli* in urinary tract infections and sepsis, and reported that in UTIs group B2 and D were dominated whereas in sepsis B2 and A group dominated. Unno *et al*., (2009)

reported that *E. coli* strains from healthy humans were nearly equally represented in each phylogenetic group, with 29%, 34%, and 36% of the strains in phylogenetic groups A, B1, and D, respectively. They also reported that there was a slightly greater number of isolates in phylogenetic group D (42.9%) from human patients than from the other phylogenetic groups (A, 23.8%; B1, 33.3%).

Houdouin *et al*., (2007) studied the phylogenetic groups among 93 *E. coli* isolates causing acute pyelonephritis, and reported that group B2 (66%) and D (25%) were dominated. In a study by Jaureguy *et al*., (2008), it was shown that human bacteremic *E. coli* strains were also dominated by B2 (50%) and D (23%), followed by A (20%) and B1 (7%). Petersen *et al*., (2009) found a strong correlation between *E. coli* of phylogenetic group B2 and inflammatory bowel disease; no correlation was found with other phylogenetic groups including group D. Further, they found a trend toward an association between the presence of B2 *E. coli* and active colitis.

Rodriguez-Siek *et al*., (2005) studied phylogenetic group distribution in *E. coli* isolates from human urinary tract infection and avian colibacillosis; distribution of the genotype varied among the two sources. For avian pathogenetic *E. coli* (APEC), group A (38%) and D (28.1%) were dominated, whereas in UTIs group B2 (65%) and D (18.5%) were dominated. In a study by Zhang *et al*., (2002a) found that group B2 (47.7%) and A (20.5%) were dominated in rectal *E. coli* strains; group B2 (58.6%) and D (19.9%) were dominated in uropathogenic *E. coli*. Zhang *et al*., (2002a) also reported that group B2 strains were the most common and group B1 strains were the least common in both the UTI and rectal specimen collections.

Pitout *et al*., (2005) found that phylogenetic group D predominated (63%) overall among ESBL-producing *E. coli* isolates from the Calgary Health Region and group B2 (21%), A (13%) and B1 (4%) were less frequently distributed. In a study by Moreno *et al*., (2006), it was shown that quinolone-susceptible isolates were statistically associated with the phylogenetic group B2 (81% versus 32%) of resistant isolates whereas quinolone-resistant isolates were significantly associated with group A (36% versus 3% of susceptible) and exhibited similar shifts towards groups B1 and D. Sannes *et al*., (2004) studied phylogenetic background of rectal and bacteremia isolates and reported that in rectal isolates, group B2 (54%) and B1 (18%) were dominated, whereas in bacteremia isolates group B2 (67%) and D (19%) were predominated. Piatti *et al*., (2008) reported a high prevalence of B2 (56%) and moderate prevalence for group A (19%) and D (17%) isolates in uropathogenetic isolates.

Duriez *et al*., (2001) examined phylogenetic groupings of commensal *E. coli* isolates from three geographic areas (Mali, France, and Croatia). They reported that commensal isolates are dominated by strains of A and B1 groups, with relatively fewer B2 strains. In an examination of commensal *E. coli* isolates in Mali, France, and Croatia using the same PCR based phylogenetic grouping method, the frequencies of B2 strains were found to be 2%, 10.5%, and 19%, respectively (Duriez *et al*., 2001). Marynard *et al*., (2004) reported that majority of the human isolates belonged to a virulent group by 45% each (group B2 or D). They also reported that A (7%) and B1 (3%) were less frequently distributed. Martinez-Medina *et al*., (2009a) reported that *E. coli* from patients with crohn's disease, dominated by phylogenetic group B2 (42.1%) and D (31.6%), whereas in healthy individuals shown an opposite distribution of group D (35%) followed by B2 (30%). Martinez-Medina *et al*.,

(2009a) also reported that group B1 and A strains was the least common in both the crohn's disease and healthy *E. coli* collections. Skurnik *et al*., (2005) reported the high prevalence of A (74%) and B1 (41%) strains than virulent groups B2 and D (27-39%) in healthy individuals.

We conclude that the recreational waters in Cochin estuary are contaminated with diverse phylogenetic groups of *E. coli*. Furthermore, high prevalence of different phylogenetic groups of *E. coli* in our study is a matter of concern, since the strains from B2 and D groups are usually pathogenic. Pathogen cycling through food is very common and fish and shellfish that harbour these strains might pose potential health risk to consumer. Cochin estuary supports good shellfish and finfish fishery which is exploited by local fishermen for livelihood. The present study contributes to understanding the distribution of different phylogenetic groups of *E. coli* from Cochin estuary. The prevalence of groups B2 and D and of the *chuA* and *yjaA* genes in water might suggest that faecal contamination by animals and human can present a high risk of extra-intestinal pathogenic *E. coli*. Thus, the correct identification of this kind of faecal contamination can also be useful to the appropriate management of environmental pollution. The large number of strains from group A and to a lesser extent from group B1 observed in the estuary is also a matter of concern since according to Escobar-Paramo *et al*. (2004b), *E. coli* from groups A and B1 can emerge as intestinal pathogenic strains. Taken all together, our data emphasize that the contamination of surface water by faecal pollution is always a potential threat to animal and human health.

Chapter 4

Antibiotic resistance of *Escherichia coli* isolates from estuarine, seafood and clinical sources

4.1 Introduction

E. coli, a member of the *Enterobacteriaceae* family, is a common inhabitant of the human and animal gut. It is the most common cause of Gram-negative nosocomial and community-acquired infections. Resistance to at least two classes of antibiotic agents in *E. coli* is nowadays an ordinary finding in human and veterinary medicine and has an increasing impact on available therapeutic options. The clinical use of antibiotics, and therefore the effective treatment of bacterial infections, is under considerable threat due to the emergence of bacteria that are resistant to many classes of commonly used antibiotics. Antibiotic agents can be found in sewage effluents, particularly in places where these drugs are extensively used, such as hospitals, pharmaceutical production plants and around farms where animal feed contains these agents.

Antibiotics present in these sources, found to play a significant role in the natural selection and survival of resistant strains. The wild dissemination of antibiotic resistance among bacterial populations is an increasing problem worldwide. Multidrug-resistant bacterial strains are prevalent in human and animal isolates all over the world. Estuarine environment offers conducive environment for horizontal gene transfer as strains from various sources reach here through land run-off, sewage inputs (partially treated and untreated) and waste discharge from animal slaughtering houses/ farms.

4.2 Review of Literature

4.2.1 Antibiotic resistance

Since the first use of antibiotics in the 1940s, bacterial resistance progressively has become a common place amongst important human and animal pathogens. Antibiotic resistant microorganisms can acquire unexpected genetic background, consequently expressing new physiologic and molecular characteristics that could interfere with the organization of infectious diseases (Linares-Rodriguez *et al*., 2005; Diniz *et al*., 2004). The escalating problem of emergence of antibiotic resistant bacteria is becoming a major global health issue (Levy, 2002; Chee-Sanford *et al*., 2001).

Antibiotic resistance varies by country, and continent. Globally, resistance to commonly used oral and parenteral antibiotics including aminoglycosides, third-generation cephalosporins, carbapenems, and β-lactam/ β-lactamase inhibitor combinations is increasing (Meier *et al*., 2011; Perez *et al*., 2007). To regulate antibiotic usage, some monitoring programs for antibiotic resistance have been established in Europe, North America and Latin America (Boerlin *et al*., 2005). However, major aquaculture production

is from Asian countries, where there are limited or nonexistent regulatory guidelines for antibiotic usage (Howgate, 1998).

After use in human beings, antibiotics and many metabolites will be emitted to the sewerage system (Daughton and Ternes, 1999) and, depending on their polarity, water solubility, and persistence, the compounds might be degraded, associate with sewage sludge, or released to rivers. Sludge-associated drugs will enter agricultural systems when the sludge is used as a fertiliser (Kinney *et al*., 2006). Antibiotics for use in human beings can also reach agricultural soils directly through irrigation with wastewaters and surface waters (Kinney *et al*., 2006). Veterinary pharmaceuticals and their metabolites can be released into the environment either directly, from use in aquaculture and the treatment of animals on pasture, or indirectly during the application of manure and slurry from intensive livestock facilities to land (Boxall *et al*., 2004). Compounds that are released to the soil system can subsequently be transported to surface water or groundwater (Topp *et al*., 2008; Blackwell *et al*., 2007) and be cycled and re-cycled within the environment.

Antibiotic-producing bacteria occur naturally throughout the environment, (Anukool *et al*., 2004) colonising plants, soil, and detritus in aquatic environments, aquatic plants, and animals. The large-scale mixing of these environmental bacteria with exogenous bacteria from anthropogenic sources such as farm drainage and waste processing provides the ideal selective and ecological conditions for new resistant strains to arise; thus, soil, water, and other nutrient-enriched habitats can act as hotspots for horizontal gene transfer (van Elsas and Bailey, 2002). Human beings can be exposed to antibiotics,

antibiotic resistance genes (ARGs), or antibiotic-resistant bacteria in the environment by several routes: (1) livestock that have accumulated veterinary drugs and resistant flora through the food chain; (2) fish exposed to pharmaceuticals released to surface waters either intentionally (aquaculture treatments) or unintentionally; (3) abstracted groundwater and surface water containing residues of pharmaceuticals that is then used for drinking water; and (4) coastal waters used for recreation or shellfish production (Wellington *et al*., 2013).

Resistance can be caused by a variety of mechanisms: (i) the presence of an enzyme that inactivates the antibiotic agent; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antibiotic agent; (iii) a mutation in the antibiotic agent's target, which reduces the binding of the antibiotic agent; (iv) posttranscriptional or posttranslational modification of the antibiotic agent's target, which reduces binding of the antibiotic agent; (v) reduced uptake of the antibiotic agent; (vi) active efflux of the antibiotic agent; and (vii) overproduction of the target of the antibiotic agent (Fluit *et al*., 2001).

Previous studies have documented that antibiotic-resistant bacteria became prevalent after the introduction of antibiotics in aquaculture (Inglis *et al*., 1993, 1991) and the use of antibiotics may accelerate the dissemination of antibiotic resistant genes and resistant bacteria (Kemper, 2008). Contamination of aquatic environments causes apprehension, since it may contribute to the prevalence of antibiotic resistant bacteria in humans, acquired directly or indirectly through the human food chain (Henriques *et al*., 2006). Once the bacteria have acquired the antibiotic resistant genes, it may exist in the

environment for a long time, even after the selection pressure ceases (Tamminen *et al*., 2011; Bean *et al*., 2005; Enne *et al*., 2001). Many bacteria have been reported to be resistant to several antibiotics simultaneously (Hatha *et al*., 2005) and capable of transferring their resistance determinants among environmental microorganisms of different genera (Agerso and Petersen, 2007; Agerso and Sandvang, 2005) and to human pathogens (Guglielmetti *et al*., 2009; Furushita *et al*., 2003), which results in adverse human health effects. The role of natural environments as reservoirs of antibiotic resistant bacteria has been addressed in several studies (Suzuki, 2011; Henriques *et al*., 2006; Ash *et al*., 2002; Goni-Urriza *et al*., 2000). It has been suggested that nutrient-rich environments such as wastewater offer optimal conditions for horizontal gene transfer, which frequently involves the passage of plasmids and transposons encoding antibiotic resistance (Kelly *et al*., 2009; Summers, 2006). Sewage microbiota is a potential habitat of resistance genes found on mobile genetic elements of bacteria which are readily transferred to other bacteria in close proximity (Knapp *et al*., 2010). Transmission of antibiotic-resistant bacteria to humans occurs via plants if agricultural lands are irrigated using surface waters or if activated sludge is applied to arable land (Wegener, 2003; Corpet, 1988).

The Study for Monitoring Antimicrobial Resistance Trends (SMART) is an ongoing, multinational surveillance program that has monitored the susceptibilities of Gram-negative bacilli from intra abdominal infections since 2002. SMART also began monitoring the susceptibilities of Gram-negative bacilli from hospitalized patients with UTIs in late 2009.

Antibiotics are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food-producing animals. Many findings suggest that inadequate selection and abuse of antibiotics may lead to resistance in various bacteria and make the treatment of bacterial infections more difficult (Kolar *et al*., 2001). Multidrug-resistant bacterial strains are prevalent in human and animal isolates all over the world (Swartz *et al*., 2002; Bass *et al*., 1999). Antibiotic resistance genes may be spread on mobile genetic elements such as plasmids, transposons and integrons. Bacteria carrying integrons might transfer antibiotic resistance genes from animals to animals as well as to humans (Aarestrup and Wegener, 1999; Recchia *et al*., 1995).

The spread of antibiotic resistance genes present in gene-transfer units and dissemination of multiresistant bacteria in nature may have consequences for human health and the evolution of environmental microbiota (Martinez, 2009). Profuse use of antibiotics in medicine and agriculture has led to appearance of antibiotic resistant phenotypes among bacteria (Borg *et al*., 2010; Shryock and Richwine, 2010). Moreover, a correlation between antibiotic use and colonization or infection with these organisms was also observed (Canton and Morosini, 2011). Antibiotic resistance may develop through mutations in chromosomal DNA or horizontal gene transfer of mobile elements. Integrons are among the main types of mobile elements currently known to be involved in the capture, mobilization and spread of antibiotic resistance genes found in Gram-negative bacteria. They are genetic platforms that are responsible for integration and rearrangements of resistance determinants called gene cassettes (Mazel, 2006). Wastewater contains microorganisms, plasmids, integrons and resistance genes, becoming a hotspot for horizontal

gene transfer in an environment that contains subinhibitory concentrations of the selective agents (Moura *et al*., 2010; Schluter *et al*., 2007).

It is widely accepted that essential components of a strategy to combat the rising threat of antibiotic resistance are: the establishment of effective surveillance systems for early detection of antibiotic resistance; measures to optimise antibiotic use; robust infection prevention and control measures to prevent the spread of antibiotic resistance; improvement of healthcare provider and public education and awareness regarding antibiotics to avert their inappropriate use; and research to guide the previously mentioned actions and to develop novel antibiotics to offer effective therapy to patients. All these should be viewed through a holistic approach encompassing both human and veterinary medicine (Paphitou, 2013).

4.2.2 Antibiotic resistance among *E. coli* isolates from environmental sources

Water testing often uses the presence of coliforms including *E. coli* as an indicator of contamination. *E. coli* is widely distributed in all warm-blooded animals including humans, domestic livestock, pets, wild animals, and birds and are of interest for the study of antibiotic resistance because they are a common carrier of resistance genes and are capable of transferring those genes to other bacteria and bacterial species. Most municipalities in developed countries treat human sewage to reduce the bacterial load before releasing it into surrounding lakes, rivers, and oceans or spreading it on land. However, studies indicate that treated sewage and the water into which it is circulated remain heavily contaminated with antibiotic resistant of resistant *E. coli* from humans (Coleman *et al*., 2012) and animals (Mariano *et al*., 2009;

Krumperman, 1983). However, in India due to population pressure and insufficient infrastructure to treat the waste, untreated or partially treated, sewage and waste water is directly released into aquatic environment.

The indiscriminate use of antibiotics in human and veterinary medicine, as well as their use as growth promoting factors in husbandry, has brought about an increase in antibiotic residues in the environment, found in human and animal wastewaters (Martinez *et al*., 2008). Antibiotics, their metabolites and resistant bacteria are excreted with urine and faeces to the wastewater, especially into hospital sewage (Chagas *et al*., 2011; Chang *et al*., 2010; Galvin *et al*., 2010; Mansouri and Abbasi, 2010; Langin, *et al*., 2009; Yang *et al*., 2009; Prado *et al*., 2008). The accumulation of these residues promotes selective pressure, enhancing the selection of resistant bacteria in these environments, since several genes coding for antibiotic resistance are located on motile genetic elements (Ash *et al*., 2002). Therefore, wastewaters constitute an ideal environment for the emergence of new pathogenic and resistant bacterial strains by the acquisition of different virulence and resistance determinants.

Estuaries and coastal water bodies, which are the major sources of seafood in India, are often contaminated by the activities of adjoining populations and partially treated or untreated sewage from the townships is released into these water bodies. The fish harvested from such areas often contain human pathogenic microorganisms. In addition, poor sanitation and cross contamination at landing centers and the open fish markets exacerbates the situation (Kumar *et al*., 2005).

Several reports have been published on antibiotic resistance among *E. coli* isolated from rivers (Korzeniewska *et al*., 2013; Tacao *et al*., 2013; Koczura *et al*., 2012; Su *et al*., 2012; Tacao *et al*., 2012; Garcia-Aljaro *et al*., 2009; Olaniran *et al*., 2009; Hu *et al*., 2008; Nazir *et al*., 2005; Goni-Urriza *et al*., 2000). Previous studies have reported that *E. coli* isolates from drinking water (Coleman *et al*., 2013; Talukdar *et al*., 2013; Sahoo *et al*., 2012; Mataseje *et al*., 2009; Sabate *et al*., 2008; Ozgumus *et al*., 2007, Gaur *et al*., 1992) can harbour resistance determinants to many classes of antibiotic agents. Several studies been reported on antibiotic resistance characterization of *E. coli* isolated from spring water (Wicki *et al*., 2011; Ozgumus *et al*., 2007), waste water (Moura *et al*., 2012; Sabate *et al*., 2008), and irrigation water (Roe *et al*., 2003). Korzeniewska *et al*., (2013) and Reinthaler *et al*., (2010) studied the antibiotic resistance of *E. coli* isolated from sewage and reported that the isolates were resistant to most of the antibiotics. Koczura *et al*., (2012) and Mokracka *et al*., (2012) studied the antibiotic resistance of *E. coli* isolated from waste water treatment plant and reported that the route of the spread of multiresistant bacteria from human communities to aquatic environment may lead through wastewater treatment plants that release treated wastewater to a water reservoir. Mutlidrug resistant *E. coli* have been reported from fish farms (Gao *et al*., 2012), beaches (Hamelin *et al*., 2006), surface water (Dolejska *et al*., 2009), and estuarine water (Chandran *et al*., 2008; Henriques *et al*., 2006; Hatha *et al*., 2005).

4.2.3 Antibiotic resistance among *E. coli* isolates from food sources

Foods contaminated with antibiotic resistant bacteria could be a major threat to public health as there is the distinct possibility that genes encoding antibiotic resistance determinants that are carried on mobile genetic elements

may be transferred to other bacteria of human clinical significance. *E. coli* is a candidate vehicle for such transfers because of its diversity and also because it survives as common flora in the gastrointestinal tracts of both humans and animals. Foodborne bacteria infections with diarrhea symptoms are usually self limiting, though systemic infection and ensuing death can occur, particularly in vulnerable groups with diminished immunity such as the elderly, infants and young children (Kennedy *et al*., 2004; Meng and Doyle, 2002; Mead *et al*., 1999). Treatment options for foodborne gastroenteritis may require fluid and electrolyte replacement and antibiotics are usually prescribed in severe cases (Huang *et al*., 2006; Hohmann, 2001; Nataro and Kaper, 1998). Although the carriage of antibiotic resistance genes is not confined to commensal *E. coli*, the capacity to threaten human consumers is significantly enhanced if foodborne strains carried virulence genes that qualified them as potential human pathogens (Schroeder *et al*., 2004; Orskov and Orskov, 1992). Resistant bacteria from the intestinal flora of food animals can contaminate carcasses of slaughtered animals and thus transfer resistance genes to the microflora of humans via the food chain (Skovgaard, 2007). Several reports have been published on antibiotic resistance among *E. coli* isolated from food producing animals (Filioussis *et al*., 2013; Jones-Dias *et al*., 2013; Ho *et al*., 2009; Jouini *et al*., 2009; Gow *et al*., 2008; Van *et al*., 2008; Meunier *et al*., 2006; Carattoli *et al*., 2005; Lanz *et al*., 2003; Sengelov *et al*., 2003; Brinas *et al*., 2002; Saenz *et al*., 2001; Normand *et al*., 2000). Antibiotic resistance in *E. coli* isolates from poultry have been reported by several works (Marchant *et al*., 2013; Randall *et al*., 2012; Guarddon *et al*., 2011; Soufi *et al*., 2011; Kozak *et al*., 2009; Miranda *et al*., 2008; Girlich *et al*., 2007; Johnson *et al*., 2007; Blanc *et al*., 2006; Miles *et al*., 2006; Guerra *et al*., 2003; van den Bogaard *et al*., 2001).

Koo and Woo, (2011) and Sunde (2005) reported antibiotic resistance among *E. coli* from meat and meat products. Several studies have reported that *E. coli* isolates from animals and from food products can harbour resistance determinants to many classes of antibiotic agents (Veldman *et al*., 2011; Hordijk *et al*., 2011; Lu *et al*., 2010; Seputiene *et al*., 2010; Slama *et al*., 2010; Costa *et al*., 2008; Cocchi *et al*., 2007a; Pomba *et al*., 2006; Brinas *et al*., 2002).

In the last few years, different reports have alerted about the antibiotic resistance of *E. coli* isolates among cattles (Endimiani *et al*., 2012; Ramos *et al*., 2012; Mevius *et al*., 2010; Scaria *et al*., 2010; Srinivasan *et al*., 2007; Guerra *et al*., 2003; Lehtolainen *et al*., 2003), as well as pigs (Marchant *et al*., 2013; Smith *et al*., 2010; Schwaiger *et al*., 2010; Moodley and Guardabassi, 2009; Blanc *et al*., 2006; Hammerum *et al*., 2006; Perreten and Boerlin, 2003; Marynard *et al*., 2003). These resistant bacteria could enter the food chain, and pose food safety problem (Tan *et al*., 2014) because they can transfer resistance genes to pathogenic bacteria.

Several studies been reported on antibiotic resistance characterization of *E. coli* isolated from seafoods (Ryu *et al*., 2012; Nawaz *et al*., 2009; Van *et al*., 2008; Kumar *et al*., 2005), swine (Endimiani *et al*., 2012; Karczmarczyk *et al*., 2011; Kozak *et al*., 2009; Varga *et al*., 2009; Guerra *et al*., 2003), beef (Ramos *et al*., 2012; Dambrosio *et al*., 2007), and broilers (Endimiani *et al*., 2012; Bortolaia *et al*., 2010; Costa *et al*., 2009; Smet *et al*., 2008).

4.2.4 Antibiotic resistance among *E. coli* isolates from clinical sources

E. coli is an important cause of serious infections in both hospitals and the community. *E. coli* can cause bloodstream infections (BSI) and respiratory

tract (RT) infections and is associated with the highest mortality (Doit *et al*., 2010). *E. coli* is the most frequently isolated bacterium and its resistance to antibiotics is regularly surveyed in general practice and in hospitals (Philippon *et al*., 1996). Treatment of humans and animals by antibiotics affects not only the targeted pathogenic bacteria, but also the complex commensal microbial communities that inhabit the skin and mucosal membranes. The emergence of resistance among commensal bacteria is a serious side effect of antibiotic usage in human and veterinary medicine because the commensals may, at a later stage, cause extraintestinal infections (Kucheria *et al*., 2005), spread to other hosts (Orskov and Orskov, 1985), or transfer genetic resistance elements to other members of the microbiota (Levy, 2000). Despite this, the dynamics of antibiotic resistance in commensals has not been studied to any great extent. For example, it is not known whether antibiotic resistance influences the capacity of a given strain to persist in the normal microbiota, or whether resistance genes are kept or lost during colonization.

Urinary tract infection (UTI) is one of the most common infectious diseases diagnosed in outpatients and also constitutes the most common nosocomial infection in many hospitals, accounting for up to 35% of all hospital-acquired infections (Gales *et al*., 2000). *E. coli* remains the principal causative pathogen of UTIs both in outpatients and inpatients (Farrell *et al*., 2003; Zhanel *et al*., 2000). Antibiotics are the typical treatment of urinary tract infections (UTIs), and thus, multidrug-resistant organisms are frequently associated with UTIs. Increasing rates of resistance among UPEC have caused growing concern in both developed and developing countries (Lina *et al*., 2007). The antibiotic susceptibility of urinary pathogens as been changing over the years and is influenced by such factors as the changing patient

population and the extensive use and misuse of antibiotic agents, which contribute to alterations in the microbial profile of urinary tract isolates (Karaca *et al*., 2005). Over the last two decades, *E. coli* has become increasingly resistant to commonly used antibiotics including ampicillin, tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole (Forward *et al*., 2001). Cephalosporins and fluoroquinolones are recommended as first-line empirical therapies for the treatment of community acquired and nosocomially acquired sepsis originating in the urinary tract, polymicrobial soft-tissue infections, and intra abdominal infections (Lipsky *et al*., 2004; Solomkin *et al*., 2003; Sobel and Kaye, 2000). These recommendations would be challenged if a significant proportion of such infections turned out to be caused by ESBL-producing *E. coli.* In fact, recent data from our area indicate that 12%-16% of patients with infections due to ESBL-producing *E. coli* are bacteremic (Rodriguez-Bano *et al*., 2006b, 2004).

Several workers have reported the antibiotic resistance among clinical *E. coli* isolates from India (Hussain *et al*., 2012; Shahid *et al*., 2012; Avasthi *et al*., 2011; Sharma *et al*., 2010; Shahid *et al*., 2008; Khan *et al*., 2002). Vigil *et al*., (2009) has reported that multidrug-resistant (MDR) *E. coli* is a serious threat to cancer patients. In the last few years, different reports have alerted about the antibiotic resistance among clinical *E. coli* isolates (Jean *et al*., 2013; Martinez *et al*., 2012; Bindayna and Murtadha, 2011; Kanamori *et al*., 2011; Villegas *et al*., 2011; Vinue *et al*., 2010; Pitout *et al*., 2009; Sawma-Awad *et al*., 2009; Arredondo-Garcia and Amabile-Cuevas, 2008; Erjavec *et al*., 2007; Pitout *et al*., 2007; Oteo *et al*., 2006; Hsu *et al*., 2006; Machado *et al*., 2005; Nijssen *et al*., 2004; Rodriguez-Bano *et al*., 2004; Yu *et al*., 2004; Khan *et al*., 2002; Ruiz *et al*., 2002; Lee *et al*., 2001;White *et al*., 2001; Jacoby and Han, 1996; Cooksey *et al*., 1990).

4.2.5 Mechanism of resistance to different classes of antibiotics

4.2.5.1 β-lactam antibiotics

All β-lactam antibiotics are bactericidal agents that inhibit cell wall synthesis. The bacterial cell wall is a complex structure composed of a tightly, cross-linked peptidoglycan net which "corsets" the cell maintaining cell shape despite a high internal osmotic pressure. The glycan component of this rigid structure consists of alternating units of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG), the former having short peptide stems attached to it. Peptides in adjacent glycan strands are cross-linked, producing the characteristic net structure of the peptidoglycan. Bacterial transpeptidases (detected as penicillin-binding proteins, PBPs) are essential enzymes that catalyze this crosslinking step. β-lactams are sterically similar to the penultimate D-Ala-D-Ala of the pentapeptide that is attached to NAM; hence PBPs mistakenly use penicillin as a substrate for cell wall synthesis and the transpeptidase (or carboxypeptidase) is acylated. The acylated PBP cannot hydrolyze the β-lactam and subsequent steps in cell wall synthesis are hindered while autolysis by cell wall degrading (autolytic) enzymes continues. Bacterial cells become permeable to water, rapidly take up fluid, and eventually lyse (Goffin and Ghuysen, 1998; Ghuysen *et al*., 1996). The extremely low toxicity of the antibiotics in these classes has resulted in their overuse in the medical community, leading to increased resistance among bacteria to this class of antibiotics.

4.2.5.1.1 β-lactamases

Resistance to β-lactam antibiotic agents in *E. coli* is primarily mediated by β-lactamases, which hydrolyze the β-lactam ring and thus inactivate the

antibiotic (Livermore, 1995). Many different β-lactamases have been described (Livermore, 1998; Bush *et al*., 1995; Livermore, 1995). Over 200 β-lactamases have been classified into four main groups and eight subgroups according to their functional and structural characteristics (Bush and Jacoby, 1997; Bush *et al*., 1995). The classical TEM-1, TEM-2, and SHV-1 enzymes are the predominant plasmid-mediated β-lactamases of gram-negative rods. Susceptibility to β-lactamase inhibitors could be affected in *E. coli* by different mechanisms. The most frequent one is the hyperproduction of classical β-lactamases or the synthesis of inhibitor-resistant TEM (IRT) β-lactamases by amino acid substitutions in TEM-1 or TEM-2. Other possible mechanisms are the hyperproduction of chromosomal AmpC β-lactamase (by gene amplification or the introduction of mutations at either the promoter or the attenuator of the structural gene) (Caroff *et al*., 1999; Nelson and Elisha, 1999; Olsson *et al*., 1982; Jaurin *et al*., 1981; Edlund *et al*., 1977) and some types of OXA β-lactamases (Naas and Nordmann, 1999; Livermore, 1995), plasmidic cephalosporinase production (Queenan *et al*., 2001; Bou *et al*., 2000; Marchese *et al*., 1998; Bauernfeind *et al*., 1997; Leiza *et al*., 1994), or even changes in membrane permeability (Martinez-Martinez *et al*., 2000). Many studies have been reported the occurrence of β-lactam producing *E. coli* in broiler chicken (Smet *et al*., 2008), and animals (Pomba *et al*., 2006; Brinas *et al*., 2002). *E. coli* isolates from waste water with high resistance against ampicillin were reported by many workers (Talukdar *et al*., 2013), river (Su *et al*., 2012; Olaniran *et al*., 2009), drinking water (Mataseje *et al*., 2009), and estuary (Henriques *et al*., 2006). Dolejska *et al*., (2009) and Roe *et al*., (2003) reported a low resistance to ampicillin among *E. coli* isolates from surface waters. *E. coli* isolates of clinical origin showed high resistance against

ampicillin (Mandal *et al*., 2012; El-Najjar *et al*., 2010; Vinue *et al*., 2010; Arredondo-Garcia and Amabile-Cuevas, 2008; White *et al*., 2001).

4.2.5.1.2 Extended-spectrum beta-lactamase

Extended-spectrum beta-lactamase (ESBL)-producing members of the family *Enterobacteriaceae* are resistant to penicillins, narrow-and extended-spectrum cephalosporins, and aztreonam (Bush and Singer, 1989). ESBL-producing organisms are also frequently resistant to aminoglycosides, trimethoprim-sulfamethoxazole, and quinolones. The oxymino-cephalosporins, such as cefotaxime, ceftazidime and ceftriaxone, have potent activity against *E. coli* clinical isolates resistant to other β-lactam agents. However, even these antibiotic agents have come to be challenged by the emergence of strains that produce extended-spectrum β-lactamases (ESBLs) (Philippon *et al*., 1989). Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to aztreonam, cefotaxime, ceftazidime, and related oxyimino-β-lactams as well as to older penicillins and cephalosporins (Jacoby, 1994; Jacoby *et al*., 1991; Payne and Amyes, 1991; Philippon *et al*., 1989). They arise by mutations in genes for common plasmid-mediated β-lactamases (especially TEM and SHV enzymes) that alter the configuration of the enzyme near its active site to increase the affinity and hydrolytic ability of the β-lactamase for oxyimino compounds while simultaneously weakening the overall enzyme efficiency (Jacoby and Han, 1996).

Although CTX-M-type ESBLs have been detected in several Gram-negative pathogens, the major clinical burden is due to CTX-M-producing *E. coli* (Kao *et al*., 2014) and *K. pneumoniae* (the latter mostly in nosocomial settings) (Ginn *et al*., 2014; Oteo *et al*., 2010; Pitout and Laupland, 2008).

CTX-M-producers have been reported as the most prevalent ESBL producers in community-onset urinary tract infections (UTI) in several settings (Doi *et al*., 2013; Smet *et al*., 2010; Woodford *et al*., 2007; Pitout *et al*., 2004; Rodriguez-Bano *et al*., 2004). CTX-M-producing *E. coli* are also an important cause of community-onset bloodstream infections (BSI), that are often secondary to an UTI (Pitout and Laupland, 2008; Rodriguez-Bano *et al*., 2006a). Colonization of the gastrointestinal tract represents a key factor in the clinical impact and epidemiology of CTX-M-producing enterobacteria. Indeed, most cases of infections caused by CTX-M-producers are preceded by colonization of the gut (Ben-Ami *et al*., 2006), and carriers are the main source of CTX-M-producers in health-care settings as well as an important vehicle for dissemination in the community (Canton and Coque, 2006) and for global dissemination via international travellers (Van der Bij and Pitout, 2012).

In the last few years, different reports have alerted about the Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* isolates from clinical isolates (Fan *et al*., 2013; Jean *et al*., 2013; Martinez *et al*., 2012; Queiroz *et al*., 2012; Avasthi *et al*., 2011; Bindayna and Murtadha, 2011; Dolejska *et al*., 2011; Kanamori *et al*., 2011; Mnif *et al*., 2010; Baudry *et al*., 2009; Diestra *et al*., 2009; Ode *et al*., 2009; Arredondo-Garcia and Amabile-Cuevas, 2008; Shahid *et al*., 2008; Lavigne *et al*., 2007; Novais *et al*., 2007; Pitout *et al*., 2007; Oteo *et al*., 2006; Machado *et al*., 2005; Eckert *et al*., 2004; Leflon-Guibout *et al*., 2004; Nijssen *et al*., 2004; Chanawong *et al*., 2002; Dutour *et al*., 2002; Forward *et al*., 2001; Jacoby and Han, 1996). Korzeniewska and Harnisz, (2013) studied β-lactamase-producing *Enterobacteriaceae* in hospital effluents and found out a statistically significant correlation between

antibiotics consumption in each hospital and the incidence of ESBL-positive isolates in hospital effluents. Mataseje *et al*., (2009) studied the cefoxitin resistance in *E. coli* isolated from beaches and private drinking water in Canada. ESBL-producing *E. coli* have been reported from rivers (Tacao *et al*., 2013; Dolejska *et al*., 2009), and sewage (Reinthaler *et al*., 2010). Many studies have been reported the occurrence of ESBL producing *E. coli* in poultry (Randall *et al*., 2012; Stuart *et al*., 2012; Girlich *et al*., 2007; Blanc *et al*., 2006), cattle (Endimiani *et al*., 2012), broiler chicken (Costa *et al*., 2009), food producing animals (Lopez-Cerero *et al*., 2011; Slama *et al*., 2010; Meunier *et al*., 2006), and pig (Moodley and Guardabassi, 2009).

4.2.5.2 Aminoglycosides

The aminoglycosides are a group of antibiotics either derived from *Streptomyces* spp. (streptomycin, neomycin and tobramycin) or *Micromonospora* spp. (gentamicin) or synthesised in vitro (netilmicin, amikacin, arbekacin and isepamicin). They exhibit antimicrobial activity against a wide spectrum of different microorganisms, including Gram-positive and Gram-negative bacteria, mycobacteria and protozoa. The most frequent clinical use of aminoglycosides is empirical therapy of serious infections such as septicaemia, nosocomial respiratory tract infections, complicated urinary tract infections (UTIs) and complicated intra-abdominal infections caused by aerobic Gram-negative bacilli. In clinical practice the molecules most frequently prescribed at present are gentamicin, tobramycin and amikacin, whilst streptomycin remains an important tool in the treatment of tuberculosis, brucellosis, tularaemia and plague (Durante-Mangoni *et al*., 2009). Aminoglycosides usage has been limited because prolonged use was found to cause kidney damage and injury to the auditory

nerves leading to deafness (Goni-Urriza *et al*., 2000). The reduced use of this class of antibiotics may explain the low resistance levels of the isolates to the antibiotics in this class, except streptomycin which is primarily used for treating tuberculosis patients.

The aminoglycosides exert their activity by binding to the aminoacyl site of 16S ribosomal RNA (rRNA) within the 30S ribosomal subunit (Mingeot-Leclercq *et al*., 1999; Fourmy *et al*., 1996). The SENTRY antimicrobial resistance surveillance programme shows that aminoglycosides still retain good activity against most Gram-negative fermenting bacilli such as *E. coli*, *K. pneumonia* and *Enterobacter* spp. (Vargas *et al*., 2006; Sader *et al*., 2003). Susceptibility rates of these bacteria to amikacin averaged 97.3%, to gentamicin 90.6% and to tobramycin 89.8% in a recent global SENTRY report (Sader *et al*., 2003).

Aminoglycoside resistance can be natural or acquired. Because aminoglycosides are transported through the bacterial cell membrane by an energy-dependent, oxygen-requiring process, anaerobic bacteria are naturally resistant to the aminoglycosides. Other organisms such as *streptococci* and *enterococci*, whose cell wall serves as a permeability barrier, are also naturally resistant to the aminoglycosides. Agents that inhibit cell wall synthesis, like β-lactams and vancomycin, breakdown this barrier and facilitate aminoglycoside uptake. Acquired aminoglycoside resistance can occur through three mechanisms: (i) alterations in membrane permeability, (ii) enzymatic modification, or (iii) alterations in the target site (Sanders and Sanders, 1995). Resistance to aminoglycosides arising from various phosphorylating, adenylating or acetylating enzymatic activities is caused by both the ability of single enzymes to modify multiple drugs and multiple enzymes with overlapping activities

acting on the same drug. These enzymes modify vulnerable amino or hydroxyl groups on the aminoglycoside antibiotics that are likely to be important for target binding and drug uptake. In addition, individual proteins can inactivate more than one aminoglycoside (Mingeot-Leclercq *et al*., 1999).

Aminoglycoside resistance most commonly results from drug-modifying enzymes that are either aminoglycoside acetyltransferases (AAC), aminoglycoside adenylyltransferases (also named aminoglycoside nucleotidyltransferases (ANT), or aminoglycoside phosphotransferases (APH). These enzymes confer high-level aminoglycoside resistance (Paulsen *et al*., 1997). The enzymes, *N*-acetyltransferases, which use acetyl-coenzyme A as donor and affect amino functions, and O-nucleotidyltransferases and O-phosphotransferases, which both use ATP as donor and affect hydroxyl functions. The functions affected in typical aminoglycosides (kanamycin and gentamicin derivatives) are on positions 3, 29, and 69 for AAC, positions 49 and 20 for ANT; and positions 39 and 20 for APH (Shaw *et al*., 1993).

Aminoglycoside-modifying enzymes are often plasmid encoded but are also associated with transposable elements. Plasmid exchange and dissemination of transposons facilitate the rapid acquisition of a drug resistance phenotype not only within a given species but among a large variety of bacterial species (Mingeot-Leclercq *et al*., 1999). Su *et al*., (2012) and Mataseje *et al*., (2009) reported a higher resistance against streptomycin and lower resistance to gentamicin in *E. coli* isolated from river, and drinking water, respectively. Roe *et al*., (2003) and Dolejska *et al*., (2009) reported *E. coli* isolates from surface water showed lower resistance against streptomycin and gentamicin. Jones-Dias *et al*., (2013) and Van *et al*., (2008) reported moderate resistance of

E. coli isolates against gentamicin in food producing animals. Many workers have been reported low resistance of *E. coli* isolates against gentamicin in poultry (Soufi *et al*., 2011; Miles *et al*., 2006; Guerra *et al*., 2003), seafood (Koo and Woo, 2011), animal (Nsofor and Iroegbu, 2012), and swine (Karczmarczyk *et al*., 2011). High resistance against streptomycin has been reported in seafood (Koo and Woo, 2011), animals (Nsofor and Iroegbu, 2012), cattle (Karczmarczyk *et al*., 2011), swine (Soufi *et al*., 2011), and food producing animals (Van *et al*., 2008). Low resistance against streptomycin has been reported in fish and seafoods (Ryu *et al*., 2012; Kumar *et al*., 2005) animals (Costa *et al*., 2008), and poultry (Obeng *et al*., 2012). Aminoglycosides resistant (streptomycin and gentamicin) *E. coli* have been reported from *E. coli* of clinical origin by several workers (Hussain *et al*., 2012; El-Najjar *et al*., 2010; Arredondo-Garcia and Amabile-Cuevas, 2008; Pitout *et al*., 2007; Hsu *et al*., 2006; White *et al*., 2001).

4.2.5.3 Quinolones

Quinolones are a family of broad-spectrum antibacterial agents that act by inhibiting the activities of DNA gyrase and topoisomerase IV. The first member of the quinolones to be synthesised was nalidixic acid (Na); new fluoroquinolones, such as ciprofloxacin (Cip), significantly enhance antibacterial potency. It has been over four decades since the first of the quinolones, nalidixic acid, was introduced for clinical use in 1962 (Ball, 2000). Fluoridation of the quinolone molecule at the C-6 position in the 1970s yielded norfloxacin, the first fluoroquinolone, which entered the clinic in 1986 (Paton and Reeves, 1988). Ciprofloxacin, perhaps the most important as well as the most used fluoroquinolone, was introduced into the clinical market in

1987. Since then, structural revisions of the quinolone molecules (with fluoroquinolones or naphthyridones as the core structures) have provided numerous new agents suited to the treatment of a variety of bacterial infections. Older quinolones are mostly active against Gram-negative bacteria and newer ones have a broad spectrum of activity, with enhanced activity against Gram-positive pathogens (Appelbaum *et al*., 2004; Ball, 2001, 2000). However, the future utility of these drugs is threatened by the increasing rate of emergence of quinolone-resistant bacteria (Davies *et al*., 2003; Karlowsky, 2003; Ling *et al*., 2003a; Zhanel *et al*., 2003). Quinolone resistance is seen widely in a number of clinical pathogens and thus poses a major public health concern (Davies *et al*., 2003; Karlowsky, 2003; Ling *et al*., 2003b; Zhanel *et al*., 2003).

Several mechanisms of quinolone resistance in bacteria have been attributed to plasmid-borne genetic elements, efflux pumps and mutations in the quinolone-resistance determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC* and *parE* genes (Fonseca *et al*., 2008; Baranwal *et al*., 2002). Mutations in QRDRs alter the affinity of DNA gyrase and topoisomerase IV for the quinolones and thus protect bacteria from death (Tavio *et al*., 1999). These enzymes are necessary for cell viability as they play vital roles in DNA replication, chromosome segregation and DNA compaction (Hardy and Cozzarelli, 2003; Hooper, 1999). In accumulated chromosomal mutations in the *gyrA* and *parC* genes as well as transferable *qnr* genes were discovered to confer resistance to quinolones (Kim *et al*., 2010).

In *Enterobacteriaceae*, quinolone resistance is mainly caused by point mutations in the quinolone resistance-determining region (QRDR) of gyrase

(*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) genes (Veldman *et al*., 2011). DNA gyrase is involved primarily in controlling DNA supercoiling and relieving topological stress caused by transcription and DNA replication. Topoisomerase IV functions in the intermolecular strand passage (decatenation) of daughter chromosomes after replication, as well as relaxing positively supercoiled DNA (Crisona *et al*., 2000; Adams *et al*., 1992). However, the emergence of plasmid-mediated quinolone resistance (PMQR) has been reported since 1998 (Martinez-Martínez *et al*., 1998). Although these PMQR determinants confer low-level resistance to quinolones, they are a favourable background for selection of additional chromosome-encoded quinolone resistance mechanisms (Poirel *et al*., 2012).

Resistance to quinolones can be either intrinsic or acquired. Naturally occurring or wild-type bacterial species display innate susceptibility or resistance to antibiotics. Levels of such intrinsic resistance to individual quinolones in bacterial species are determined by the susceptibility of the drug targets to the drugs (Ince *et al*., 2002; Jorgensen *et al*., 2000; Pestova *et al*., 2000) as well as the expression status of endogenous multidrug efflux pumps (Zhang *et al*., 2001; Harding and Simpson, 2000; Giraud *et al*., 2000; Li *et al*., 1994). Acquired resistance to quinolones is mainly mediated by chromosomal mutations that either alter the targets of the quinolone drugs (i.e. DNA gyrase and topoisomerase IV) (Hooper, 2002, 2001a, 2000; Oizumi, 2001) or activate expression of the multidrug efflux pumps for which quinolones are substrates (Li and Nikaido, 2004; Drlica and Malik, 2003; Thomas *et al*., 2001; Poole, 2000). Decreased uptake as a result of porin reduction also contributes to quinolone resistance (Li and Nikaido, 2004; Martinez-Martinez *et al*., 2002) and this often interplays synergistically with other resistance mechanisms such

as efflux pumps to increase resistance levels (Li and Nikaido, 2004). The target alterations most frequently occur to the *gyrA* gene, particularly within a small, limited region of the gene called the "quinolone resistance-determining region" (QRDR) (Piddock, 1999). Batard *et al*., (2013) reported that the level of hospital use of quinolones influences the incidence of quinolone resistance in *E. coli* hospital isolates and the consumption of two other classes of antibiotics, cephalosporins and tetracyclines, is also associated with quinolone resistance.

E. coli isolates with high resistance against quinolones were reported by many workers in waste water (Talukdar *et al*., 2013; Mokracka *et al*., 2012). Low resistance of quinolones resistant *E. coli* have been reported from estuary (Henriques *et al*., 2006), river (Su *et al*., 2012) and drinking water (Mataseje *et al*., 2009) surface water (Dolejska *et al*., 2009). Quinolone resistance has been reported in *E. coli* isolates from broiler chicken (Bortolaia *et al*., 2010), poultry (Soufi *et al*., 2011), meat (Van *et al*., 2008), food producing animals (Filioussis *et al*., 2013; Jones-Dias *et al*., 2013) and from other animals (Veldman *et al*., 2011; Gibson *et al*., 2010). Costa *et al*., (2008) and Karczmarczyk *et al*., (2011) reported low level of resistance against ciprofloxacin and nalidixic acid among *E. coli* isolates from healthy pets and cattle, respectively. Quinolones resistant *E. coli* have been reported from *E. coli* of clinical origin by several workers (Nakano *et al*., 2013; Mandal *et al*., 2012; Bansal and Tandon, 2011; Galani *et al*., 2010; Mihu *et al*., 2010; Vinue *et al*., 2010; Pitout *et al*., 2009; Arredondo-Garcia and Amabile-Cuevas, 2008; Pitout *et al*., 2007; Karaca *et al*., 2005; Machado *et al*., 2005; Ruiz *et al*., 2002; Vila *et al*., 2000; Canawati *et al*., 1997).

4.2.5.4 Chloramphenicol

Chloramphenicol is an antibiotic available for clinical use since 1948 and is active against Gram positive and many Gram-negative bacteria including *Rickettsiae* (Shaw, 1984). Chloramphenicol was used extensively in veterinary medicine until concerns over its toxicity emerged (Settepani, 1984). Currently, only a fluorinated derivative of chloramphenicol, florfenicol, is approved for veterinary use in food animals, but florfenicol is not approved for use in swine in the United States (Bischoff *et al*., 2002). Chloramphenicol inhibits translation during protein synthesis and causes aplastic anemia in a small percentage of patients, and its use is very minimal in non life-threatening situations. The observed rare bacterial resistance to chloramphenicol has been attributed to the restricted use of the drug (Goni-Urriza *et al*., 2000). Chloramphenicol binds to the 50S ribosomal subunit and inhibits the peptidyltransferase step in protein synthesis. Resistance to chloramphenicol may be mediated either enzymatically through the chemical inactivation of the drug or nonenzymatically through drug efflux. Chloramphenicol acetyltransferase catalyzes the acetylation of the 3′- OH of chloramphenicol and is responsible for most enzymatic resistance to chloramphenicol (Fluit *et al*., 2001; Vassort-Bruneau *et al*., 1996; Shaw, 1983). Chloramphenicol acetyltransferases (CAT) have a trimeric structure composed of identical subunits (Schwarz and Cardoso, 1991; Leslie, 1990; Shaw, 1983).

The most frequently found chloramphenicol resistance mechanism in Gram-negative bacteria is the plasmid-mediated production of chloramphenicol acetyltransferase (CAT) (Shaw, 1984). Other mechanisms of chloramphenicol resistance include mutations that alter ribosomes in such a way that the cell

becomes insensitive to chloramphenicol, a relative permeability barrier to the influx of the antibiotic (Davies and Smith, 1978) and finally, membrane borne efflux pumps that transport toxic antibiotic compounds off the cell (Grkovic *et al*., 2002). Active efflux of chloramphenicol has been described in *E. coli* by Edgar and Bibi, (1997), Nielsen *et al*., (1996), and McMurry *et al*., (1994). *E. coli* isolates with low resistance against chloramphenicol from various sources such as spring waters (Ozgumus *et al*., 2007) river (Koczura *et al*., 2012; Su *et al*., 2012; Mataseje *et al*., 2009), waste water (Talukdar *et al*., 2013), and surface water (Dolejska *et al*., 2009) were reported by many workers. *E. coli* isolates with low resistance against chloramphenicol were reported by many workers in poultry (Miles *et al*., 2006), seafood (Koo and Woo, 2011; Kumar *et al*., 2005), healthy pets (Costa *et al*., 2008), cattle (Karczmarczyk *et al*., 2011), poultry (Soufi *et al*., 2011; Guerra *et al*., 2003), food producing animals (Jones-Dias *et al*., 2013).

4.2.5.5 Tetracyclins

It is one of the earliest broad spectrum antibiotics, and since it was developed in the 1940s, it has been produced at the largest scale of all antibiotics (Roberts, 1996). Not only has it been used in human and veterinary medicine, but it has also been used as a growth promoter in animal husbandry (van den Bogaard and Stobberingh, 2000) Its efficacy, low cost, and the lack of side effects make it the most popularly used antibiotic in livestock farming, including aquaculture. First-generation tetracyclines, such as tetracycline, chlortetracycline, and oxytetracycline, have been widely used as animal growth promoters for decades. Second-generation tetracyclines, such as minocycline and doxycycline, are commonly employed in the prophylactic and therapeutic

treatment of human and animal infections. Tetracycline is never given to children, due to its effect on growing bones and teeth (Sanchez *et al*., 2004). Tetracycline is not used to treat *E. coli* infections in humans, but resistance to tetracycline is still common in *E. coli* (Dominguez *et al*., 2002; Calva *et al*., 1996), which suggests that resistance has been selected by a bystander effect on commensal *E. coli*, during treatment of other pathogens in humans or animals. Such widespread use of tetracycline antibiotics has resulted in selection for resistant bacteria, and its imprudent use has caused a high prevalence of tetracycline resistance (Roberts, 2003, 1996; Chopra and Roberts, 2001).

Tetracycline resistance in bacteria is mediated by four mechanisms such as efflux, ribosomal protection, enzymatic inactivation, and target modification (Chopra and Roberts, 2001). Tetracycline efflux, first identified in 1953 in *Shigella dysenteriae* (Akiba *et al*., 1960), and ribosomal protection, first identified in *Streptococcus* spp. (Burdett, 1986), are now prevalent in both Gram-negative and Gram-positive bacteria (Chopra and Roberts, 2001). These resistance mechanisms are widely distributed in bacteria due to their association with mobilizable DNA elements, which have facilitated the spread to more than 50 genera, and are often coupled with multidrug-resistance (Roberts, 2005, 1996; Clewell *et al*., 1995).

The leading tetracycline resistance mechanism in *E. coli* is the extrusion of drug from the cytoplasm via efflux (Chopra and Roberts, 2001). Tetracycline-specific efflux pumps are members of the major facilitator superfamily (MFS) of efflux pumps (Paulsen *et al*., 1996). MFS pumps specific for tetracycline operate by transporting tetracycline in an energy-dependent fashion, via proton exchange, thereby reducing the intracellular concentration of the drug (Chopra

and Roberts, 2001). Tet pumps are divided into six groups based on amino acid sequence, with Tet (A), Tet (B), Tet (C), Tet (D), and Tet (E) placed in group 1 due to amino acid sequence similarity (Roberts, 2005; Chopra and Roberts, 2001). Most tetracycline-specific efflux pumps confer resistance to tetracycline only; however, *tet (B)* encodes a pump that is able to extrude both tetracycline and minocycline (Petersen *et al*., 1999; Guay and Rothstein, 1993). The prevalence of tetracycline resistance represents a useful marker to monitor resistance genes (Ozgumus *et al*., 2007), and it can provide a good model for ecological studies of antibiotic resistance (Karami *et al*., 2006). Tetracycline-resistant *E. coli* can serve as a useful indicator for antibiotic resistant bacteria in the food chain due to its high occurrence.

Several studies have demonstrated the prevalence and characterization of tetracycline resistant *E. coli* from food-producing animals (Gow *et al*., 2008; Lanz *et al*., 2003; Sengelov *et al*., 2003), humans (Schwaiger *et al*., 2010; Tuckman *et al*., 2007; Karami *et al*., 2006), companion animals (Costa *et al*., 2008), aquatic environments (Ozgumus *et al*., 2007), and raw seafood (Nawaz *et al*., 2009). Tetracycline resistance has been reported in *E. coli* isolates from poultry (van den Bogaard *et al*., 2001), pig (Schwaiger *et al*., 2010), and meat (Koo and Woo, 2011). Gao *et al*., (2012) reported the occurrence of tetracycline-resistant bacteria in aquaculture environment. Many studies have reported the occurrence of tetracycline resistant *E. coli* of clinical origin (Fan *et al*., 2007; Erjavec *et al*., 2007; Tuckman *et al*., 2007; Karami *et al*., 2006). *E. coli* isolates with high resistance against tetracycline were reported from various sources such as waste water (Talukdar *et al*., 2013; Mokracka *et al*., 2012), river (Koczura *et al*., 2012; Su *et al*., 2012; Olaniran *et al*., 2009), drinking water (Mataseje *et al*., 2009), and estuary (Henriques *et al*., 2006).

4.2.5.6 Sulphonamides

Sulphonamides have been widely used to treat bacterial and protozoal infections ever since their clinical introduction in 1935 (Perreten and Boerlin, 2003). To overcome the rapid emergence and spread of resistance that has strongly limited the use of these inexpensive antibacterial drugs, sulphonamides have generally been combined with diaminopyrimidines, such as trimethoprim (Perreten and Boerlin, 2003; Huovinen, 2001; Skold, 2000; Huovinen *et al.,* 1995). Sulfamethoxazole in combination with trimethoprim (co-trimoxazole) remains an antibiotic alternative in the treatment of several infectious diseases and is, according to WHO, the drug of choice for some conditions (Grape *et al*., 2003). The combination trimethoprim-sulfamethoxazole, is still commonly used in human medicine for the treatment of urinary tract infections. In veterinary medicine, sulphonamides alone or in combination with other antibiotic compounds are widely used to prevent and treat diarrhea and other infectious diseases in intensive animal husbandry. Sulphonamides compete with the structural analog *p*-aminobenzoic acid for binding to dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, thus inhibiting the formation of dihydrofolic acid (Skold, 2000).

Resistance to sulphonamides in *E. coli* can result from mutations in the chromosomal DHPS gene (*folP*) (Vedantam *et al*., 1998; Swedberg *et al*., 1993) or more frequently from the acquisition of an alternative DHPS gene (*sul*) (Radstrom and Swedberg, 1988, Sundstrom *et al*., 1988), whose product has a lower affinity for sulphonamides (Perreten and Boerlin, 2003; Skold, 2000; Swedberg and Skold, 1980). Sulphonamides resistance genes can be transferred from commensal bacteria, *via* integrons, transposons or plasmids,

into more virulent bacteria in the human intestine (Guerra *et al*., 2003). Sulphonamide deserves special attention because of its widespread use, high excretion rate, high solubility and persistence in the environment (Lamshoft *et al*., 2007). Even without selective pressure, sulphonamide-resistant bacteria can remain stable in the aqueous environment for 5 or 10 years. The sulphonamide-resistant bacteria have been verified to be more persistent than the sulphonamide itself (Bean *et al*., 2005; Enne *et al*., 2001). Sulphonamide resistance has been reported in *E. coli* isolates in poultry (Soufi *et al*., 2011), and pig (Hammerum *et al*., 2006; Perreten and Boerlin, 2003). Sulphonamide resistant *E. coli* has been reported from *E. coli* of clinical origin by several workers (Hussain *et al*., 2012; Vinue *et al*., 2010; Pitout *et al*., 2009; Arredondo-Garcia and Amabile-Cuevas, 2008; Frank *et al*., 2007; Pitout *et al*., 2007; Hsu *et al*., 2006; Karaca *et al*., 2005; Yu *et al*., 2004; Lee *et al*., 2001). Many studies have been reported the occurrence of sulphonamide resistant *E. coli* in water (Su *et al*., 2012; Hu *et al*., 2008; Moura *et al*., 2007; Roe *et al*., 2003). *E. coli* isolates with high resistance against sulphonamides were reported by many workers in river (Koczura *et al*., 2012; Su *et al*., 2012) and waste water (Talukdar *et al*., 2013; Mokracka *et al*., 2012). *E. coli* with moderate level of sulphonamide resistance have been reported from estuary (Henriques *et al*., 2006), and drinking water (Mataseje *et al*., 2009).

4.2.5.7 Trimethoprim

Trimethoprim is a broad spectrum antibiotic agent active against enteric pathogens such as *E. coli* and *Shigella* species (Huovinen *et al*., 1995). Trimethoprim is an analog of dihydrofolic acid, an essential component in the synthesis of amino acid and nucleotides that competitively inhibits the enzyme

dihydrofolate reductase (DHFR). Bacteria may become resistant to trimethoprim by several mechanisms, including the development of permeability barriers, efflux pumps, and the existence of naturally insensitive target DHFR enzymes, mutational and regulation changes in target enzymes and the acquirement of drug-resistant target enzymes (Huovinen, 2001). At least 15 DHFR enzyme types are known based on their properties and sequence homology (Schmitz and Fluit, 1999). The most common resistance mechanism is the acquirement of a trimethoprim-insensitive DHFR variants resulting in high-level trimethoprim resistance in various bacteria.

Trimethoprim resistant *E. coli* has been reported from clinical sources (Frank *et al*., 2007; Karaca *et al*., 2005; Yu *et al*., 2004; Lee *et al*., 2001). *E. coli* isolates with high degree of resistance against trimethoprim were reported from river (Koczura *et al*., 2012; Su *et al*., 2012) and waste water (Mokracka *et al*., 2012). Many studies reported trimethoprim resistant *E. coli* in cattle (Karczmarczyk *et al*., 2011), livestock (Nsofor and Iroegbu, 2012), poultry (Obeng *et al*., 2012; Guerra *et al*., 2003), and meat (Van *et al*., 2008).

Prevalence of drug resistance among *E. coli* isolates are well documented from estuarine, seafood and clinical sources as evidenced by the review of relevant literature. However most of this research was carried out in developed countries and there is dearth of such research in emerging countries like India. The published literatures on this subject from the study area are restricted to few (Chandran *et al*., 2008; Hatha *et al*., 2005). Moreover aquatic environment is highly dynamic and the conditions are ever changing. Estuaries are characterised by high degree of pollution and is a melting pot for commensal/ opportunistic pathogens from various sources. These natural waters also provide a conducive

environment for gene transfer among bacteria which could lead to changes in the resistance patterns over time. Furthermore the resistance pattern in any particular area/country is determined by the prevailing usage patterns/ frequency of the antibiotics and need to be monitored specifically for these study area. Hence the study was taken up with following objectives.

4.3 Objectives

1) To determine the prevalence of antibiotic resistance among *E. coli* isolates from environmental, seafood and clinical sources

2) To determine the MAR (multiple antibiotic resistance) index of *E. coli* isolates and elucidation of resistance pattern.

4.4 Materials and Methods

In the present study a total of 300 *E. coli* isolates of estuarine origin, 100 *E. coli* of clinical and 100 *E. coli* of seafood origin were tested for antibiotic sensitivity by disc diffusion method.

4.4.1 Antibiotic susceptibility testing

Antibiotic resistance patterns of all *E. coli* isolates were determined by disc diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar (Hi-Media, India). The antibiotics tested were listed in the Table 4.1. Pure cultures of all *E. coli* isolates were enriched in nutrient broth at 37° C for 6-8 hrs. The cultures were then streaked over surface dried sterile Muller Hinton agar plates using a sterile cotton swab. After 15-30 min of pre incubation time antibiotic discs were aseptically placed over the seeded Muller Hinton agar plates, sufficiently separated from each other so as to avoid overlapping of

inhibition zone. After overnight incubation at 37 °C, diameter of the inhibition zone was measured and the isolates were classified as sensitive, intermediate or resistant, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standards Institute, 2007).

Table 4.1 List of antibiotics used, abbreviation and their concentration

Sl.No	Name of the antibiotic	Abbreviation	Concentration
1	Ampicillin	Amp	10 µg
2	Cefotaxime	Ctx	30 µg
3	Cefoxitin	Cx	30 µg
4	Cefpodoxime	Cpd	10 µg
5	Ceftazidime	Caz	30 µg
6	Ceftriaxone	Ctr	30 µg
7	Cefuroxime	Cxm	30 µg
8	Chloramphenicol	C	30 µg
9	Ciprofloxacin	Cip	5 µg
10	Co-Trimoxazole	Co	25 µg
11	Gentamicin	Gen	10 µg
12	Nalidixic acid	Na	30 µg
13	Streptomycin	S	10 µg
14	Tetracycline	Te	30 µg
15	Trimethoprim	Tr	5 µg

4.4.2 MAR indexing of the *E. coli* isolates

Multiple antibiotic resistances (MAR) index of the individual isolates were done according to Krumperman, (1983). MAR index of an individual isolate was calculated by dividing the number of antibiotic to which the isolate was resistant by the total number of antibiotics to which the isolate was exposed. The isolates with MAR index greater than 0.2 is considered to have originated from high risk source of contamination such as human, poultry or diary cattle where antibiotics are frequently used.

4.4.3 Statistical analysis

Statistical analysis in this study was performed using SPSS software 13 (Statistical Package for the Social Science). One-Way Analysis of Variance (ANOVA, Games-Howell) was applied to test difference in the bacterial resistance among antibiotic agents, different stations, and different sources and in antibiotic resistance among different phylogenetic groups within five stations and between different sources. Pearson correlation coefficient was used to analyze the correlation between antibiotic resistance phenotypes. Significance level was set at $\alpha = 0.05$.

4.5 Results

4.5.1 Antibiotic resistance of *E. coli* isolates from Cochin estuary

4.5.1.1 Overall antibiotic resistance of *E. coli* isolates from Cochin estuary

Prevalence of antibiotic resistance was evaluated in a total of three hundred *E. coli* isolated from Cochin estuary (Figure 4.1). Results revealed significant differences in bacterial resistance to the 15 different antibiotics used in this study (ANOVA, $p = 0.000$). Games-Howell analysis showed that the prevalence of resistance to ampicillin was significantly higher than to cefotaxime, cefpodoxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, gentamicin, and streptomycin ($p < 0.05$). Games-Howell analysis also showed that the prevalence of resistance to cefoxitin and cefpodoxime were significantly higher than to cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, and gentamicin ($p < 0.05$). Statistical analysis of the result revealed a highly significant association was found between ceftazidime with ceftriaxone ($p = 0.002$). Furthermore, a significant association was found between co-trimoxazole with

trimethoprim and nalidixic acid ($p < 0.05$). There was also a significant association seen between cefotaxime with cefuroxime and gentamicin ($p < 0.05$).

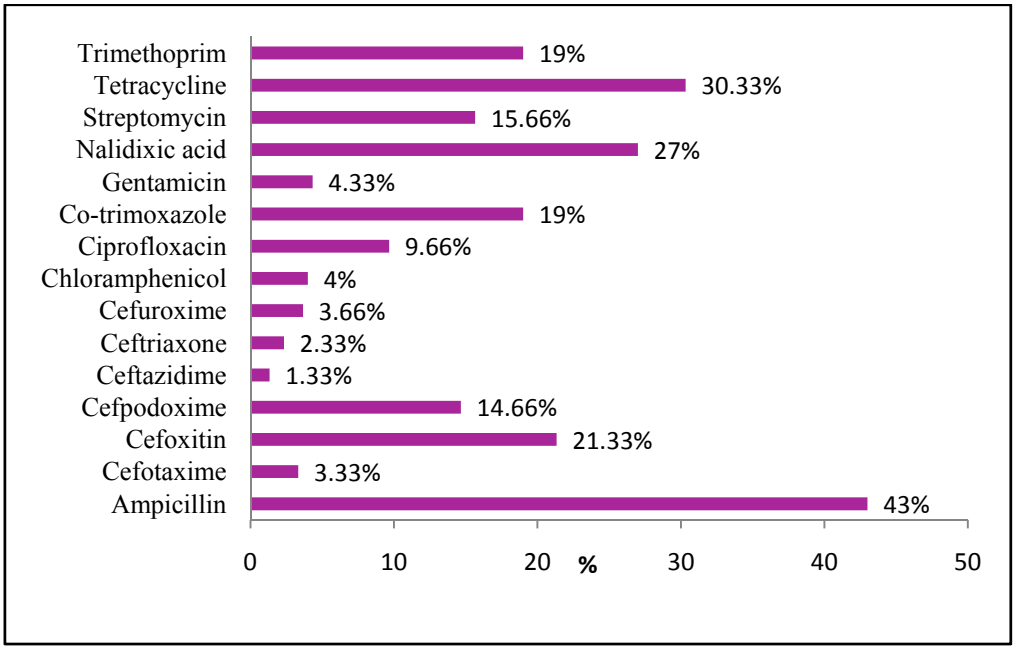


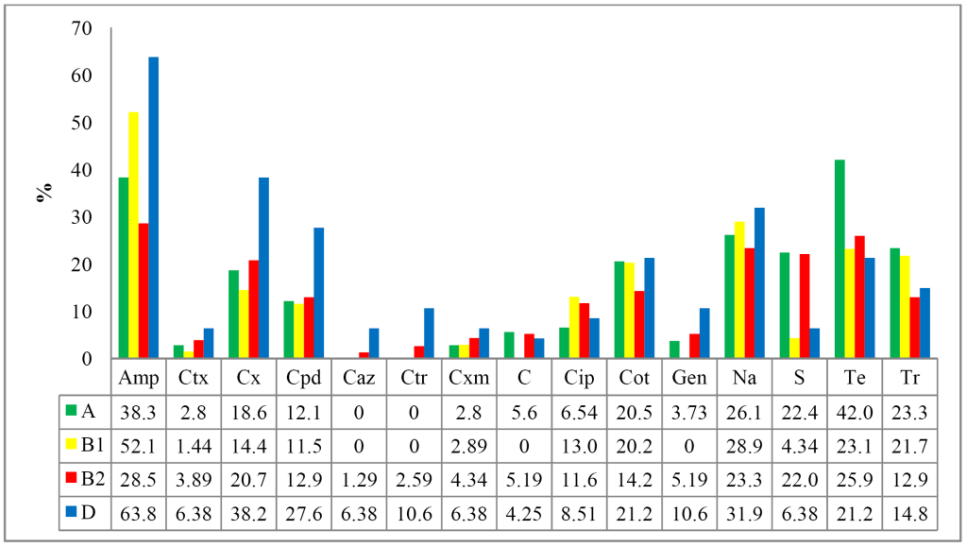
Figure 4.1 Percentage of antibiotic resistance among *E. coli* isolates from Cochin estuary (n = 300)

Though the individual strains vary in the resistance to various antibiotics collectively, *E. coli* strains isolated from Cochin estuary showed resistance to all the antibiotics tested. Among individual antibiotics, the highest percentage of antibiotic resistance was to ampicillin (43%), followed by tetracycline (30.33%), nalidixic acid (27%), and cefoxitin (21.33%). Between 20% and 10% of *E. coli* were resistant to co-trimoxazole (19%), trimethoprim (19%), streptomycin (15.66%), and cefpodoxime (14.66%). Resistance to ciprofloxacin, gentamicin, chloramphenicol, cefuroxime, cefotaxime, ceftriaxone and ceftazidime were lower, with percentages of 9.66%, 4.33%, 4%, 3.66%, 3.33%, 2.33% and

1.33%, respectively. There was no significant difference in antibiotic resistance seen among *E. coli* from the five different stations (ANOVA, p = 0.318).

4.5.1.1.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from Cochin estuary

Among *E. coli* isolates of different phylogenetic groups, group D strains showed relatively higher resistance to ampicillin (63.82%), cefoxitin (38.29%), nalidixic acid (31.91%), cepfodoxime (27.65%), co-trimoxazole (21.27%), gentamicin (10.63%), ceftriaxone (10.63%) (Figure 4.2). Resistance to ciprofloxacin (13.04%) was higher among *E. coli* belonging to phylogenetic group B1. Phylogenetic group A strains showed highest resistance against tetracycline (42.05%) and trimethoprim (23.36%). Collectively, phylogenetic group B2 and D strains showed resistance against all the antibiotics tested. All the isolates belonging to phylogenetic group A were sensitive to ceftazidime and ceftriaxone. Phylogenetic group B1 isolates were sensitive to ceftazidime, ceftriaxone, chloramphenicol and gentamicin.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	38.3	2.8	18.6	12.1	0	0	2.8	5.6	6.54	20.5	3.73	26.1	22.4	42.0	23.3
B1	52.1	1.44	14.4	11.5	0	0	2.89	0	13.0	20.2	0	28.9	4.34	23.1	21.7
B2	28.5	3.89	20.7	12.9	1.29	2.59	4.34	5.19	11.6	14.2	5.19	23.3	22.0	25.9	12.9
D	63.8	6.38	38.2	27.6	6.38	10.6	6.38	4.25	8.51	21.2	10.6	31.9	6.38	21.2	14.8

Figure 4.2 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from Cochin estuary (n = 300)

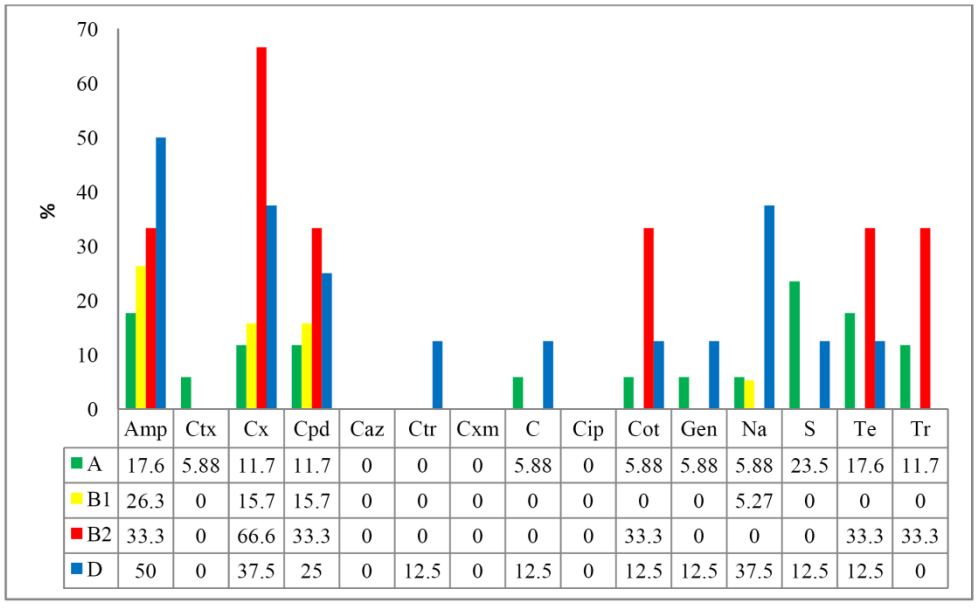
4.5.1.2 Antibiotic resistance of *E. coli* isolates from station 1 (Chitoor)

Significant difference in bacterial resistance to the 15 different antibiotics were found in station 1, Chitoor (p =0.001). But there was no significant difference in antibiotic resistance were seen among the 4 different phylogenetic groups (ANOVA, p = 0.100).

In station 1 (Chittor), the highest percentage of antibiotic resistance was for ampicillin (27.66%), followed by cefoxitin (21.27%), cefpodoxime (17.02%), nalidixic acid (10.64%), streptomycin (10.64%), and tetracycline (10.64%) (Table 4.2). Between 10% and 2% resistance was found for co-trimoxazole (6.38%), trimethoprim (6.38%), chloramphenicol (4.25%), gentamicin (4.25%), cefotaxime (2.13%) and ceftriaxone (2.12%). None of the isolates were showed resistance to ceftazidime, cefuroxime, and ciprofloxacin.

4.5.1.2.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from station 1 (Chitoor)

Phylogenetic group A strains showed highest resistance against streptomycin only. Phylogenetic group A isolates were sensitive against ceftazidime, and ceftriaxone, cefuroxime, and ciprofloxacin. All isolates belonging to phylogenetic group B1 and B2 were sensitive to cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, gentamicin, and streptomycin. Similarly, phylogenetic group D isolates were sensitive to cefotaxime, ceftazidime, cefuroxime, ciprofloxacin, and trimethoprim (Figure 4.3).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	17.6	5.88	11.7	11.7	0	0	0	5.88	0	5.88	5.88	5.88	23.5	17.6	11.7
B1	26.3	0	15.7	15.7	0	0	0	0	0	0	0	5.27	0	0	0
B2	33.3	0	66.6	33.3	0	0	0	0	0	33.3	0	0	0	33.3	33.3
D	50	0	37.5	25	0	12.5	0	12.5	0	12.5	12.5	37.5	12.5	12.5	0

Figure 4.3 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from station 1 (Chitoor)

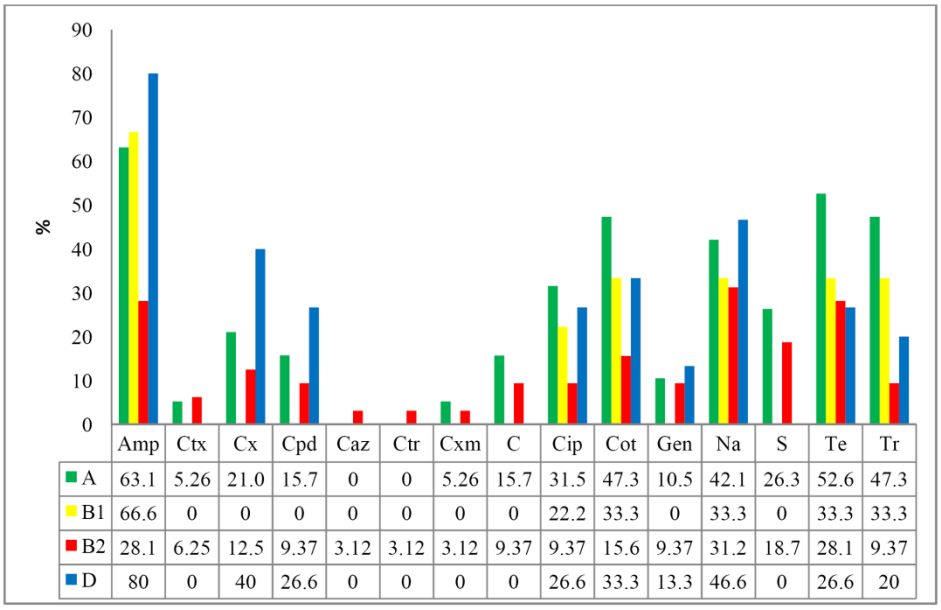
4.5.1.3 Antibiotic resistance of *E. coli* isolates from station 2 (Bolgatty)

Significant difference in bacterial resistance to the 15 different antibiotics were found in station 2, Bolgatty ($p = 0.000$). Games-Howell analysis showed that the percentage of bacterial resistance to nalidixic acid was significantly higher than to cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, and gentamicin ($p < 0.05$). But there was no significant difference in antibiotic resistance were seen among the 4 different phylogenetic groups (ANOVA, $p = 0.274$).

Among *E. coli* isolated from station 2 (Bolgatty), the highest percentage of antibiotic resistance was for ampicillin (52%), followed by nalidixic acid (37.33%), tetracycline (34.67%), co-trimoxazole (29.34%), trimethoprim (24%), ciprofloxacin (20%), cefoxitin (18.67%), streptomycin (14.65%) and cefpodoxime (13.33%) (Table 4.2). Five to 10% of strains were resistant to gentamicin (9.34%), and chloramphenicol (8%); resistance to cefotaxime, cefuroxime, ceftazidime, and ceftriaxone were lower, with percentages of 4%, 2.66%, 1.33%, and 1.33% respectively.

4.5.1.3.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty)

Nearly 86% of phylogenetic group D strains showed resistance to ampicillin (80%), followed by nalidixic acid (46.7%) cefoxitin (40%) and cefpodoxime (26.7%) (Figure 4.4).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	63.1	5.26	21.0	15.7	0	0	5.26	15.7	31.5	47.3	10.5	42.1	26.3	52.6	47.3
B1	66.6	0	0	0	0	0	0	0	22.2	33.3	0	33.3	0	33.3	33.3
B2	28.1	6.25	12.5	9.37	3.12	3.12	3.12	9.37	9.37	15.6	9.37	31.2	18.7	28.1	9.37
D	80	0	40	26.6	0	0	0	0	26.6	33.3	13.3	46.6	0	26.6	20

Figure 4.4 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty)

Phylogenetic group D isolates were sensitive against cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, and streptomycin. Phylogenetic group A strains showed relatively higher resistance to tetracycline (52.6%), co-trimoxazole (47.4%), trimethoprim (47.4%), ciprofloxacin (31.6%), and chloramphenicol (15.7%). The entire phylogenetic group A isolates were sensitive to ceftazidime, and ceftriaxone, while phylogenetic group B2 isolates showed resistance to all the antibiotics tested. Phylogenetic group B1 isolates were sensitive to cefotaxime, cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, gentamicin and streptomycin.

4.5.1.4 Antibiotic resistance of *E. coli* isolates from station 3 (Off Marine Science Jetty)

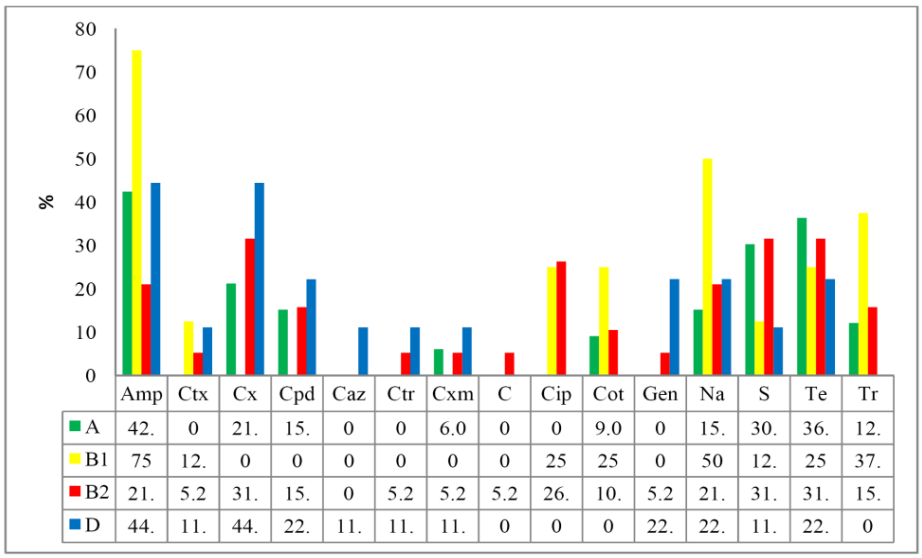
Significant difference in bacterial resistance to the 15 different antibiotics were found in station 3, Off Marine Science Jetty ($p = 0.000$). Games-Howell analysis showed that the percentage of bacterial resistance to tetracycline was significantly higher than to ceftazidime, ceftriaxone, cefuroxime, and chloramphenicol ($p < 0.05$). But there was no significant difference in antibiotic resistance were seen among the 4 different phylogenetic groups (ANOVA, $p = 0.867$).

E. coli isolates from station 3 (Off Marine Science Jetty), were resistant to ampicillin (42.02%), tetracycline (31.88%), streptomycin (26.08%), cefoxitin (24.63%), nalidixic acid (21.73%), trimethoprim (14.49%), cepfodoxime (14.44%), co-trimoxazole (10.15%) and ciprofloxacin (10.15%) (Table 4.2). Between 2-6% of isolates were resistant to cefuroxime (5.79%), cefotaxime (4.35%), gentamicin (4.35%), and ceftriaxone (2.89%). The least resistance was detected against ceftazidime and chloramphenicol (1.45%). Among different phylogenetic groups, phylogenetic group B1 strains showed highest resistance against ampicillin (75%), nalidixic acid (50%), trimethoprim (37.5%), co-trimoxazole (25%), and cefotaxime (12.5%).

4.5.1.4.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from station 3 (Off Marine Science Jetty)

Phylogenetic group B2 strains showed relatively higher resistance to streptomycin (31.6%) and ciprofloxacin (26.3%) (Figure 4.5). Resistance to tetracycline (36.4%) was relatively high among *E. coli* belonging to group A, while phylogenetic group D strains showed resistance to cefoxitin (44.4%), cefpodoxime (22.2%), gentamicin (22.2%), ceftazidime (11.1%), ceftriaxone (11.1%), and cefuroxime (11.1%). Whereas all the phylogenetic group A isolates

were sensitive to cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, and gentamicin, phylogenetic group B1 isolates were sensitive to cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol and gentamicin. Phylogenetic group B2 isolates showed resistance against all the antibiotics tested except ceftazidime. Phylogenetic group D isolates were sensitive against chloramphenicol, ciprofloxacin, co-trimoxazole and trimethoprim.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	42.	0	21.	15.	0	0	6.0	0	0	9.0	0	15.	30.	36.	12.
B1	75	12.	0	0	0	0	0	0	25	25	0	50	12.	25	37.
B2	21.	5.2	31.	15.	0	5.2	5.2	5.2	26.	10.	5.2	21.	31.	31.	15.
D	44.	11.	44.	22.	11.	11.	11.	0	0	0	22.	22.	11.	22.	0

Figure 4.5 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from station 3 (Off Marine Science Jetty).

4.5.1.5 Antibiotic resistance of *E. coli* isolates from station 4 (Thevara)

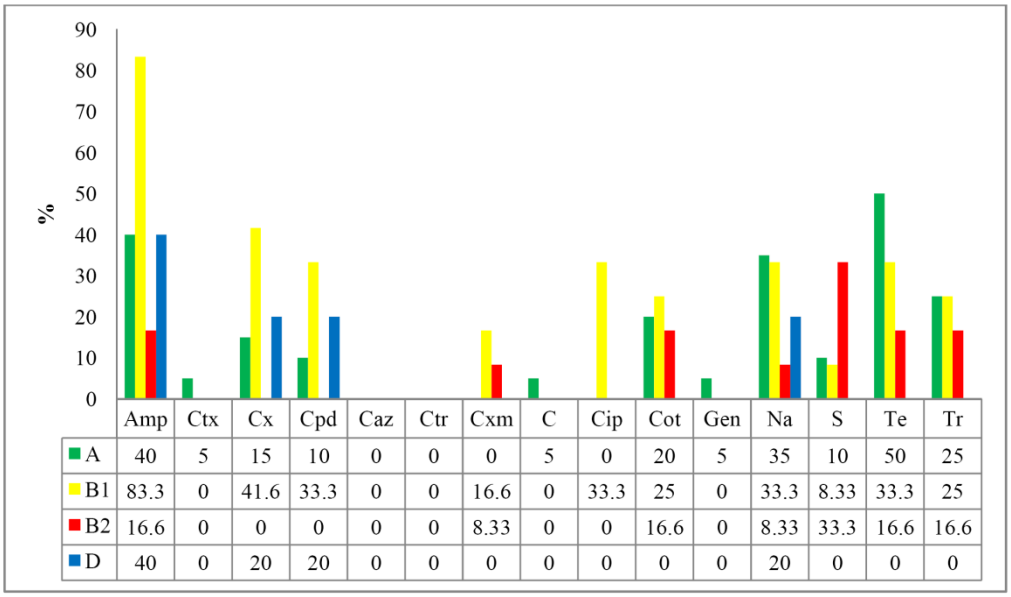
Significant difference in bacterial resistance to the 15 different antibiotics were found in station 4, Thevara ($p = 0.001$). There was also significant difference in antibiotic resistance were seen among the 4 different phylogenetic groups (ANOVA, $p = 0.039$).

Among *E. coli* isolated from station 4 (Thevara), the highest percentage of antibiotic resistance was for ampicillin (44.89%), followed by tetracycline (32.65%), nalidixic acid (26.53%) and trimethoprim (20.41%) (Table 4.2).

Between 5-20% of strains were resistant to cefoxitin (18.36%), co-trimoxazole (18.36%), cefpodoxime (14.28%), streptomycin (14.28%), ciprofloxacin (8.16%), cefuroxime (6.12%); and 2.04% were resistant to cefotaxime, chloramphenicol, and gentamicin. All isolates were sensitive to ceftazidime and ceftriaxone.

4.5.1.5.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from station 4 (Thevara)

Among different phylogenetic groups, more than 80% of phylogenetic group B1 strains showed resistant to ampicillin followed by, cefoxitin (41.7%), cepfodoxime (33.3%), ciprofloxacin (33.3%), co-trimoxazole (25%), and cefuroxime (16.7%) (Figure 4.6). Phylogenetic group A strains showed relatively higher resistance to tetracycline and nalidixic acid, while phylogenetic group B2 strains were highly resistant to streptomycin. All the phylogenetic group A isolates were sensitive against ceftazidime, ceftriaxone, cefuroxime, and ciprofloxacin. Phylogenetic group D isolates were sensitive against cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, co-trimoxazole, gentamicin, streptomycin, tetracycline and trimethoprim. Phylogenetic group B1 isolates were sensitive against ceftazidime, ceftriaxone, ciprofloxacin and gentamicin. Phylogenetic group B2 isolates were sensitive against cefotaxime, cefoxitin, cepfodoxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, and gentamicin.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	40	5	15	10	0	0	0	5	0	20	5	35	10	50	25
B1	83.3	0	41.6	33.3	0	0	16.6	0	33.3	25	0	33.3	8.33	33.3	25
B2	16.6	0	0	0	0	0	8.33	0	0	16.6	0	8.33	33.3	16.6	16.6
D	40	0	20	20	0	0	0	0	0	0	0	20	0	0	0

Figure 4.6 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from station 4 (Thevara)

4.5.1.6 Antibiotic resistance of *E. coli* isolates from station 5 (Barmouth)

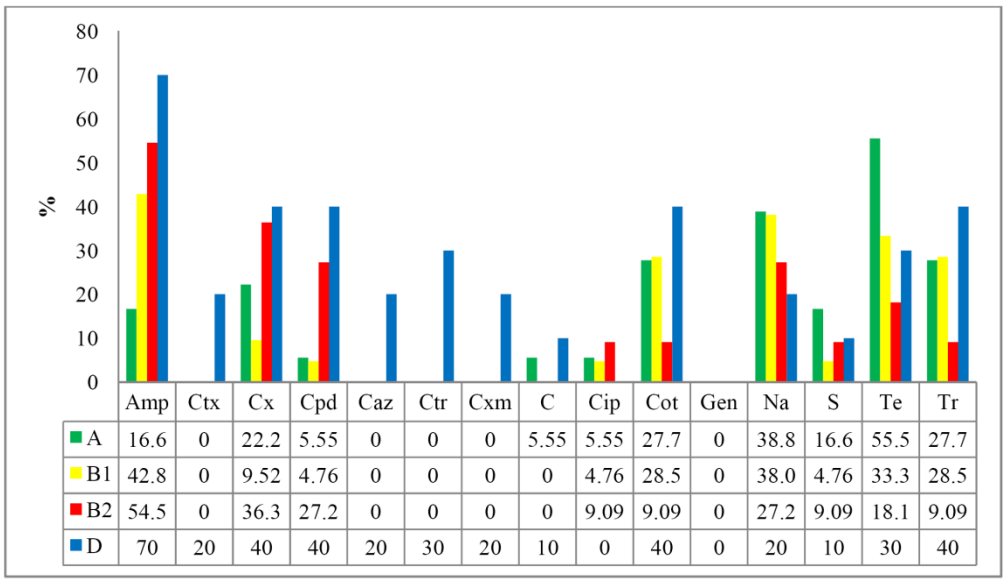
Significant difference in bacterial resistance to the 15 different antibiotics were found in station 5, Barmouth ($p = 0.000$). But there was no significant difference in antibiotic resistance were seen among the 4 different phylogenetic groups (ANOVA, $p = 0.126$).

Most of the *E. coli* strains isolated from station 5 (Barmouth) were found to be resistant to ampicillin (41.66%), tetracycline (36.67%), nalidixic acid (33.34%), co-trimoxazole (26.67%) trimethoprim (26.67%) and cefoxitin (23.33%) (Table 4.2). Between 15% and 5% resistance was found for cefpodoxime (15%) and streptomycin (10%), ceftriaxone (5%), and ciprofloxacin (5%). Moreover, 3% were resistant to cefotaxime, chloramphenicol, ceftazidime

and cefuroxime. None of the isolates were showed resistance against gentamicin.

4.5.1.6.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from station 5 (Barmouth)

Among different phylogenetic groups, phylogenetic group D strains showed relatively higher resistance to ampicillin (70%), cefoxitin (40 %), cepfodoxime (40%), co-trimoxazole (40%), trimethoprim (40%), and ceftriaxone (30%), cefotaxime (20%), ceftazidime (20%), cefuroxime (20%), and chloramphenicol (10%). Phylogenetic group A strains were highly resistant to tetracycline (55.6%) nalidixic acid (38.9%), and streptomycin (16.7%). Among phylogenetic group B2 isolates resistance to ciprofloxacin was only (9.09%). Phylogenetic group A, B1 and B2 isolates were sensitive to cefotaxime, ceftazidime, ceftriaxone, cefuroxime and gentamicin. Phylogenetic group D isolates were sensitive to ciprofloxacin and gentamicin (Figure 4.7).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	16.6	0	22.2	5.55	0	0	0	5.55	5.55	27.7	0	38.8	16.6	55.5	27.7
B1	42.8	0	9.52	4.76	0	0	0	0	4.76	28.5	0	38.0	4.76	33.3	28.5
B2	54.5	0	36.3	27.2	0	0	0	0	9.09	9.09	0	27.2	9.09	18.1	9.09
D	70	20	40	40	20	30	20	10	0	40	0	20	10	30	40

Figure 4.7 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from station 5 (Barmouth)

4.5.1.7 Incidence of antibiotic resistance: a comparison from all stations in Cochin estuary

Table 4.2 shows the prevalence of antibiotic resistance among *E. coli* isolates from different stations at Cochin estuary. *E. coli* isolates from station 2 showed higher prevalence of resistance against ampicillin, chloramphenicol, ciprofloxaxin, co-trimoxazole, gentamicin and nalidixic acid. While isolates from station 3 higher prevalence of resistance against cefoxitin and streptomycin, whereas *E. coli* isolates from station 4, exhibited higher prevalence of resistance against cefuroxime only. Tetracycline and trimethoprim resistant *E. coli* isolates were prevalent in station 5.

Table 4.2 Percentage of antibiotic resistance among *E. coli* isolates from different stations at Cochin estuary

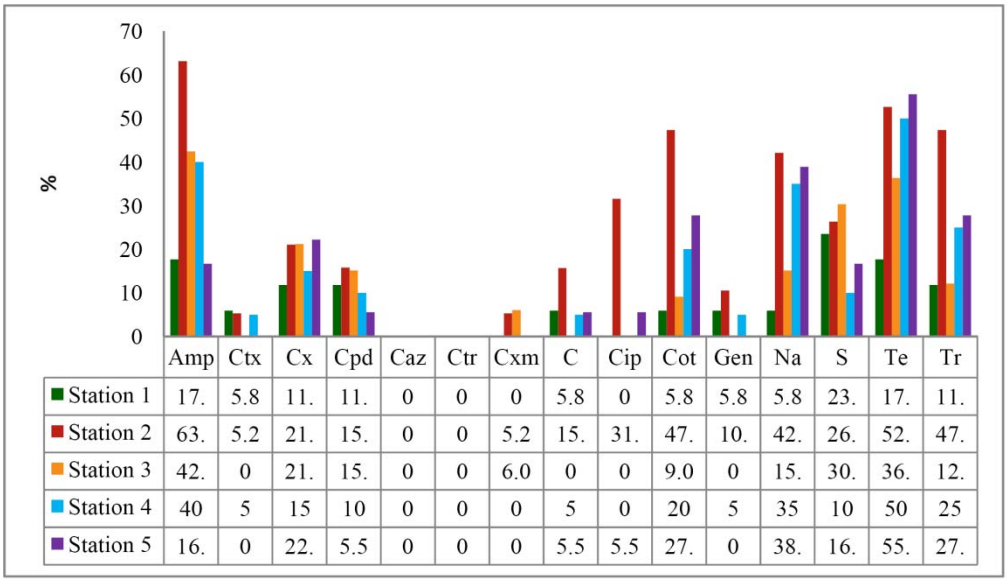
Antibiotics	Stations No*				
	1	2	3	4	5
Ampicillin	27.66%	52%	42.02%	44.89%	41.66%
Cefotaxime	2.13%	4%	4.35%	2.04%	3.34%
Cefoxitin	21.27%	18.67%	24.63%	18.36%	23.33%
Cefpodoxime	17.02%	13.33%	14.44%	14.28%	15%
Ceftazidime	0	1.33%	1.45%	0	3.33%
Ceftriaxone	2.12%	1.33%	2.89%	0	5%
Cefuroxime	0	2.66%	5.79%	6.12%	3.33%
Chloramphenicol	4.25%	8%	1.45%	2.04%	3.34%
Ciprofloxacin	0	20%	10.15%	8.16%	5%%
Co-trimoxazole	6.38%	29.34%	10.15%	18.36%	26.67%
Gentamicin	4.25%	9.34%	4.35%	2.04%	0
Nalidixic acid	10.64%	37.33%	21.73%	26.53%	33.34%
Streptomycin	10.64%	14.65%	26.08%	14.28%	10%
Tetracycline	10.64%	34.67%	31.88%	32.65%	36.67%

*1, Chitoor; 2, Bolgatty; 3, Off Marine Science Jetty; 4, Thevara; 5, Barmouth

4.5.1.8 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates: a comparison from all stations in Cochin estuary

E. coli isolates belonging to phylogenetic group A isolates from different stations, were sensitive to third generation cephalosporins such as ceftazidime and ceftriaxone, however *E. coli* strains from different stations showed significant variation in their resistance to various antibiotics (Figure 4.8).

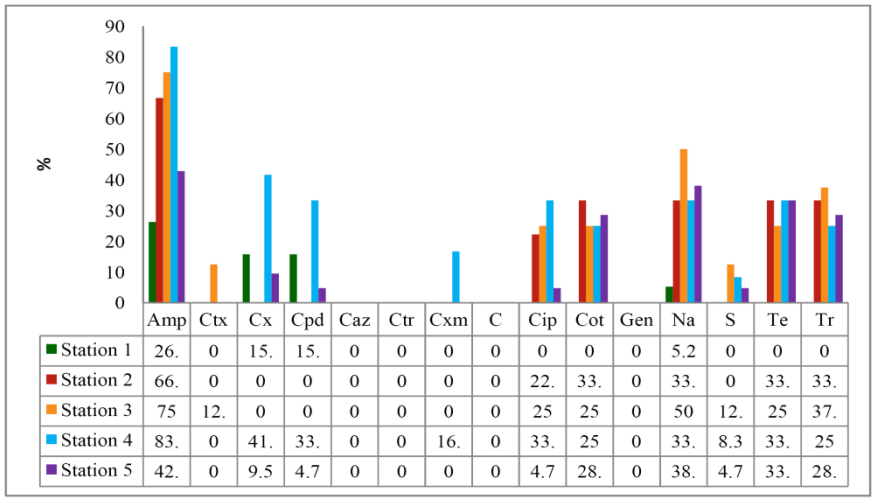
Antibiotic resistance of *E. coli* belonged to phylogenetic group A was significantly varied among all stations (p = 0.050). Games-Howell analysis showed that antibiotic resistance of *E. coli* isolates belonged to group A was significantly higher in station 2, (Bolgatty) when compared to those from station 1, 3, 4, and 5.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Station 1	17.	5.8	11.	11.	0	0	0	5.8	0	5.8	5.8	5.8	23.	17.	11.
Station 2	63.	5.2	21.	15.	0	0	5.2	15.	31.	47.	10.	42.	26.	52.	47.
Station 3	42.	0	21.	15.	0	0	6.0	0	0	9.0	0	15.	30.	36.	12.
Station 4	40	5	15	10	0	0	0	5	0	20	5	35	10	50	25
Station 5	16.	0	22.	5.5	0	0	0	5.5	5.5	27.	0	38.	16.	55.	27.

Figure 4.8 Percentage of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'A' from different stations at Cochin estuary

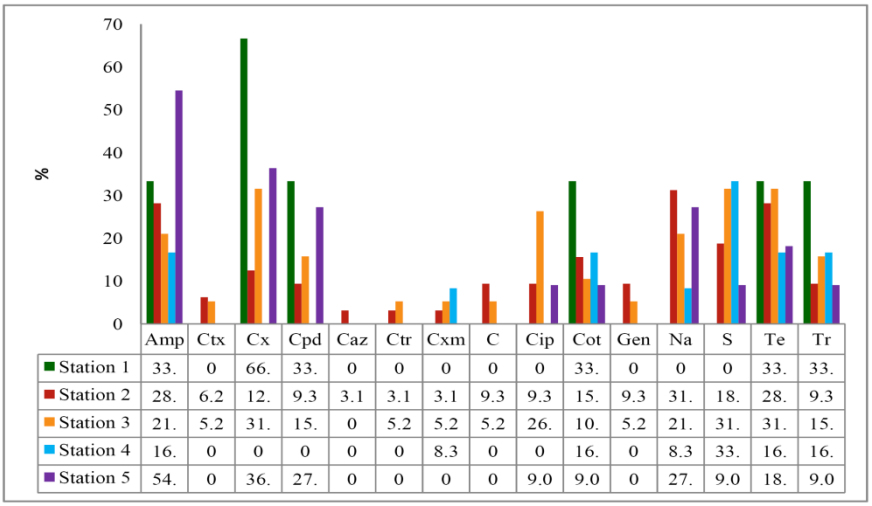
Phylogenetic group B1 isolates from different stations, were sensitive to as ceftazidime, ceftriaxone, chloramphenicol, and gentamicin (Figure 4.9). However, there was no significant difference in antibiotic resistance of *E. coli* belonged to phylogenetic group B1 (p = 0.130).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Station 1	26.	0	15.	15.	0	0	0	0	0	0	0	5.2	0	0	0
Station 2	66.	0	0	0	0	0	0	0	22.	33.	0	33.	0	33.	33.
Station 3	75	12.	0	0	0	0	0	0	25	25	0	50	12.	25	37.
Station 4	83.	0	41.	33.	0	0	16.	0	33.	25	0	33.	8.3	33.	25
Station 5	42.	0	9.5	4.7	0	0	0	0	4.7	28.	0	38.	4.7	33.	28.

Figure 4.9 Percentage of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'B1' from different stations at Cochin estuary

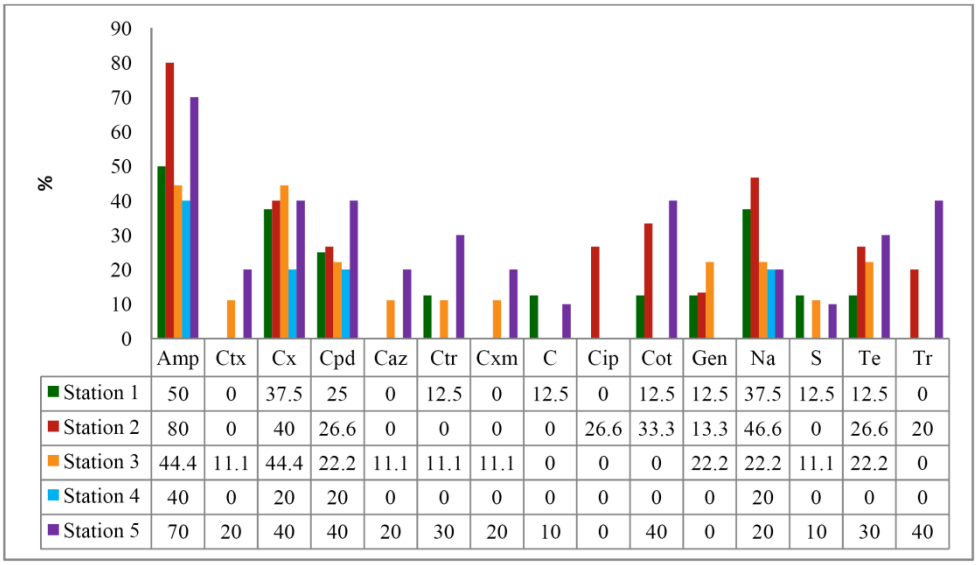
Collectively, strains isolated from station 2 and 3 showed resistance to all the antibiotics tested except for ceftazidime (Figure 4.10). But there was no significant difference in antibiotic resistance of *E. coli* belonged to phylogenetic group B2 (p = 0.583).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Station 1	33.	0	66.	33.	0	0	0	0	0	33.	0	0	0	33.	33.
Station 2	28.	6.2	12.	9.3	3.1	3.1	3.1	9.3	9.3	15.	9.3	31.	18.	28.	9.3
Station 3	21.	5.2	31.	15.	0	5.2	5.2	5.2	26.	10.	5.2	21.	31.	31.	15.
Station 4	16.	0	0	0	0	0	8.3	0	0	16.	0	8.3	33.	16.	16.
Station 5	54.	0	36.	27.	0	0	0	0	9.0	9.0	0	27.	9.0	18.	9.0

Figure 4.10 Percentage of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'B2' from different stations at Cochin estuary

Antibiotic resistance of *E. coli* belonged to phylogenetic group D was significantly varied among all stations (p = 0.047) (Figure 4.11). Games-Howell analysis showed that antibiotic resistance of *E. coli* isolates belonged to group D was significantly higher in station 5, (Barmouth) than to station 1, 2, 3, and 4.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Station 1	50	0	37.5	25	0	12.5	0	12.5	0	12.5	12.5	37.5	12.5	12.5	0
Station 2	80	0	40	26.6	0	0	0	0	26.6	33.3	13.3	46.6	0	26.6	20
Station 3	44.4	11.1	44.4	22.2	11.1	11.1	11.1	0	0	0	22.2	22.2	11.1	22.2	0
Station 4	40	0	20	20	0	0	0	0	0	0	0	20	0	0	0
Station 5	70	20	40	40	20	30	20	10	0	40	0	20	10	30	40

Figure 4.11 Percentage of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'D' from different stations at Cochin estuary

4.5.2 Prevalence of antibiotic resistance among *E. coli* isolates from clinical sources

Antibiotic resistance was evaluated in a total of one hundred *E. coli* strains isolated from clinical origin (Figure 4.12). Significant differences in bacterial resistance to the 15 different antibiotics were found (ANOVA, p = 0.000). Games-Howell analysis showed that the percentage of bacterial resistance to ampicillin, cefoxitin and cefpodoxime were significantly higher when compared to cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, co-trimoxazole, gentamicin, and streptomycin ($p < 0.05$).

Statistical analysis of the result revealed a highly significant association was found between cefotaxime with ceftazidime and cefuroxime ($p < 0.05$). Furthermore, a significant association was found between ceftriaxone with gentamicin, nalidixic acid, and tetracycline ($p < 0.05$). Collectively, *E. coli* strains from clinical origin showed resistance to all the antibiotics tested. While most of the strains were resistant to ampicillin (98%), followed by cefoxitin (91%), cefpodoxime (82%), nalidixic acid (78%), co-trimoxazole (55%), trimethoprim (54%), ciprofloxacin (47%) tetracycline (46%), cefuroxime (45%), and cefotaxime (41%). Between 20-40% of isolates were resistant to streptomycin (38%), ceftazidime (27%), ceftriaxone (23%), and gentamicin (22%). The least resistance was observed against chloramphenicol (12%). There was no significant differences in antibiotic resistance among *E. coli* belonging to different phylogenetic groups (ANOVA, $p = 0.102$).

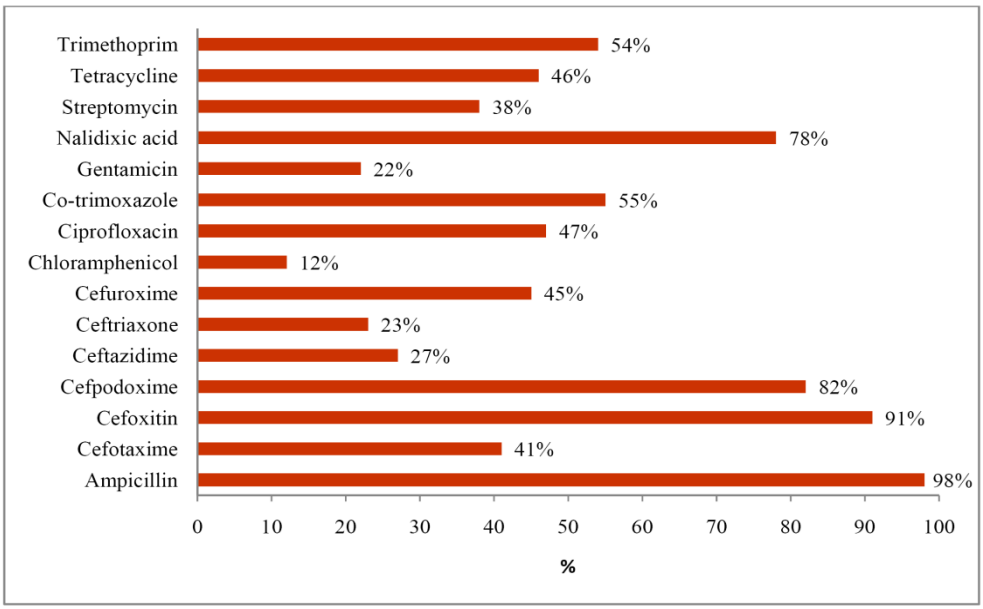
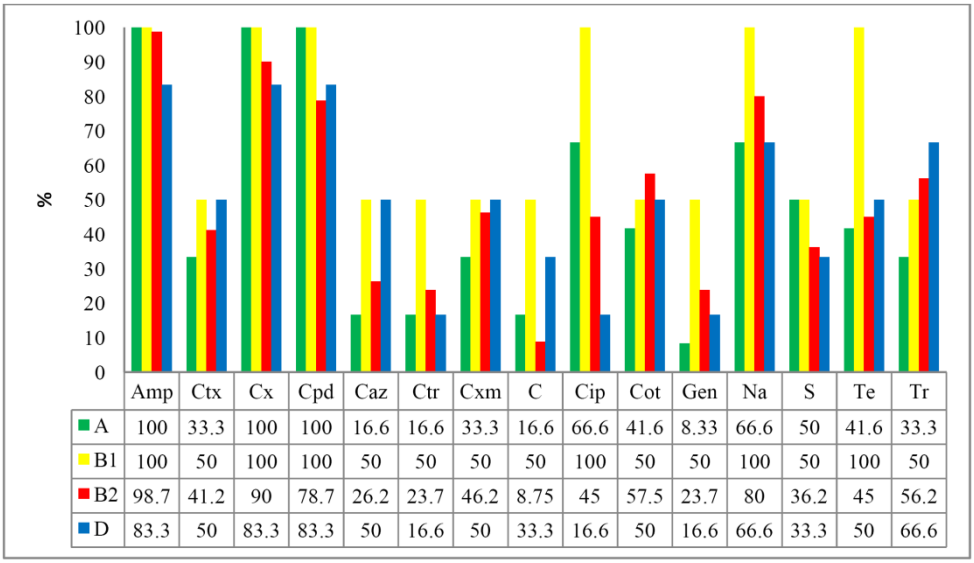


Figure 4.12 Percentage of antibiotic resistance among *E. coli* isolates from clinical sources (n = 100)

4.5.2.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from clinical sources

E. coli strains from clinical sources belonged to group A and B1 were resistant to ampicillin, cefoxitin and cepfodoxime. All the *E. coli* strains belonging to group B1 were resistant to ciprofloxacin, nalidixic acid and tetracycline. B1 isolates also showed higher resistance (50%) to ceftriaxone, chloramphenicol and gentamicin. Among B2 group *E. coli* showed highest resistance against co-trimoxazole (57.5%). D group isolates showed higher resistance against trimethoprim. Strains belonged to non pathogenic groups such as A and B1 showed more antibiotic resistance than pathogenic groups B2 and D. Fifty percentage of group B1 and D isolates showed relatively higher resistance to cefotaxime, ceftazidime, and cefuroxime (Figure 4.13).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	100	33.3	100	100	16.6	16.6	33.3	16.6	66.6	41.6	8.33	66.6	50	41.6	33.3
B1	100	50	100	100	50	50	50	50	100	50	50	100	50	100	50
B2	98.7	41.2	90	78.7	26.2	23.7	46.2	8.75	45	57.5	23.7	80	36.2	45	56.2
D	83.3	50	83.3	83.3	50	16.6	50	33.3	16.6	50	16.6	66.6	33.3	50	66.6

Figure 4.13 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from clinical sources (n = 100)

4.5.3 Antibiotic resistance among *E. coli* isolates from seafood sources

Antibiotic resistance was evaluated in a total of one hundred *E. coli* isolates from seafood (shellfish and fish) origin (Figure 4.14). Significant differences in bacterial resistance to the 15 different antibiotics were found (ANOVA, p = 0.000). Among individual antibiotics, the highest percentage of antibiotic resistance was to ampicillin (51%), followed by cefoxitin (14%), tetracycline (12%), cefpodoxime (11%), and streptomycin (11%). Between 10% and 5% resistance was found for nalidixic acid (8%), trimethoprim (8%), co-trimoxazole (8%), and cefuroxime (5%); resistance to cefotaxime (3%), ciprofloxacin (3%), gentamicin (3%), ceftazidime (2%), and chloramphenicol (2%). All the strains were sensitive to ceftriaxone. Although no significant differences in antibiotic resistance were seen among different phylogenetic groups (ANOVA, p = 0.083), antibiotic resistance of *E. coli* belonged to A was significantly higher than phylogenetic group D (p = 0.009).

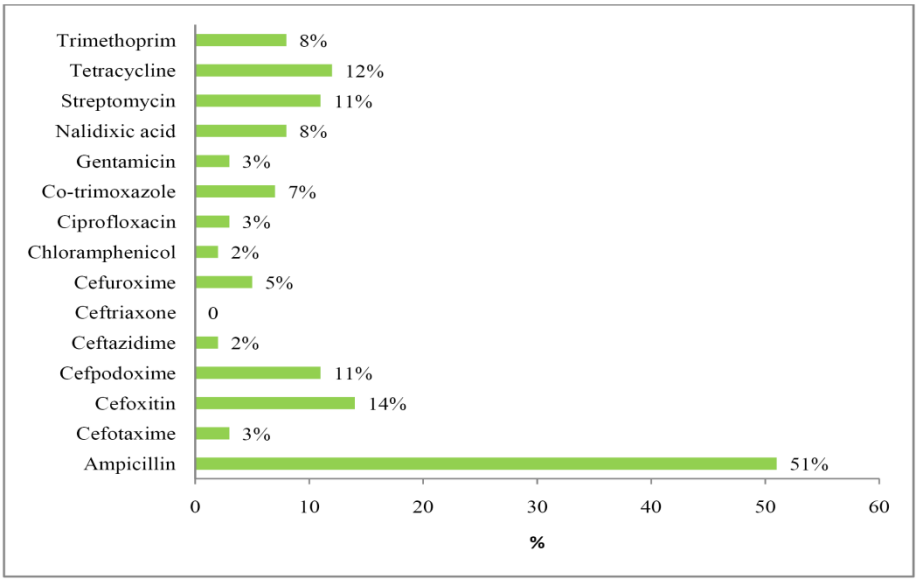
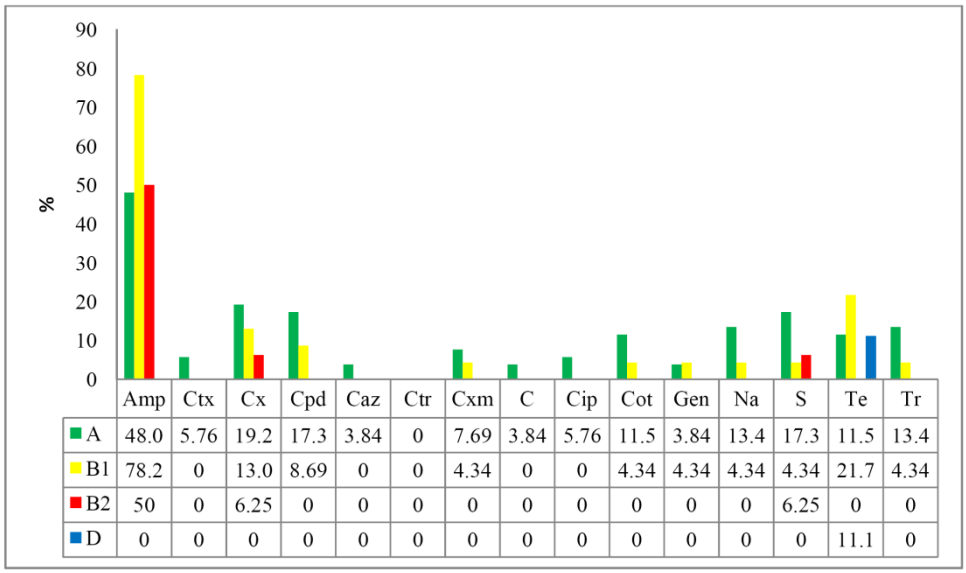


Figure 4.14 Percentage of antibiotic resistance among *E. coli* isolates from seafood sources (n = 100)

4.5.3.1 Overall incidence of antibiotic resistance among various phylogenetic groups of *E. coli* isolates from seafood sources

E. coli isolates belonging to group A showed resistance against all the antibiotic tested except ceftriaxone (Figure 4.15). These strains also showed relatively higher resistance to cefoxitin (19.2%), streptomycin (19.2%) and cepfodoxime (17.3%). *E. coli* belonged to B1 showed resistance to ampicillin, tetracycline, cefoxitin, cepfodoxime, ceftriaxone, co-trimoxazole, gentamicin, nalidixic acid, streptomycin and trimethoprim, of which resistance to ampicillin (78.1%) and tetracycline (21.7%) was relatively high. B2 strains showed resistance to ampicillin, cefoxitin and streptomycin. It was interesting to note that *E. coli* strains belonging to phylogenetic group D showed resistance to tetracycline only. Strains belonging to non pathogenic groups such as A and B1 showed more antibiotic resistance than pathogenic groups B2 and D.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
A	48.0	5.76	19.2	17.3	3.84	0	7.69	3.84	5.76	11.5	3.84	13.4	17.3	11.5	13.4
B1	78.2	0	13.0	8.69	0	0	4.34	0	0	4.34	4.34	4.34	4.34	21.7	4.34
B2	50	0	6.25	0	0	0	0	0	0	0	0	0	6.25	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0	0	11.1	0

Figure 4.15 Percentage of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from seafood sources (n = 100)

4.5.4 Relative prevalence of antibiotic resistance of *E. coli* isolates from different sources

There was a significant difference in antibiotic resistance were seen among the *E. coli* isolates of different origin (Figure 4.16). Antibiotic resistance of *E. coli* strains from clinical sources was significantly higher than those from seafood and estuary ($p = 0.000$). However, no significant difference was observed in bacterial resistance to the 15 different antibiotics were found among *E. coli* of different origin ($p = 0.171$).

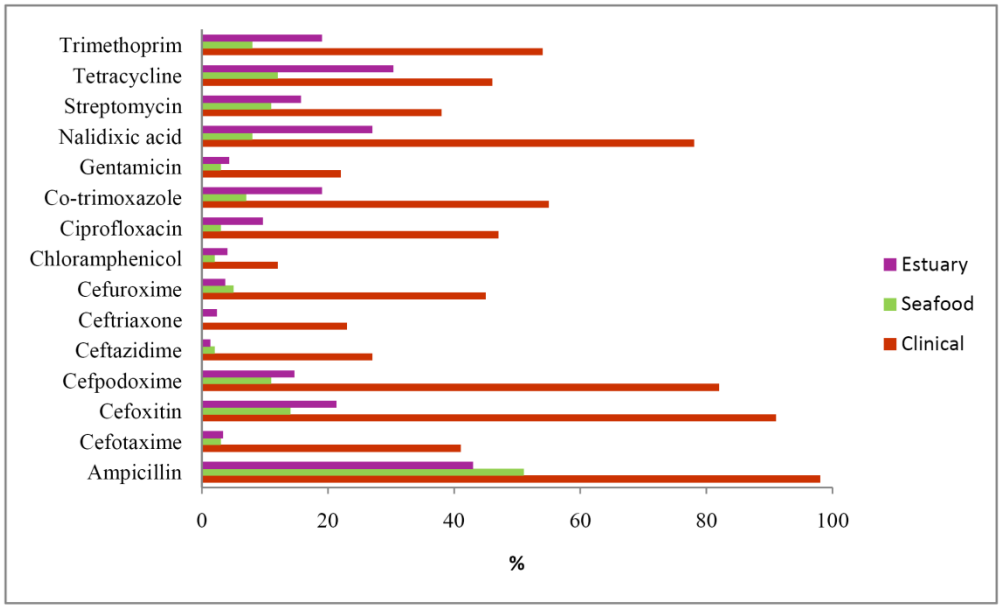
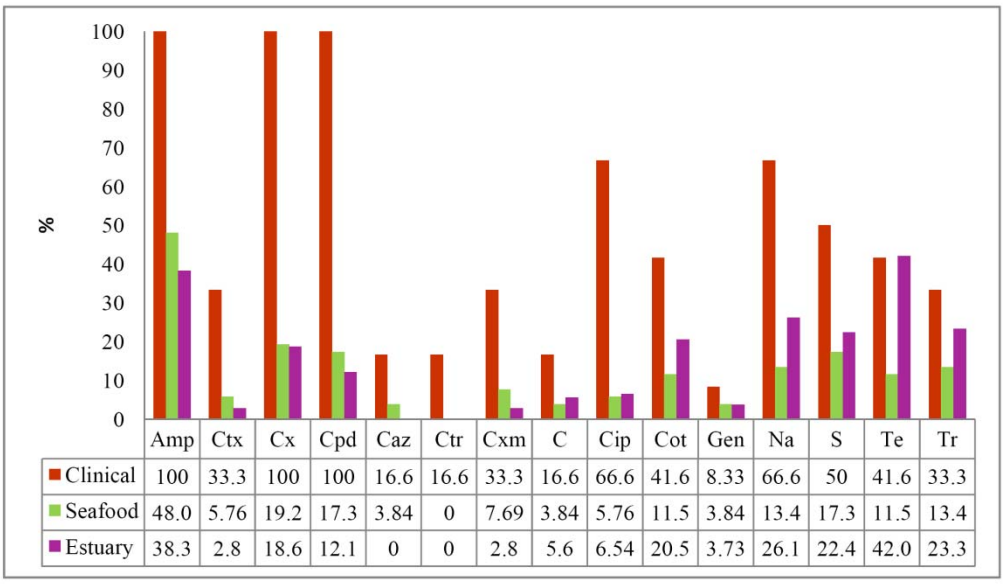


Figure 4.16 Percentage of antibiotic resistance among *E. coli* isolates from different sources

4.5.4.1 Relative incidence of antibiotic resistance in various phylogenetic groups of *E. coli* isolates from different sources (estuary, seafood and clinical)

Antibiotic resistance of *E. coli* belonged to phylogenetic group A, was significantly higher in *E. coli* of clinical origin than those from seafood and

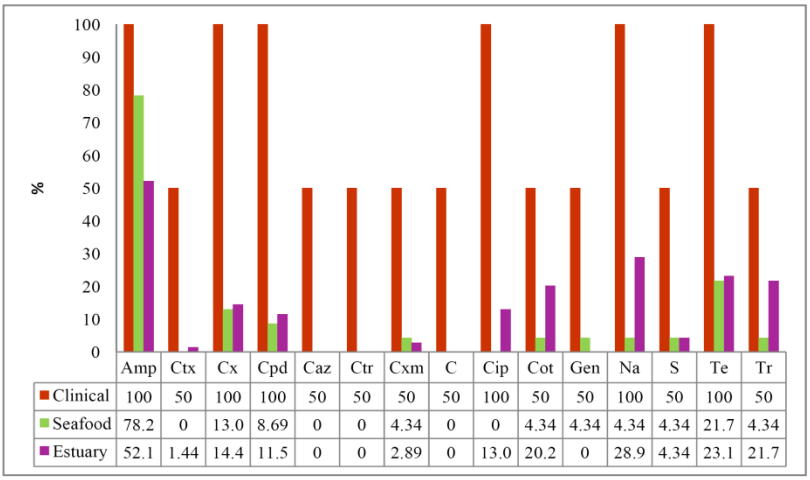
estuary ($p < 0.05$). *E. coli* of estuarine origin showed moderate resistance to all the antibiotics tested except ceftazidime, and ceftriaxone (Figure 4.17). Seafood isolates showed least resistance against all the antibiotics tested except ampicillin (48.07%). All the seafoodborne *E. coli* isolates were sensitive to ceftriaxone.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Clinical	100	33.3	100	100	16.6	16.6	33.3	16.6	66.6	41.6	8.33	66.6	50	41.6	33.3
Seafood	48.0	5.76	19.2	17.3	3.84	0	7.69	3.84	5.76	11.5	3.84	13.4	17.3	11.5	13.4
Estuary	38.3	2.8	18.6	12.1	0	0	2.8	5.6	6.54	20.5	3.73	26.1	22.4	42.0	23.3

Figure 4.17 Relative prevalence of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'A' from different sources

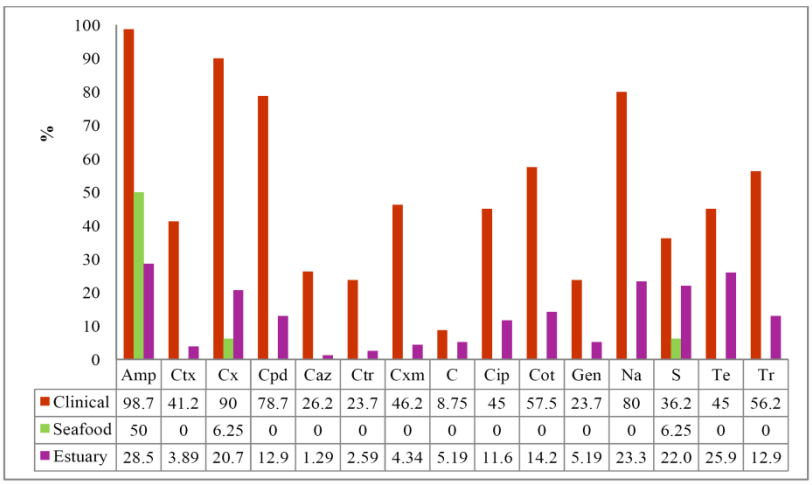
Antibiotic resistance of *E. coli* belonged to phylogenetic group B1, was significantly higher in *E. coli* of clinical origin than those from seafood and estuary ($p = 0.000$). *E. coli* of estuarine origin showed moderate resistance to all the antibiotics tested except ceftazidime, ceftriaxone and chloramphenicol (Figure 4.18). Seafood isolates were sensitive to cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, and ciprofloxacin.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Clinical	100	50	100	100	50	50	50	50	100	50	50	100	50	100	50
Seafood	78.2	0	13.0	8.69	0	0	4.34	0	0	4.34	4.34	4.34	4.34	21.7	4.34
Estuary	52.1	1.44	14.4	11.5	0	0	2.89	0	13.0	20.2	0	28.9	4.34	23.1	21.7

Figure 4.18 Relative prevalence of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'B1' from different sources

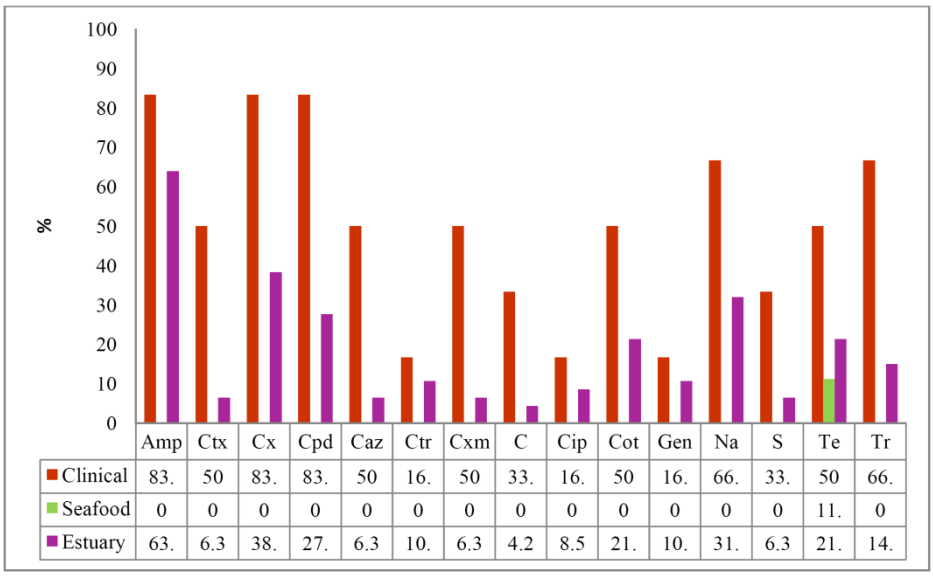
Antibiotic resistance of *E. coli* belonged to phylogenetic group B2, was significantly higher in *E. coli* of clinical origin than those from seafood and estuary (p = 0.000). *E. coli* of estuarine origin showed moderate resistance to all the antibiotics tested (Figure 4.19). Seafood isolates were sensitive to all antibiotics tested except ampicillin, cefoxitin and trimethoprim.



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Clinical	98.7	41.2	90	78.7	26.2	23.7	46.2	8.75	45	57.5	23.7	80	36.2	45	56.2
Seafood	50	0	6.25	0	0	0	0	0	0	0	0	0	6.25	0	0
Estuary	28.5	3.89	20.7	12.9	1.29	2.59	4.34	5.19	11.6	14.2	5.19	23.3	22.0	25.9	12.9

Figure 4.19 Relative prevalence of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'B2' from different sources

Antibiotic resistance of *E. coli* belonged to phylogenetic group D, was significantly higher in *E. coli* of clinical origin than those from seafood and estuary (p = 0.000). *E. coli* of estuarine origin showed moderate resistance to all the antibiotics tested (Figure 4.20). Seafood isolates were sensitive to all antibiotics tested except tetracycline (11.1%).



	Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Clinical	83.	50	83.	83.	50	16.	50	33.	16.	50	16.	66.	33.	50	66.
Seafood	0	0	0	0	0	0	0	0	0	0	0	0	0	11.	0
Estuary	63.	6.3	38.	27.	6.3	10.	6.3	4.2	8.5	21.	10.	31.	6.3	21.	14.

Figure 4.20 Relative prevalence of antibiotic resistance among *E. coli* isolates belonged to phylogenetic group 'D' from different sources

4.5.5 MAR Index and resistance patterns of *E. coli* isolates from Cochin estuary

4.5.5.1 MAR Index and resistance patterns of *E. coli* isolates from station 1 (Chitoor)

No significant difference in MAR index were seen among *E. coli* from 5 different stations (ANOVA, p = 0.129). More than 10% of isolates from station 1 showed resistance to three or more antibiotics. MAR index and resistance pattern of *E. coli* isolates from station 1 is given in Table 4.3. MAR

index varied from 0.2 to 0.66. There was not much diversity among the resistance patterns indicating possible origin of these isolates from a common source. AmpCxCpd was the frequently encountered pattern of antibiotic resistance among these isolates.

Table 4.3 MAR index and resistance pattern of *E. coli* (n = 47) isolates from station 1 (Chitoor)

MAR index	Resistance Pattern	% incidence
0.66	AmpCxCpdCtrCCotGenNaSTe	2.12
0.53	AmpCtxCxCpdGenSTeTr	2.12
0.4	AmpCxCpdCotTeTr	2.12
0.33	AmpCotNaTeTr	2.12
0.26	AmpCxCpdNa	2.12
0.2	AmpCxCpd	6.38
0.2	CSTe	2.12

4.5.5.2 MAR Index and resistance patterns of *E. coli* isolates from station 2 (Bolgatty)

About 30% of isolates from station 2 showed resistance to more than three antibiotics, while 19% of isolates showed resistance against more than five antibiotics. MAR index and resistance pattern of *E. coli* isolates from station 2 is given in Table 4.4. MAR index varied from 0.2 to 1. One isolate showed resistance to all the antibiotics tested. There was very high diversity among the resistance patterns among *E. coli* isolates from Bolgatty, indicating possible origin of these isolates from different sources. AmpNaTe was the frequently encountered pattern of antibiotic resistance among these isolates.

Table 4.4 MAR index and Resistance pattern of *E. coli* (n = 75) isolates from station 2 (Bolgatty)

MAR index	Resistance Pattern	% incidence
1	AmpCtxCxCpdCazCtrCxmCipCCot GenNaSTeTr	1.33
0.8	AmpCtxCxCpdCxmCCipCotNaSTeTr	1.33
0.66	AmpCxCpdCCotGenNaSTeTr	1.33
0.6	AmpCCipCotGenNaSTeTr	1.33
0.53	AmpCxCpdCipCotGenNaTe	1.33
0.46	AmpCipCotNaSTeTr	1.33
0.46	AmpCxCpd CipNa STe	1.33
0.46	AmpCCotGenNaSTe	1.33
0.4	AmpCipCotNaTeTr	2.66
0.4	AmpCxCpdCipNaTe	1.33
0.4	AmpCipCotGenNaTe	1.33
0.4	AmpCxCpdCotNaTr	1.33
0.4	AmpCxCpdNaSTe	1.33
0.33	AmpCipCotTeTr	1.33
0.33	AmpCxCpdCotTr	1.33
0.26	AmpCxCGen	1.33
0.26	AmpNaSTe	1.33
0.26	AmpCipCotTr	2.66
0.26	AmpCtxCxS	1.33
0.26	AmpCipNaTe	1.33
0.26	AmpCxCotTr	1.33
0.2	AmpCotTr	2.66
0.2	CotTeTr	1.33
0.2	AmpNaTe	4
0.2	CotTeTr	1.33
0.2	CotNaTe	1.33

4.5.5.3 MAR Index and resistance patterns of *E. coli* isolates from station 3 (Off marine Science Jetty)

Nearly 9% of isolates from station 3 showed resistance against more than five antibiotics and 20% of isolates showed resistance to three or more antibiotics.

MAR index and resistance pattern of *E. coli* isolates from station 3 is given in Table 4.5. MAR index varied from 0.2 to 0.8. There was a moderate diversity among the resistance patterns among *E. coli* isolates from station 3. As in the case of isolates from station2, AmpNaTe was the frequently encountered pattern of antibiotic resistance among isolates from station 3.

Table 4.5 MAR index and resistance pattern *E. coli* (n = 69) isolates from station 3 (Off Marine Science Jetty)

MAR index	Resistance Pattern	% incidence
0.8	AmpCxCpdCtrCipCCotGenNaSTeTr	1.44
0.66	AmpCtxCxCpdCipCotNaSTeTr	1.44
0.6	AmpCtxCxCpdCazCtrCxmSTe	1.44
0.53	AmpCxCpdCxmNaSTeTr	1.44
0.4	AmpCxCpdCxmCotTr	1.44
0.4	AmpCipCotNaSTr	1.44
0.33	AmpCxCpdSTe	1.44
0.33	AmpCxCpdNaTe	1.44
0.33	AmpCotSTeTr	1.44
0.26	AmpCxCpdTe	1.44
0.26	CotSTeTr	1.44
0.26	AmpCxCotTr	1.44
0.26	AmpCtxCxCip	1.44
0.26	AmpCxCpdNa	1.44
0.2	AmpNaTe	4.34
0.2	AmpCxCpd	1.44
0.2	AmpCxCxm	1.44
0.2	AmpSTe	1.44
0.2	STeTr	2.89
0.2	AmpCxTe	1.44
0.2	NaTeTr	1.44
0.2	CipNaTe	1.44
0.2	AmpCxGen	1.44

4.5.5.4 MAR Index and resistance patterns of *E. coli* isolates from station 4 (Thevara)

About 12% and 18% isolates from station 4 showed resistance against more than 5 and 3 antibiotics, respectively. MAR index and resistance pattern of *E. coli* isolates from station 4 is given in Table 4.6. MAR index varied from 0.2 to 0.6. There was not much diversity among the resistance patterns among *E. coli* isolates from station 4. AmpCxCpd was the frequently encountered pattern of antibiotic resistance among isolates from station 4.

Table 4.6 MAR index and resistance pattern of *E. coli* (n = 49) isolates from station 4 (Thevara)

MAR index	Resistance Pattern	% incidence
0.6	AmpCxCpdCxmCipCotNaTeTr	2.04
0.46	AmpCipCotNaSTeTr	2.04
0.46	AmpCxCpdCxmCipNaTe	2.04
0.4	AmpCipCotNaTeTr	2.04
0.4	AmpCxmCotSTeTr	2.04
0.4	AmpCtxCxCotTeTr	2.04
0.26	AmpCotNaTr	2.04
0.26	CotNaTeTr	2.04
0.26	AmpCxCpdNa	2.04
0.2	AmpCotTr	2.04
0.2	CotTeTr	2.04
0.2	NaTeTr	2.04
0.2	NaGenTe	2.04
0.2	AmpCxCpd	4.08

4.5.5.5 MAR Index and resistance patterns of *E. coli* isolates from station 5 (Barmouth)

About 12% and 20% isolates from station 5 showed resistance against more than 5 and 3 antibiotics, respectively. MAR index and resistance pattern of *E. coli* isolates from station 5 is given in Table 4.7. MAR index varied from 0.2 to 0.73. There was a low diversity among the resistance patterns among

E. coli isolates from station 5. NaSTe was the frequently encountered pattern of antibiotic resistance among isolates from station 5.

Table 4.7 MAR index and resistance pattern of *E. coli* (n = 60) isolates from station 5 (Barmouth)

MAR index	Resistance Pattern	% incidence
0.73	AmpCtxCxCpdCazCtrCxmCCotSTr	1.66
0.53	AmpCxCpdCCotNaTeTr	1.66
0.46	AmpCxCpdCotNaTeTr	1.66
0.46	AmpCxCipCotNaTeTr	1.66
0.46	AmpCtxCxCpdCazCtrCxm	1.66
0.4	AmpCipCotNaTeTr	1.66
0.4	AmpCotCipNaTeTr	1.66
0.33	AmpCotNaTeTr	3.33
0.33	AmpCxCpd Ctr Na	1.66
0.26	CotNaTeTr	1.66
0.26	AmpCxCpdNa	1.66
0.26	AmpCotTeTr	5
0.2	CotTeTr	6.66
0.2	NaSTe	8.33
0.2	AmpCxCpd	5
0.2	Amp CxNa	1.66

4.5.6 MAR Index and resistance patterns of *E. coli* isolates from clinical sources

Games-Howell analysis showed that MAR index of clinical isolates were significantly higher when compared to that of *E. coli* from seafood and clinical origin ($p = 0.000$). Fifty five percentages of isolates showed resistance against more than 5 antibiotics. Sixty seven percentages of isolates showed resistance against more than 3 antibiotics. MAR index and resistance pattern of *E. coli* isolates from clinical sources is given in Table 4.8. MAR index varied from 0.2 to 1. There was a high diversity among the resistance patterns

among *E. coli* isolates from clinical sources. AmpCxCpd was the frequently encountered pattern of antibiotic resistance among isolates from clinical sources.

Table 4.8 MAR index and resistance pattern of *E. coli* isolates from clinical sources (n = 100)

MAR index	Resistance Pattern	% incidence
1	AmpCtxCxCpdCazCtrCxmCCipCotGenNaSTe Tr	1
0.93	AmpCtxCxCpdCazCtrCxmCCipCotGenNaTeTr	1
0.93	AmpCtxCxCpdCazCtrCxmCipCotGenNaSTeTr	1
0.86	AmpCtxCxCpdCazCtrCxmCotGenNaSTeTr	3
0.86	AmpCtxCxCpdCazCtrCxmCCipGenNaSTe	2
0.86	AmpCtxCxCpdCazCtrCxmCipCotNaSTeTr	2
0.86	AmpCtxCxCpdCtrCxmCCipCotGenNaTeTr	1
0.86	AmpCtxCxCazCtr CxmCCipCotNaSTeTr	1
0.86	AmpCtxCxCpdCazCxmCipCotGenNaSTeTr	1
0.8	AmpCtxCxCpdCazCtrCxmCCotNaSTr	1
0.8	AmpCtxCxCpdCazCtrCxmCCipCotNaTeTr	2
0.8	AmpCtxCxCpdCxmCipCotGenNaSTeTr	1
0.8	AmpCtxCxCpdCazCtrCxmCipCotGen NaTr	1
0.8	AmpCtxCxCpdCazCxmCotCipNaSTeTr	1
0.8	AmpCtxCxCxmCpdCipCotGenNaSTeTr	2
0.8	AmpCtxCxCpdCazCtrCxmCCotNaSTr	1
0.73	AmpCtxCxCpdCazCtrCxmCipGenNaTe	1
0.73	AmpCtxCxCpdCxmCotGenNaSTeTr	2
0.73	AmpCtxCxCpdCxmCipCotGenNaTeTr	1
0.73	AmpCtxCxCpdCxmCipCotNaSTeTr	1
0.66	AmpCxCpdCCipCotNaSTeTr	1
0.66	AmpCtxCxCpdCazCxmCCotNaTeTr	1
0.6	AmpCxCpdCipCotNaTeTr	1
0.6	AmpCtxCxCpdCxmCipGenNaTe	1
0.6	AmpCtxCxCpdCazCtrCxmCipNa	1
0.6	AmpCxCxmCpdCipCotNaTeTr	1
0.6	AmpCtxCxCxmCipCotNaTeTr	1
0.6	AmpCtxCxCpdCxmCotNaTeTr	1
0.6	AmpCtxCxCpdCxmCipCotNaTr	1
0.53	AmpCxCpdCipCotNaSTe	1

Table 4.8 cont…

0.53	AmpCtxCxCpdCxmCipNaTe	2
0.53	AmpCtxCxCpdCxmGenNaS	1
0.53	AmpCtxCxCpdCazCtrCxmNa	1
0.53	AmpCxCipCotNaSTeTr	1
0.53	AmpCxCpdCipCotNaSTr	1
0.53	AmpCxCpdCipCotNaTeTr	3
0.53	AmpCxCpdCotCxmCNaS	1
0.46	AmpCtxCxCpdCxmCipNa	1
0.46	AmpCxCpdCipNaCotTr	1
0.46	AmpCxCpdCotNaTeTr	2
0.46	AmpCxCpdCotSTeTr	1
0.46	AmpCpdCotNaSTeTr	1
0.46	AmpCxCotNaSTeTr	1
0.46	AmpCtxCxCpdCtrCxmGen	1
0.46	AmpCxCpdCazCotNaTr	2
0.46	AmpCtxCxCpdCxmCipNa	1
0.46	AmpCxCpdCipNaS	1
0.46	AmpCtxCazCxmCipNa	1
0.46	AmpCxCipCotNaTeTr	1
0.4	AmpCxCpdCazCtrCipNa	1
0.4	AmpCxCpdCot GenNaTr	1
0.4	AmpCxCpdCotNaTr	3
0.4	AmpCxCpdCotTr	1
0.4	AmpCxCpdNaTeTr	1
0.33	AmpCxCpdCipNa	1
0.33	AmpCxCpdNaS	1
0.33	AmpCxCpdCazCtr	1
0.26	AmpCxCpdS	4
0.26	AmpCotNaTr	1
0.26	AmpCxCipNa	4
0.26	AmpCxCpdCxm	2
0.26	AmpCxCpdNa	3
0.26	AmpCxCpdS	2
0.26	CotNaTeTr	1
0.2	AmpCxCpd	1
0.2	AmpCxNa	1
0.2	AmpCotTr	2
0.2	AmpCxCpd	6

4.5.7 MAR Index and resistance patterns of *E. coli* isolates from seafood sources

Only 13% of isolates showed resistance against more than 2 antibiotics. Four percentages of isolates showed resistance against more than 5 antibiotics. MAR index and resistance pattern of *E. coli* isolates from seafood sources is given in Table 4.9. MAR index varied from 0.2 to 0.86. There was a low diversity among the resistance patterns among *E. coli* isolates from seafood sources. AmpCxCpdS was the frequently encountered pattern of antibiotic resistance among isolates from seafood sources.

Table 4.9 MAR index and resistance pattern of *E. coli* isolates from seafood sources (n = 100)

MAR index	Resistance Pattern	% incidence
0.86	AmpCxCpdCazCtxCxmCCipCotGenNaTeTr	1
0.8	AmpCtxCxCpdCazCxmCCotNaSTeTr	1
0.73	AmpCtxCxCpdCxmCipCotNaSTeTr	1
0.4	AmpCotGenNaSTr	1
0.33	CotNaSTeTr	1
0.33	AmpCxCpdCxmTr	1
0.33	AmpCpdCotTeTr	1
0.26	AmpCotSTr	1
0.26	AmpCxCpdS	3
0.2	AmpNaTe	1
0.2	AmpCipS	1
0.2	AmpCpdCxm	1
0.2	AmpCxCpd	1

4.6 Discussion

4.6.1 Antibiotic resistance of *E. coli* isolates from Cochin estuary

The resistance to tested antibiotics found to vary among the strains, though collectively they exhibited resistance to all the antibiotics. Among the different antibiotics tested, resistance to ampicillin was relatively frequent (43%) when compared to others. Only 1.3% of isolates were resistant to ceftazidime.

E. coli isolates from Cochin estuary showed higher resistance to ampicillin, followed by tetracycline and nalidixic acid. Similar values of high resistance were observed by many workers in *E. coli* isolated from water samples (Talukdar *et al*., 2013; Olaniran *et al*., 2009). Ampicillin resistance was found in 43% of *E. coli* in the present study, which is consistent with the finding of Su *et al*., (2012). In contrast, Mataseje *et al*., (2009) and Henriques *et al*., (2006) who reported ampicillin resistance in 97% and 100% of *E. coli* isolates. In addition, very high percentages of resistance to chloramphenicol, tetracycline, streptomycin, trimethoprim and co-trimoxazole have been detected in *E. coli* isolated from river (Su *et al*., 2012) and waste waters (Garcia-Aljaro *et al*., 2009). However, isolates from Cochin estuary were mostly sensitive to chloramphenicol which is an important class of antibiotics. Koczura *et al*., (2012) reported that integron-harboring *E. coli* strains isolated from a river in Poland showed higher resistance to streptomycin, co-trimoxazole, trimethoprim and chloramphenicol. Previous studies reported very low percentages of resistance for ampicillin, tetracycline, streptomycin and sulphonamides in *E. coli* isolated from irrigation water and sediments (Roe *et al*., 2003) and surface waters (Dolejska *et al*., 2009), when compared

to our findings. This is quite possible as the selective pressure for resistant strains vary greatly at different geographical locations.

The moderate percentages of co-trimoxazole (19%) resistance among *E. coli* isolates from Cochin estuary are similar to those previously detected in *E. coli* isolates of water origin (Korzeniewska *et al*., 2013; Mataseje *et al*., 2009; Ozgumus *et al*., 2007). Cephalosporins, such as cefoxitin (21.33%) and cefpodoxime (14.66%) showed moderate resistance, whereas cefuroxime (3.66%), cefotaxime (3.33%), ceftriaxone (2.33%) and ceftazidime (1.33%) showed least resistance among *E. coli* isolated from Cochin estuary. Hospitals are the major source of the cephalosporin group of antibiotics in wastewater (Kummerer, 2009). Hospital sewage also brings with itself a huge amount of antibiotic-resistant bacteria and their gene pool (Unno *et al*., 2010). Galvin *et al*., (2010) reported resistance to cefotaxime of 7% *E. coli* strains isolated from hospital effluent. Our results are different than those obtained by Reinthaler *et al*., (2003), who reported that cephalosporins (cefotaxime and ceftazidime) and aminoglycosides were 100% effective for *E. coli* isolated from sewage in three different waste water treatment plants. Many studies reported that *E. coli* isolates of river were sensitive to cephalosporins (Su *et al*., 2012; Olaniran *et al*., 2009). Koczura *et al*., (2012) reported that integron-harboring *E. coli* strains isolated from a river in Poland showed lower resistance to cefotaxime, cefuroxime, and ceftazidime. Results of our indicates, the lowest resistance against ceftazidime (1.33%), which is similar to the observations by Mokracka *et al*., (2012), Dolejska *et al*., (2009) and Henriques *et al*., (2006). Mataseje *et al*., (2009) and Roe *et al*., (2003) also reported low level of resistance to ceftriaxone among *E. coli* isolated from water. Data from the SENTRY Asia-Pacific Surveillance Program (1998-

2002) shows that the prevalence and distribution of ESBLs varies considerably throughout the Asia-Pacific region. In the Philippines, phenotypic ESBL-producing *E. coli* and *K. pneumoniae* account for 5% and 21.9%, respectively (Hirakata *et al*., 2005). The CTX-M type has largely replaced the TEM and SHV types in Asia, like many other regions around the world (Hawkey, 2008). Korzeniewska *et al*., (2013) reported similar values of resistance for chloramphenicol and co-trimoxazole and higher values for cefotaxime, ceftazidime, and gentamicin in *E. coli* isolated from river water. Ozgumus *et al*., (2007) also reported lower resistance for chloramphenicol in *E. coli* isolated from tap and spring waters in a coastal region. Koczura *et al*., (2012) also reported that integron-harboring *E. coli* strains isolated from a river in Poland showed lower resistance to chloramphenicol.

It is interesting to point out that the isolates recovered from Cochin estuary showed in general low level of resistance to gentamicin, chloramphenicol, cefuroxime, cefotaxime, ceftriaxone and ceftazidime, and these values were lower than those previously reported for *E. coli* from environment (Sahoo *et al*., 2012). The low level of resistance of the *E. coli* isolates for gentamicin, chloramphenicol, cefotaxime, and ceftriaxone (2-4%) identified in our study, are similar to those previously reported in nondiarrheagenic *E. coli* isolated from different water sources (Batabyal *et al*., 2013). Our results on resistance of the *E. coli* to gentamicin and cefotaxime also similar to those previously reported in *E. coli* isolated from different sources of water supply in Kashmir, India (Rather *et al*., 2013). Similarly, low level of resistance of the *E. coli* isolates for cefuroxime, cefotaxime, and ceftazidime observed in our study are similar to those previously reported in *E. coli* isolated from waste water (Garcia-Aljaro *et al*., 2009). Coleman *et al*., (2013) reported that *E. coli* isolates from Canadian

drinking water, showed lower resistance to tetracyclines, aminoglycosides, β-lactams, sulphonamides, quinolones and choramphenicol. Ferreira da Silva *et al*., (2007) demonstrated higher percentage of amoxicillin and tetracycline resistance in *E. coli* isolated from the treated effluent than in *E. coli* isolated from the inflow of the same waste water treatment plant.

Nearly 10% of our *E. coli* isolates from estuary were resistant to ciprofloxacin. This is contrary to the observations of Nazir *et al*., (2005), who reported that *E. coli* isolates recovered from a river, ponds and tap waters in Bangladesh were sensitive to ciprofloxacin. Goni-Urriza *et al*., (2000) studied the antibiotic resistance of freshwater bacterial populations recovered from Arga River (Spain) and reported that the isolates belonging to *Enterobacteriaceae* were resistant to nalidixic acid, tetracycline and β-lactams and sensitive to quinolones. Mokracka *et al*., (2012) reported that isolates belonging to *Enterobacteriaceae* showed higher resistance against ampicillin, ciprofloxacin, co-trimoxazole, chloramphenicol, trimethoprim, and tetracycline.

In the present study, we found that more than 57% of the *E. coli* isolates were resistant to at least one of the 15 antibiotics tested and 42% of these isolates were multidrug-resistant, defined as resistant to three or antibiotics. Talukdar *et al*., (2013) found that about 49% isolates were multidrug-resistant.

Previous studies from our research group also showed high prevalence of antibiotic resistance in *E. coli* isolates from same region, although resistance levels were higher than the ones obtained in the present study (Chandran *et al*., 2008). Among the antibiotics high resistance was observed against kanamycin (85%), tetracycline (83%) and streptomycin (80%). The least resistances detected against gentamicin (14%) and chloramphenicol (10%). Several interesting

relationship emerged when the result of our study were compared with the previous study conducted by our researcher's in the same study area. Most of the *E. coli* isolates showed in general low percentage of resistance to chloramphenicol, ciprofloxacin, gentamicin, streptomycin and tetracycline and these values are lower than those previously reported by our research groups ($F = 14.9$, $p = 0.000$). This could point to a reduced use of these antibiotics during the last 10 years. *E. coli* isolates from two study period showing resistance to ampicillin and nalidixic acid were statistically significant ($p < 0.05$). All other antibiotics such as chloramphenicol, ciprofloxacin, gentamicin, streptomycin and tetracycline were showed significant difference ($p > 0.05$) over two study period.

4.6.2 Antibiotic resistance of *E. coli* isolates from seafood sources

Among individual antibiotics, the highest percentage of antibiotic resistance was observed against ampicillin (51%), followed by cefoxitin (14%), tetracycline (12%), cefpodoxime (11%), and streptomycin (11%). Between 10% and 5% resistance was found for nalidixic acid (8%), trimethoprim (8%), co-trimoxazole (8%), and cefuroxime (5%); resistance to cefotaxime (3%), ciprofloxacin (3%), gentamicin (3%), ceftazidime (2%), and chloramphenicol (2%). In general the prevalence of antibiotic resistance among the *E. coli* strains from seafood was much lower when compared to those from estuary. All the strains were sensitive to ceftriaxone. Although no significant differences in antibiotic resistance were seen among different phylogenetic groups (ANOVA, $p = 0.083$), antibiotic resistance of *E. coli* belonged to A was significantly higher than phylogenetic group D ($p = 0.009$). *E. coli* isolates from seafood sources collectively showed resistance against all the antibiotics tested except ceftriaxone. When the result of antibiotic

resistance for our *E. coli* isolates were compared with those previously reported (Bortolaia *et al*., 2010; Kumar *et al*., 2005; Sunde, 2005), the resistance level of our isolates were higher. Miles *et al*., (2006) also reported lower resistance than our results in *E. coli* except for nalidixic acid. Several studies also reported a lower resistance against *E. coli* isolates than our results except for streptomycin and tetracycline (Obeng *et al*., 2012; Ryu *et al*., 2012; Ghanbarpour and Akhtardanesh, 2010; Costa *et al*., 2008). Tetracycline and Streptomycin resistance in our study was found in 12% and 11% of *E. coli* isolates respectively, which was lower than that reported by several studies (Nsofor and Iroegbu, 2012; Stuart *et al*., 2012; Zakeri and kasheli, 2012; Scaria *et al*., 2010; Slama *et al*., 2010; Ho *et al*., 2009; Smet *et al*., 2008; Van *et al*., 2008; Vulfson *et al*., 2001).

Our findings are in agreement with data from several previous studies which found that resistance to tetracycline, streptomycin (aminoglycosides), and penicillins is common among *E. coli* isolated from food animals (Jouini *et al*., 2009; Vasilakopoulou *et al*., 2009; Van *et al*., 2008; Srinivasan *et al*., 2007; Schroeder *et al*., 2003). However resistance level in *E. coli* isolates of this study was lower than that reported by Sifuna *et al*., (2008) and Van *et al*., (2008).

Resistance of *E. coli* isolates from seafood to cephalosporins, such as cefuroxime, cefotaxime, ceftazidime was in all cases below 6%, and no resistant isolates were detected for ceftriaxone. However, resistance to cefoxitin and cepfodoxime was found in 14% and 11% of *E. coli* isolates respectively. Jones-Dias *et al*., (2013) reported moderate resistance (12% - 3%) for cephalosporins in *E. coli* isolated from animals and food products. In

addition very low percentages of resistance (0% - 1%) to cephalosporins have been detected in *E. coli* isolates of poultry (Soufi *et al*., 2011). Srinivasan *et al*., (2007) also reported a lower resistance against cephalosporins than our results, except cefuroxime (22%). We have observed that 5% *E. coli* isolates were resistant to cefuroxime.

Our results revealed ampicillin resistance in 51% of *E. coli*, which is consistent with Koo and Woo, (2011) who isolated *E. coli* from fish and fishery products. Kumaran *et al*., (2010) reported that 56% *E. coli* strains from seafood in India were resistant to ampicillin and more than 35% resistant to ciprofloxacin. Though the level of resistance to ampicillin was similar to our findings, ciprofloxacin resistance was much lower in our *E. coli* isolates from seafood. When the result of ampicillin resistance for our *E. coli* isolates were compared with those previously reported (Costa *et al*., 2008; Miles *et al*., 2006; Guerra *et al*., 2003; Marynard *et al*., 2003) the resistance rate for our isolates were higher. However, frequency of ampicillin in *E. coli* isolates of this study was lower than that reported by several studies (Nsofor and Iroegbu, 2012, Karczmarczyk *et al*., 2011; Ho *et al*., 2009; and Srinivasan *et al*., 2007). In addition, very high resistance to tetracycline and ampicillin have been reported in faecal isolates of healthy food-producing animals (Saenz *et al*., 2001). In India, Kumar *et al*., (2005) however reported a lower resistance of *E. coli* from seafoods (0% - 6%) to all the antibiotic agents.

Our studies revealed that 8% of *E. coli* isolates were resistant to co-trimoxazole and trimethoprim, which is comparable to the level seen in *E. coli* isolated from cattle (Guerra *et al*., 2003) and higher than previously reported from elsewhere (Ryu *et al*., 2012; Costa *et al*., 2008; Sunde, 2005).

Seputiene *et al*., (2010) and Hammerum *et al*., (2006) reported 18% - 25% of sulphonamide resistance against *E. coli* isolated from human and animal origin. Boerlin *et al*., (2005) reported that the frequency of resistance to all the antibiotic agents, except streptomycin, was consistently higher among isolates from cases of diarrhea than among those from healthy finisher pigs.

Nalidixic acid and ciprofloxacin resistance in our study was found in 8% and 3% of *E. coli* isolates respectively. Similar to our findings, resistance to ciprofloxacin in *E. coli* isolates from animal sources was reported as either low (Teshager *et al*., 2000) or non-existent (Klein and Bulte, 2003; Schroeder *et al*., 2003; Meng *et al*., 1998) in developed countries, perhaps due to restricted uses of fluoroquinolones in animal husbandry in these countries. In a country such as Canada, fluoroquinolones are not registered for use in pigs, therefore *E. coli* isolated from this source showed very little resistance to these antibiotics (Boerlin *et al*., 2005). Fluoroquinolones are critically important for treating serious infections in humans, and the likelihood that resistance to fluoroquinolones in *E. coli* was induced by the use of these antibiotics in food animals is a concern.

4.6.3 Antibiotic resistance among *E. coli* isolates from clinical sources

When the result of antibiotic resistance for our clinical *E. coli* isolates were compared with those previously reported (Villegas *et al*., 2011; Vinue *et al*., 2010; Machado *et al*., 2005; Nijssen *et al*., 2004; Khan *et al*., 2002; Ruiz *et al*., 2002; Cooksey *et al*., 1990), the resistance rates for our isolates were found to be higher. Clinical *E. coli* isolates showed higher resistance to ampicillin. Similar values of high resistance were observed Kanamori *et al*., (2011). Arredondo-Garcia and Amabile-Cuevas, (2008) studied the antibiotic

resistance of uropathogenic *E. coli*, and reported resistance was lower than our results, except for co-trimoxazole. Results of the present study revealed that 54% and 55% of clinical *E. coli* isolates were resistant to co-trimoxazole and trimethoprim, respectively. But, Yu *et al*., (2004) and Lee *et al*., (2001) reported higher trimethoprim resistances in 69% and 63% of clinical *E. coli* isolates. Bindayna and Murtadha, (2011) reported high percentages of resistance for ciprofloxacin (98%) and trimethoprim (93%). *E. coli* isolates from clinical specimens are mostly resistant to multiple antibiotics and a substantial proportion of *E. coli* isolates from the urinary tract is resistant to trimethoprim (Lee *et al*., 2001; Seol *et al*., 1997; Huovinen *et al*., 1995).

Hussain *et al*., (2012) studied the antibiotic resistance of uropathogenic *E. coli* recovered from an Indian hospital and reported that the isolates were resistant to co-trimoxazole, ciprofloxacin, chloramphenicol, tetracycline, gentamicin, which was higher than the level encountered in our results, except for chloramphenicol. Sawma-Awad *et al*., (2009) worked on isolates collected from Lebanon and reported also a high level of resistance to ampicillin (68%), 43% resistance to tetracycline and 38% to co-trimoxazole. Antibiotic resistance of our clinical isolates were higher than those observed in commensal *E. coli* strains (Shakya *et al*., 2013; Infante *et al*., 2005; Kang *et al*., 2005a; Rodrigues-Bano *et al*., 2004; Cooksey *et al*., 1990).

Our results revealed resistance of clinical isolates of *E. coli* against aminoglycosides, at 38% and 22% against streptomycin, gentamicin respectively. Karaca *et al*., (2005) studied the co-trimoxazole and quinoline resistance in *E. coli* isolated from urinary tract infections over ten years and reported that the ratio for co-trimoxazole resistance showed high levels at the beginning of

the period, with a peak in 1996 (69.3%), and then decreased to the minimum level in 2003 (38.5%). Karaca *et al*., (2005) also reported that quinolones showed an opposite distribution, with the lowest resistance ratio occurring in 1996 (5.2%) and the resistance ratio increased over the years, showing the highest ratio in 2002. In our study clinical *E. coli* isolates showed resistance against quinolones, with 78% and 47% against nalidixic acid and ciprofloxacin, respectively. Ruiz *et al*., (2002) reported that 25% of clinical *E. coli* isolates showed resistance against nalidixic acid and ciprofloxacin, which was lower than our results.

Batard *et al*., (2013) studied the relationship between hospital antibiotic use and quinolone resistance in *E. coli* and reported that the incidence of quinolone-resistant isolates was independently associated with the consumption of tetracyclines, cephalosporins and quinolones. Tetracycline resistance was found in 46% of clinical *E. coli* in the present study. Baudry *et al*., (2009) reported that the highest co-resistance among ESBL-producing *E. coli* was to the fluoroquinolones. A major concern regarding ESBL-producing *E. coli* is its high rate of co resistance to non-β-lactam antibiotics, particularly quinolones, trimethoprim-sulfamethoxazole, and aminoglycosides (Azap *et al*., 2010). Studies from Israel in 2004 and Spain in 2006 reported that ciprofloxacin resistance was 39% and 31.5% in ESBL-producing *E. coli* isolates, respectively (Calbo *et al*., 2006; Colodner *et al*., 2004). In Turkey, an extremely high rate of ciprofloxacin resistance (84%) among ESBL-producing *E. coli* was reported (Azap *et al*., 2010).

In our study 12% of *E. coli* isolates found to be resistant to chloramphenicol, which is consistent with *E. coli* isolated from blood origin (Vinue *et al*., 2010) and with *E. coli* isolated from healthy adults (Kang *et al*., 2005a; Kronvall *et al*., 2005). However, higher resistance for chloramphenicol has been reported in clinical isolates by several workers (Martinez *et al*., 2012; Erjavec *et al*., 2007; Hsu *et al*., 2006; Machado *et al*., 2005).

E. coli isolates of clinical origin showed higher resistance against cephalosporins. The observed 91% resistance to cefoxitin in this study corroborates the finding of Hoban *et al*., (2012), who reported 88.4% and 94.5% cefoxitin resistance in America and Europe respectively. In contrast, numerous studies reported lower resistance for cefoxitin than our results (Kanamori *et al*., 2011; Oteo *et al*., 2006; Nijssen *et al*., 2004; Rodriguez-Bano *et al*., 2004; Jacoby and Han, 1996). In our study resistance for cefotaxime and ceftazidime were 41% and 27%, respectively. Huang *et al*., (2012) based on the 2011 CLSI criteria, reported that 40.4% of ESBL-producing *E. coli*, were susceptible to ceftazidime. Huang *et al*., (2012) also reported that the proportion of ESBL-producing *E. coli* isolates increased from 14.5% in 2002 to 40.4% in 2010. In addition, very high percentages of resistance to cefotaxime and ceftazidime have been detected in clinical isolates (Jean *et al*., 2013). Oteo *et al*., (2006) reported that about 28% of ESBL *E. coli* isolates showed resistance to cefotaxime and ceftazidime. Pitout *et al*., (2004) studied the antibiotic resistance of ESBL producing *E. coli* isolates and reported that all *E. coli* isolates were resistant to cepfodoxime, 71% were resistant to cefotaxime, and 17% were resistant to ceftazidime.

Data from the SENTRY Asia-Pacific Surveillance Program (1998-2002) showed that the prevalence and distribution of ESBLs varies considerably throughout the Asia-Pacific region. In the Philippines, phenotypic ESBL-producing *E. coli* account for 5% (Hirakata *et al*., 2005). Our results revealed that 82% of clinical *E. coli* isolates were showed resistance against cefpodoxime. Kanamori *et al*., (2011) studied antibiotic resistance of clinical *Enterobacteriaceae* isolates and reported that all isolates were resistant to cefpodoxime. Korzeniewska and Harnisz, (2013) studied the prevalence of ESBL-producing *Enterobacteriaceae* in sewage samples from three hospitals in Poland and reported that (95.2%), (81.6%) and (73.5%) isolates were resistant to cefotaxime, ceftazidime and cefpodoxime, respectively. Results of the present study revealed that 45% and 23% of clinical *E. coli* isolates were resistant to cefuroxime and ceftriaxone, respectively. Giufre *et al*., (2012) reported that multidrug-resistant strains isolated from urinary tract infections have frequently showed triple resistance to ciprofloxacin, ampicillin and trimethoprim/ sulfamethoxazole. In a study performed with clinical strains obtained worldwide (Winoku *et al*., 2001) it was showed that co-resistance to aminoglycosides, tetracyclines, quinolones and the combination sulfamethoxazole/ trimethoprim was common among ESBL positive strains from all geographic regions under study.

Perrin *et al*., (1999) reported that nosocomial strains were significantly more resistant to co-trimoxazole and first generation quinolones. Pitout *et al*., (2009, 2007) reported higher resistance for co-trimoxazole, gentamicin, and ciprofloxacin. Shahid *et al*., (2008) studied the antibiotic resistance of *E. coli* recovered from an Indian tertiary care hospital and reported that the isolates were resistant to gentamicin, cefotaxime and ceftriaxone, which was lower than our

results except for gentamicin. White *et al*., (2001) reported that urinary isolates were resistant to ampicillin, tetracycline, trimethoprim, and streptomycin, which was lower than our results except for streptomycin. Bartoloni *et al*., (2009) studied the antibiotic resistance in a very remote human community of the Peruvian Amazonas with minimal antibiotic exposure, and reported high levels of acquired resistance to the oldest antibiotics such as ampicillin, tetracycline, trimethoprim/sulfamethoxazole, streptomycin and chloramphenicol.

Present study on antibiotic resistance of *E. coli* from three different sources such as estuary, seafood and clinical revealed clearly different level of drug resistance. While *E. coli* from estuarine water had considerably high and diverse patterns of antibiotic resistance when compared to those from seafood, whereas the clinical strains of *E. coli* had significantly higher level of resistance to all the antibiotics tested in the study. Results on antibiotic resistance patterns also revealed the difference on the sources of the *E. coli* strains. In general estuary offers a conducive environment for gene transfer, which is reflected in much more diverse patterns of resistance among the *E. coli* from this source. For majority of the antibiotics tested, we observed source-dependent differences in frequency of resistant isolates. Resistance to aminoglycosides, cephalosporins, quinolones, sulphonamides, and trimethoprim occurred more often among clinical isolates. Since all of the clinical isolates were cultured from hospital inpatients, it can be assumed that elevated frequency of resistance may be partially due to selection imposed by hospital environment selection imposed by hospital environment.

Antibiotic resistance of *E. coli* belonged to phylogenetic group A and D was significantly varied among all stations in Cochin estuary. But there was no

significant difference in antibiotic resistance of *E. coli* belonged to phylogenetic group B1and B2. But *E. coli* isolates of seafood and clinical origin belonging to non pathogenic groups such as A and B1 showed more antibiotic resistance than pathogenic groups B2 and D. There was a significant difference in antibiotic resistance were seen among the *E. coli* isolates of different origin. Antibiotic resistance of *E. coli* belonged to phylogenetic group A, B1, B2 and D was significantly higher in *E. coli* of clinical origin than those from seafood and estuary.

E. coli isolates from station 2 (Bolgatty) and station 3 (Off Marine Science Jetty), compared to those recovered from the other stations, were more frequently resistant to cefotaxime, co-trimoxazole, trimethoprim and fluoroquinolones. We conclude that the high prevalence of multidrug resistance detected in stations close to Cochin City is a matter of concern, since there is a large reservoir of antibiotic resistance genes within the community, and that the resistance genes and plasmid-encoded virulent genes were easily transferable to other strains. The indiscriminating use of antibiotics in clinical as well as in veterinary and agriculture has brought about an increasing antibiotic residues in the environment. Hence the use of antibiotics should be restricted to the treatment of invasive illness and not used for uncomplicated diseases and agricultural purpose. There should be a strict monitoring for the waste disposal to the water bodies.

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Chapter 5

Prevalence of antibiotic resistance genes in *Escherichia coli* isolates from estuarine, seafood and clinical sources

5.1 Introduction

E. coli, a dominant facultative anaerobe in the gut and an important human pathogen, may be a major reservoir of antibiotic resistance determinants on conjugative plasmids. The escalating problem of emergence of antibiotic resistant bacteria and their resistant genes is becoming a major global health issue (Levy, 2002; Chee-Sanford *et al*., 2001). Antibiotic resistance genes may be spread on mobile genetic elements such as plasmids (Schuurmans *et al*., 2014), transposons and integrons. Bacteria carrying integrons might transfer antibiotic resistance genes from animals to animals as well as to humans (Aarestrup and Wegener, 1999; Recchia and Hall, 1995). Many gene cassettes

of integrons contain antibiotic resistance genes and thus play an important role in the dissemination of antibiotic resistance genes and the development of multidrug resistance. The spread of antibiotic resistance genes present in gene-transfer units and dissemination of multiresistant bacteria in nature may have consequences for human health and the evolution of environmental microbiota (Martinez, 2009). Antibiotic resistance may develop through mutations in chromosomal DNA or horizontal gene transfer of mobile elements.

Resistance genes carried by the commensal bacteria in environment are of clinical importance because resistant strains probably originate from the natural environment. They are then carried to a clinical setting by the discharges of carriers such as microbiota of the healthy people under antibiotic selective pressure. There is growing evidence that the environment plays a role in the spread of antibiotic resistance among pathogenic strains. Many questions have been raised concerning the impact of the release of antibiotics and antibiotic-resistant bacteria on the environment or on human and animal health (Aminov, 2010). Previous investigations showed prevalence of antibiotic resistance genes and integrons on surface water in the United States (Storteboom *et al*., 2010), France (Laroche *et al*., 2009), Portugal (Henriques *et al*., 2006), Czech Republic (Dolejska *et al*., 2009), Korea (Kim *et al*., 2008), Vietnam (Phan *et al*., 2011; Takasu *et al*., 2011; Hoa *et al*., 2008), Thailand (Takasu *et al*., 2011), India (Kumar *et al*., 2005), and China (Chen *et al*., 2010). The spreading of multiple resistance bacteria such as *E. coli* in aquatic environments, especially in drinking water source, is also a threat to human health. The main concern is that those antibiotic resistance genes in surface water may persist in water treatment processes and revive in the final drinking water. Several studies have attempted to evaluate the impacts of antibiotic use

on antibiotic resistance, and antibiotic resistance genes in the environment have been studied as an emerging contaminant (Biswal *et al*., 2014; Pruden *et al*., 2006). Sixteen tetracycline resistance genes (Pruden *et al*., 2006; Pei *et al*., 2006; Kim *et al*., 2004; Smith *et al*., 2004; Aminov *et al*., 2002; Aminov, 2001), three sulphonamide resistance genes (Pruden *et al*., 2006), and 10 β-lactamase resistance genes (Henriques *et al*., 2006) have been detected in river sediment, seawater, irrigation ditches, dairy lagoons, and the effluents of wastewater recycling and drinking water, indicating the ubiquitous occurrence of these resistance genes. The food chain probably also takes place in the transit of integrons from the environments to the human. Indeed, bacteria harbouring integrons have been recovered from a variety of aquatic living organisms, such as in prawns, with an *Enterobacter cloacae* harbouring a *class1 integrons* (Gillings *et al*., 2009). All these results underline the link, *via* the food chain, between the environmental integrons and the human or animal integrons.

5.2 Review of literature

5.2.1 Antibiotic resistance genes

5.2.1.1 TEM

The first plasmid-mediated β-lactamase in gram-negatives, TEM-1, was described in the early 1960s (Datta and Kontomichalou, 1965). The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM (Medeiros, 1984). Being plasmid and transposon mediated has facilitated the spread of TEM-1 to other species of bacteria. Within a few years after its first isolation, the TEM-1 β-lactamase spread worldwide and is now found in many different species of members of the family *Enterobacteriaceae,*

Pseudomonas aeruginosa, *Haemophilus influenza* and *Neisseria gonorrhoeae*. Another common plasmid mediated β-lactamase found in *Klebsiella pneumoniae* and *E. coli* was SHV-1.

The genes encoding β-lactamases can be located on the bacterial chromosome, on plasmids, or on transposons. The genetic environment of the β-lactamase (*bla*) gene dictates whether the β-lactamases are produced in a constitutive or inducible manner. Recently, an increasing number of *bla* genes are being discovered on integrons (Weldhagen, 2004). Mobile genetic elements that contain integrons are an important source for the spread of *bla* genes and for the dissemination of other resistance determinants. Serving as a "sink" for resistance genes, integrons are not mobile; their location in mobile genetic elements (plasmids, transposons) enables their movement (Babic *et al*., 2006). ESBLs are capable of hydrolyzing third-generation cephalosporins and monobactams, and are plasmid-mediated β-lactamases that are easily transferable among different bacteria (Bush, 2010; Pitout and Laupland, 2008). ESBLs are categorized into three types, which are TEM, SHV, and CTX-M. The CTX-M β-lactamases have become the most prevalent in the world since 1995, and have been divided into five groups known as CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (Pitout and Laupland 2008; Bonnet, 2004).

TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Livermore, 1995). This enzyme is also responsible for the ampicillin and penicillin resistance that is seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. TEM-1 is able to hydrolyze penicillins

and early cephalosporins such as cephalothin and cephaloridine. TEM-2, the first derivative of TEM-1, had a single amino acid substitution from the original β-lactamase (Barthelemy *et al*., 1985). TEM, SHV, and CTX-M are the 3 main families of ESBLs described. While TEM and SHV ESBLs arise via substitutions in strategically positioned amino acids from the natural narrow-spectrum TEM-1/-2 or SHV-1 β-lactamase, all CTX-M variants demonstrate an ESBL phenotype (Gniadkowski, 2008; Paterson and Bonomo, 2005). Until the 1990s, most ESBLs identified were of SHV- and TEM-types. At present, the CTX-M (especially CTX-M-15) are the most prevalent ESBLs worldwide (Seiffert *et al*., 2013; Rossolini *et al*., 2008; Livermore *et al*., 2007).

To date, hundreds of discrete β-lactamases have been described. Most important are plasmid-bound cephalosporinases, and the ESBLs. ESBLs, predominantly variants of the TEM and SHV-lactamases family, have been first described in 1983 in Germany (Knothe *et al*., 1983). These variants show single-nucleotide polymorphisms within the TEM and SHV gene, which change the activity spectrum of β-lactamases (Chaibi *et al*., 1999). They are most prevalent in *Klebsiella spp*. and *E. coli* and are capable of inactivating a wide variety of β-lactam antibiotics including third-generation cephalosporins and monobactams.

Other mechanisms contributing to β-lactam resistance involve AmpC-type cephalosporinases, alteration of porin channels with possible antibiotic efflux, hyperproduction of specific β-lactamases and inhibitor-resistant mutants (Kaye *et al*., 2004; Bush, 2001). The TEM enzymes and SHV β-lactamases have been known as ESBL-producing groups of β-lactamases for a long time (Bush, 2010). In contrast to them, CTX-M β-lactamases as a group

have only increased in significance in recent years (Eisner *et al*., 2006; Paterson and Bonomo, 2005). Previous studies have shown that ESBL mediating plasmids may carry more than one β-lactamase gene such as CTX-M and TEM (Woodford *et al*., 2009), and that they may be responsible for high level β-lactamase resistance phenotypes (Kiratisin *et al*., 2008). TEM β-lactamases have been known as an ESBL-producing group of β-lactamases for a long time. Many studies have reported the occurrence of bla_{TEM} gene in aquatic environment (Blaak *et al*., 2014; Ojer-Usoz *et al*., 2014; Korzeniewska *et al*., 2013; Korzeniewska and Harnish, 2013; Hu *et al*., 2008; Hamelin *et al*., 2006; Henriques *et al*., 2006). Several studies have reported the presence of bla_{TEM} gene in *E. coli* isolated from cattle (Karczmarczyk *et al*., 2011; Scaria *et al*., 2010; Guerra *et al*., 2003), poultry (Bagheri *et al*., 2014; Ahmed *et al*., 2013; Obeng *et al*., 2012; Soufi *et al*., 2011; Brinas *et al*., 2002), house hold pigeons (Hasan *et al*., 2014) pig (Smith *et al*., 2010; Blanc *et al*., 2006; Marynard *et al*., 2003), and fish (Ryu *et al*., 2012; Van *et al*., 2008). Previous studies reported the occurrence of bla_{TEM} in clinical *E. coli* isolates (Hussain *et al*., 2012; Shahid *et al*., 2012; Martinez *et al*., 2012; Bindayna and Murtadha, 2011; Sharma *et al*., 2010; Lavigne *et al.,* 2007; Velasco *et al*., 2007; Karisik *et al*., 2006; Machado *et al*., 2005; Eckert *et al*., 2004; Pallecchi *et al*., 2004; Chanawong *et al*., 2002; Cooksey *et al*., 1990).

5.2.1.2 $bla_{CTX\text{-}M}$

In recent years a new family of plasmid-mediated ESBLs, called CTX-M, that preferentially hydrolyze cefotaxime has arisen. These enzymes are not very closely related to TEM or SHV

β-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases (Tzouvelekis *et al*., 2000). The most common cause of resistance to β-lactams is the production of extended-spectrum β-lactamases (ESBLs), especially of the CTX-M type (Pitout, 2012). The CTX-M ESBLs (resistant to cefotaxime and ceftriaxone and usually susceptible to ceftazidime) comprise a rapidly emerging group. There are now more than 40 unique CTX-M type β-lactamases that are divided into five groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M- 25 group) (Delmas *et al*., 2006; Bonnet, 2004). Unlike the TEM and SHV family ESBLs, evidence is lacking that CTXM ESBLs arose through single amino acid mutations. It is thought, CTX-M ESBLs arose by plasmid acquisition of pre existing chromosomal ESBL genes from the *Kluyvera* spp. (Bonnet, 2004).

The first reports describing CTX-M β-lactamases were from Western Europe. CTX-M-1 was found in an *E. coli* clinical strain isolated in Germany in 1989 (Bauernfeind *et al*., 1990). The CTX-M-type β-lactamases belong in a quite heterogeneous lineage of molecular class A active site-serine β-lactamases, which includes at least six sublineages or groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC, named after the arche-typal enzymes of each group) that differ from each other by ≥10% amino acid residues. Unlike many acquired β-lactamases for which the original sources remains unknown, the source of bla_{CTX-M} genes has been identified in some species of genus *Kluyvera*, a member of the family *Enterobacteriaceae* that is rarely associated with human infections (Sarria *et al*., 2001). In fact, close homologs of acquired bla_{CTX-M} genes have been detected in the chromosome of strains of *Kluyvera cryocrescens* (genes encoding CTX-M variants of the CTX-M-1 and KLUC groups) (Decousser *et al*., 2001), *Kluyvera ascorbata* (genes encoding

CTX-M variants of groups 1 and 2) (Rodriguez *et al*., 2004; Humeniuk *et al*., 2002), and *Kluyvera georgiana* (genes encoding CTX-M variants of groups 8, 9 and 25) (Rodriguez *et al*., 2010; Olson *et al*., 2005; Poirel *et al*., 2002). Each CTX-M group is likely derived from one or more different *Kluyvera* strains, and the existence of several groups of transferable $bla_{CTX\text{-}M}$ genes is consistent with a history of multiple gene capture events. Minor allelic variants within each group could reflect either different gene capture events or post-capture protein microevolution occurred in secondary hosts and likely influenced by the selective pressure encountered in clinical and veterinary settings. Finally, the CTX-M enzymes with hybrid structure are apparently derived from recombination events between genes of different groups or of different sources. In fact, co-existence of multiple CTX-M variants in the same host has been reported (Morosini *et al*., 2010; Sun *et al*., 2010) and could favour the emergence of such hybrid enzymes. The occurrence of at least three hybrids between CTX-M-15 and CTX-M-14 (i.e. CTX-M-64, CTX-M-123 and CTX-M-132) could depend on the high prevalence of these variants (Zhao and Hu, 2013; Canton *et al*., 2012). The association of CTX-M-encoding plasmids with highly successful virulent clonal lineages of *E. coli* and *K. pneumonia* has generated a number of the so-called "high-risk" multiresistant and virulent clones (Woodford *et al*., 2011) that have further contributed to the rapid and global dissemination of CTX-M-type ESBLs.

The $bla_{CTX\text{-}M}$ gene was identified as the predominant ESBL genotype in clinical isolates (Dzierzanowska *et al*., 2010; Mugnaioli *et al*., 2006), fecal isolates of humans (Vinue *et al*., 2009), sewages sludge (Ojer-Usoz *et al*., 2014; Reinthaler *et al*., 2010) and recreational waters (Blaak *et al*., 2014). The CTX-M-1-like (e.g., CTX-M-1, -15, and -32) ESBLs are widely distributed

among *E. coli* isolates of animal and human origin (Carattoli, 2008; Coque *et al*., 2008a; Perez *et al*., 2007). In particular, CTX-M-1 is first in rank in poultry from the Netherlands (Leverstein-van Hall *et al*., 2011), France (Girlich *et al*., 2007), and Belgium (Smet *et al*., 2008); in pigs from Spain (Cortes *et al*., 2010) and Portugal (Goncalves *et al*., 2010); and in animal companions in the Czech Republic, Italy, and Portugal (Dolejska *et al*., 2011; Carattoli *et al*., 2008).

5.2.1.3 Tetracycline resistance genes (*tetA* and *tetB*)

Historically, the genotyping of tetracycline resistance genes in bacterial isolates has been performed by hybridization with conventional probes generated from known tetracycline resistance determinants or polynucleotides prepared from restriction fragments of bacterial DNA or plasmids containing a portion of the target gene (Frech and Schwarz, 2000; Andersen and Sandaa, 1994; DePaola *et al*., 1993, 1988; Marshall *et al*., 1983). This method is not sufficiently sensitive or ‘‘user friendly’’ for clinical and environmental studies, but has lead to the identification of *tet* gene classes based on having over 80% DNA homology (Levy *et al*., 1999). More recently, PCR-based approaches were developed for the detection of tetracycline efflux pumps of Gram-negative bacteria in environmental samples and in clinical isolates (Hartman *et al*., 2003; Aminov *et al*., 2002; Ng *et al*., 2001; Guardabassi *et al*., 2000). Some studies have tried to develop multiplex conventional PCR for DNA based simultaneous detection of a group of different *tet* gene classes that resulted in a significant savings of labour and cost in screening a large number of samples (Strommenger *et al*., 2003; Hartman *et al*., 2003; Aminov *et al*., 2002; Ng *et al*., 2001; Guardabassi *et al*., 2000; Warsa *et al*., 1996; Roberts *et al*., 1993).

The *tet* genes are found in a variety of bacteria isolated from humans, animals, and the environment. The majority of the *tet* genes are associated with either conjugative or mobilizable elements, which may partially explain their wide distribution among bacterial species (Recchia and Hall, 1995; Jones *et al*., 1992; Mendez *et al*., 1980). The Gram-negative *tet* efflux genes are found on transposons inserted into a diverse group of plasmids from a variety of incompatibility groups (Jones *et al*., 1992; Mendez *et al*., 1980). Gram-positive efflux genes are associated with small plasmids (Schwarz *et al*., 1998, 1992; Khan and Novick, 1983). The ribosomal protection genes *tet*(S) and *tet*(O) can be found on conjugative plasmids, or in the chromosome, where they are not self-mobile (Luna and Roberts, 1998; Charpentier *et al*., 1994, 1993). The *tet*(M) and *tet*(Q) genes are generally associated with conjugative chromosomal elements, which code for their own transfer (Clewell *et al*., 1995; Li *et al*., 1995; Salyers *et al*., 1995a, b). These conjugative transposons transfer mobilizable plasmids to other isolates and species and even unlinked genomic DNA (Clewell *et al*., 1995; Li *et al*., 1995; Manganelli *et al*., 1995; Needham *et al*., 1994; Showsh and Andrews, 1992; Brown and Roberts, 1987).

Tetracycline resistance originates in most cases from acquiring resistance genes and not from mutations. Two relevant mechanisms of tetracycline resistance have been identified (i) efflux pumps and (ii) ribosomal protection. Of the 33 distinct tetracycline resistance genes (*tet* genes), 23 code for efflux pumps enabling bacteria to transport tetracycline actively out of the cell. The remaining 10 genes encode ribosomal protection proteins. The relevant mechanism in *E. coli* is the active efflux mechanism, encoded by all seven tet genes, so far identified in *E. coli* (Roberts, 2003; Chopra and Roberts, 2001).

The *tet*(E) gene differs from the *tet*(A), *tet*(B), *tet*(C), and *tet*(D) genes because it is associated with large plasmids which are neither mobile nor conjugative (DePaola and Roberts, 1995; Sorum *et al*., 1992).

The major mechanisms of tetracycline resistance are known to be efflux pump activity, ribosomal protection, and enzymatic inactivation. Various *tet* genes confer resistance by these mechanisms. Among the forty tetracycline resistance genes discovered thus far (Thaker *et al*., 2010), the genes associated with an efflux mechanism, namely *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, and *tet(E)* (Chopra and Roberts, 2001) confer tetracycline resistance in *Escherichia* spp. Most tetracycline resistance genes have been found on mobile elements, plasmids or transposons (Roberts, 1996).Tetracycline resistance genes such as, *tet(A)* and *tet(B)* were reported to be common in *E. coli* isolates from humans and animals in many countries (Schwaiger *et al*., 2010; Costa *et al*., 2008; Bryan *et al*., 2004; Lanz *et al*., 2003). Schwaiger *et al*., (2010) and Marynard *et al*., (2004) found that *tet(A)* was common in *E. coli* isolates of animal origin. However, *tet(B)* was the most frequently observed in clinical isolates (Tuckman *et al*., 2007). Tetracycline resistant gene *tet(B)* was found to be predominant in *E. coli* O157:H7 isolates from humans and bovines (Wilkerson *et al*., 2004), as well as *E. coli* isolates from catfish in the U.S (Nawaz *et al*., 2009). The *tet(B)* confers a wider spectrum of resistance to tetracyclines (Roberts, 1996). In a study by Koo and Woo, (2011) demonstrated that *tet(A)* and *tet(B)* are widespread in meat sources.

Previous studies of *E. coli* isolated from raw meat samples also reported a similar prevalence and pattern of *tet* gene expression (Soufi *et al*., 2009; Van *et al*., 2008; Sunde and Norstrom, 2006). Occurrence of each *tet* gene may be

associated with different patterns of antibiotic use, due to co-selection (Lanz *et al*., 2003; Chopra and Roberts, 2001). It has been found that long-term use of tetracycline selects not only for tetracycline-resistant bacteria but also for multiple resistant strains because tet genes are present in the same mobile elements as other resistance genes (Levy, 1992). The *tet(A)* and *tet(B)* genes are known to be located on conjugative plasmids of different incompatibility group (Jones *et al*., 1992).

5.2.1.4 Sulphonamide resistance genes (*sul1* and *sul2*)

Sulphonamide resistance in Gram-negative bacilli generally arises from the acquisition of *sul1*, *sul2* or *sul3* genes, encoding forms of the target dihydropteroate synthase that are not inhibited by the drug (Perreten and Boerlin, 2003; Skold, 2000). The *sul1* gene has been detected as part of the 3′-conserved segment (3′-CS) of class 1 integrons, which are the most frequently detected integrons in *Enterobacteriaceae* (Carattoli, 2001). The *sul2* gene is usually located on small non-conjugative or large transmissible multi-resistance plasmids (Enne *et al*., 2001; Skold, 2000), frequently linked to the streptomycin resistance gene pair *strA-strB*. This structure is not recognized as a mobile element by itself, but has been associated with transposition, mediated by elements such as IS26 and ISCR2 (Toleman *et al*., 2006; Waldor *et al*., 1996). The *sul3* gene has been described as a genetic organization *qacH–IS440–sul3* (Perreten and Boerlin, 2003), sometimes also linked to non-classic class 1 integrons lacking the 3′-CS.

Sulphonamides resistance genes can be transferred from commensal bacteria, *via* integrons, transposons or plasmids, into more virulent bacteria in the human intestine (Guerra *et al*., 2003). *SulI* gene is also one of the most

commonly detected sulphonamide resistant genes in the environment (Pei *et al*., 2006). *SulI* gene is one of the first discovered plasmid-borne and drug resistant genes (Akiba *et al*., 1960), encoding forms of enzyme dihydropteroate synthase (DHPS) that are not inhibited by antibiotic sulphonamides. Although the use of sulphonamides was limited for several years due to the quite rapid occurrence of resistance and dissemination among treated patients (Skold, 2000), *sulI* gene was still commonly found to be associated with persistent sulphonamide resistance in Gram-negative bacteria (Radstrom *et al*., 1991). Previous investigations showed prevalence of *sul* genes on aquatic environments (Gao *et al*., 2012; Su *et al*., 2012; Dolejska *et al*., 2009; Hu *et al*., 2008; Hamelin *et al*., 2006). Previous studies also reported occurrence of *sul* genes in cattle (Karczmarczyk *et al*., 2011; Srinivasan *et al*., 2007; Guerra *et al*., 2003), food producing animals (Ho *et al*., 2009), swine (Kozak *et al*., 2009), pig (Schwaiger *et al*., 2010; Smith *et al*., 2010; Kozak *et al*., 2009), poultry (Soufi *et al*., 2011; Kozak *et al*., 2009) and shellfish (Van *et al*., 2008). Several researchers also reported the occurrence of *sul* gene in clinical *E. coli* (Hussain *et al*., 2012; Baudry *et al*., 2009; Blahna *et al*., 2006; Frank *et al.,* 2007; Oteo *et al*., 2006; Infante *et al*., 2005; Grape *et al*., 2003; Kerrn *et al*., 2002; Enne *et al*., 2001).

5.2.1.5 Trimethoprim resistance genes (*dhfrIa* and *dhfrVII*)

Plasmid-mediated trimethoprim resistance was first described in 1972 (Fleming *et al*., 1972) and is caused by non-allelic and drug-insusceptible variants of chromosomal DHFR (Amyes and Smith, 1974; Skold and Widh, 1974). The genes for some of these enzymes may be temporarily located on the chromosome by virtue of transposons movement, but they are still referred to as plasmid-borne or transferable DHFRs.

The two initially observed plasmid-borne DHFRs mediating resistance to trimethoprim were found by Pattishall *et al*., (1977) to be distinct from each other. At present 16 different types have been found in Gram-negative facultative rods (Then, 1993), and most of these have been defined by amino acid sequence analysis, nucleotide sequencing, or both. Phylogeny analysis has revealed that two subgroups of the transferable DHFRs are related (64 to 88% identity) significantly over the background level (20 to 40% identity in different species) and here are called families (Jansson, 1993; Sundstrom, 1989). The first of these, family 1, includes enzyme types I, V, VI, VII, and Ib (Young *et al*., 1994; Sundstrom *et al*., 1993; Wylie and Koornhof, 1991; Sundstrom *et al*., 1988; Fling and Richards, 1983; Simonsen *et al*., 1983). These DHFRs are 64 to 88% identical, and the polypeptide length is invariably 157 amino acids. The family 2 DHFRs include types IIa, IIb, and IIc, which are completely unrelated to other DHFRs in prokaryotes and eukaryotes but which are closely related to one another (78 to 86% amino acid identity) (Flensburg and Steen, 1986).

The emergence of trimethoprim resistance genes among pathogenic bacteria is very likely due to the recruitment of metabolic genes from unidentified organisms by horizontal genetic exchange. One could speculate that these genes encode the housekeeping DHFR in the cells to which they originally belong. The diversity among chromosomal DHFRs in bacteria is rather broad and includes variants with low levels of susceptibility to trimethoprim (Jansson, 1993; Then and Hermann, 1984.). Trimethoprim resistance genes use sophisticated transfer mechanisms including site-specific recombination (Sundstrom *et al*., 1993, 1988; Sundstrom and Skold, 1986). In fact, similarly elaborated mechanisms are involved in evolutionarily old

phenomena such as phage lysogeny, variation of surface antigens, and monomerization of circular chromosomes (Craig, 1988). Previous studies reported the occurrence of *dhfr* genes among food and food producing animals (Smith *et al*., 2010; Blahna *et al*., 2006; Marynard *et al*., 2003) and clinical isolates (Baudry *et al*., 2009; Frank *et al*., 2007).

5.2.1.6 Quinolone resistance genes

In *E. coli*, the *gyrA* quinolone resistance-determining region (QRDR) spans amino acids 67–106, with alteration at positions 83 and 87 often associated with clinical resistance. Substitutions at Ser-83 or Asp-87 in *gyrA* and at Ser-80 and Glu-84 in *parC* are the most common mutations in *E. coli* (Friedman *et al*., 2001). Bansal and Tandon, (2011) reported that ciprofloxacin resistant *E. coli* isolates found to carry the mutations in *gyrA*, *parC* and *parE*. DNA gyrase (*GyrA* and *GyrB*) and topoisomerase IV (*ParC* and *ParE*) are the two essential type II topoisomerases in *E. coli*. These enzymes act *via* inhibition of DNA replication.

In *Enterobacteriaceae,* quinolone resistance is mainly caused by point mutations in the quinolone resistance-determining region (QRDR) of gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) genes (Veldman *et al*., 2011). However, the emergence of plasmid-mediated quinolone resistance (PMQR) has been reported since 1998 (Martinez-Martinez *et al*., 1998). Although these PMQR determinants confer low-level resistance to quinolones, they are a favourable background for selection of additional chromosome-encoded quinolone resistance mechanisms (Poirel *et al*., 2012). The reason is that the presence of a single mutation in the QRDR of *gyrA* usually results in high-level resistance to nalidixic acid, but to obtain high levels of resistance to

fluoroquinolones, the presence of additional mutation(s) in *gyrA* and/or in another target such as *parC* is required (Ruiz *et al*., 2002).

The DNA topoisomerases II (gyrase) and IV are essential for bacterial replication by controlling DNA supercoiling and chromosome partitioning. They consist of two subunits A and B, encoded by *gyrA* and *gyrB* (DNA gyrase) and *parC* and *parE* (topoisomerase IV), respectively. In *E. coli* the predominant resistance mechanisms are single-nucleotide polymorphisms (SNPs) in a small region of *gyrA*, consequently designated the quinolone resistance-determining region (QRDR). Mutations in *gyrB* are of less significance, whereas mutations in *parC* are most of all responsible for quinolone resistance in Gram-positive bacteria (Munoz and de la Campa, 1996). Single mutations in *gyrA* confer low-level fluoroquinolone resistance, for the development of high-level fluoroquinolone resistance several mutations are necessary. Quinolones resistance among *Enterobacteriaceae* is usually mediated by chromosomal mutations in the quinolone-resistance determining region (QRDR) that encode DNA gyrase (*gyrA* and *parC*) genes (Jacoby, 2005; Hooper, 2001b). Nevertheless, low-level resistance can also arise from the expression of plasmid-mediated quinolone resistance (PMQR) determinants such as: i) *qnrA*, -B, -S, -C, -D genes that encode proteins protecting the DNA gyrase from the quinolones action; ii) an aminoglycoside acetyltransferase encoded by the *aac(6')-Ib-cr* gene that also acetylates quinolones; and iii) plasmid-mediated quinolones efflux-pumps (qepA) (Strahilevitz *et al*., 2009).

5.2.1.7 Aminoglycoside resistance genes

Aminoglycoside resistance in *Enterobacteriaceae* is generally due to enzymatic inactivation, which is mediated by 3 different classes of aminoglycoside-

modifying enzymes (AMEs): acetyltransferases, nucleotidyltransferases, and phosphotransferases (Magnet and Blanchard, 2005). More recently, a new aminoglycosides resistance mechanism that consists of ribosomal protection through enzymatic methylation of specific residues within the 16S rRNA (impeding binding of drugs to the 30S ribosomal subunits) has been described. These 16S rRNAmethylases (ArmA, RmtA, RmtB, RmtC, RmtD, RmtF, RmtG, and NpmA) confer high-levels of resistance to aminoglycosides and can be mobilized among different species (Bueno *et al*., 2013; Hidalgo *et al*., 2013; Doi and Arakawa, 2007). Previous studies reported the occurrence of aminoglycoside resistance genes among poultry (Soufi *et al*., 2011); cattle (Scaria *et al*., 2010), pigs (Marynard *et al*., 2003) and clinical isolates (Baudry *et al*., 2009).

5.2.1.8 Chloramphenicol resistance genes (*cat1*)

Chloramphenicol binds to the 50S ribosomal subunit and inhibits the peptidyltransferase step in protein synthesis. Resistance to chloramphenicol is generally due to inactivation of the antibiotic by chloramphenicol acetyltransferase. The *cat* genes of Gram-negative and Gram-positive bacteria show little homology, and a variety of different enzymes have been described. The gene is most commonly found on plasmids. Sometimes decreased outer membrane permeability or active efflux is observed in gram-negative bacteria (Schmitz and Fluit, 1999). Detection of chloramphenicol resistance by molecular techniques has not been widely studied. Detection of chloramphenicol resistance determinants has received little attention, because chloramphenicol is little used for the treatment of severe infections. Only a limited number of small-scale studies have used molecular techniques to investigate the distribution of *cat* genes.

Chloramphenicol resistance is due to inactivation of the drug mediated by chloramphenicol acetyltransferases (CAT) that have a trimeric structure composed of identical subunits (Schwarz and Cardoso, 1991; Leslie, 1990; Shaw, 1983). There are several types of *cat* genes with different nucleic acid sequences, and the resulting CAT enzymes have different structures (Roberts *et al*., 1982). Zaidenzaig and Shaw, (1976), using enzymologic, biochemical and immunological methods, have classified the CATs produced by the R plasmid in Gram-negative bacteria into enzyme types I, II, and III. The CAT of R plasmids from *Vibrio anguillarum* have been classified into two types (Aoki, 1988; Masuyoshi *et al*., 1988). One type, from an R plasmid first detected in 1980, was classified as CAT II, but the other type, detected before 1977, could not be classified as either CAT I, II, or III and therefore has been designated as a new type, CAT IV (Zhao and Aoki, 1992; Aoki, 1988). The *cat* or other resistance genes have been analyzed by molecular biological methods including PCR (Arcangioli *et al*., 2000). Previous studies reported the occurrence of *cat* genes among food and food producing animals (Van *et al*., 2008; Marynard *et al*., 2003; Bischoff *et al*., 2002) and beach (Hamelin *et al*., 2006).

5.2.1.9 *Class 1 integrons*

Integrons are genetic elements of variable length that contain a 5′ conserved integrase gene (*int*), gene cassettes with other antibiotic resistance genes, and an integration site for the gene cassette, *attI* (Babic *et al*., 2006). To date, four classes of integrons (classes 1, 2, 3 and 4) have been found to be associated with resistance gene cassettes. *Class 1 integrons* are most frequently found among multiresistant Gram-negative bacteria and more than 100 gene cassettes that

confer resistance have been identified (Fluit and Schmitz, 2004). Integrons are among the main types of mobile elements currently known to be involved in the capture, mobilization and spread of antibiotic resistance genes found in Gram-negative bacteria. They are genetic platforms that are responsible for integration and rearrangements of resistance determinants called gene cassettes (Mazel, 2006). Wastewater contains microorganisms, plasmids, integrons and resistance genes, becoming a hotspot for horizontal gene transfer in an environment that contains subinhibitory concentrations of the selective agents (Moura *et al*., 2010; Schluter *et al*., 2007). Integrons are capable of mobilizing or integrating gene cassettes encoding antibiotic resistance determinants such as resistance to trimethoprim, aminoglycosides, chloramphenicol or tetracyclines.

Integrons were first reported in clinical isolates in the 1980s and continue to be extensively studied in clinical environments, due to their association with other mobile genetic elements and multiresistance phenotypes (Leverstein-van Hall *et al*., 2002a, b; Hall *et al*., 1999). However, in the last decade special attention has been given to integrons from natural environments in order to gather information on their ecology and diversity, and to understand their role in bacterial adaptation. Evidence that stress response may trigger the expression of *intI* (Guerin *et al*., 2009) and gene cassettes (Michael and Labbate, 2010) has been recently reported, suggesting that integrons may constitute important adaptive systems in bacterial evolution.

The integron consists of an integrase gene of the tyrosine recombinase family, a primary recombination site called *attI*, and a promoter PC that directs transcription of the captured genes. Several cassettes may be inserted into the same integron (Partridge, 2011). The resistance integrons are linked to mobile

DNA elements such as transposons and conjugative plasmids that enable spreading horizontally through bacterial populations (Fluit and Schmitz, 2004). Upon the homology of the integrase genes five classes of resistance integrons and group of chromosomally encoded integrons have been defined (Mazel, 2006). Chromosomal integrons typically contain long arrays of cassettes with related *attC* sites that exhibit some species specificity (Partridge *et al*., 2009). Three classes of integrons are responsible for multidrug resistance, with class 1 being most ubiquitous among resistant bacteria and considered to play the main role in the emergence and wide dissemination of resistance genes (Cambray *et al*., 2010; Nemergut *et al*., 2008). Recently, class 1 integrons without resistance genes have been detected in non-pathogenic environmental Betaproteobacteria (Gillings *et al*., 2008).

Chromosomal and plasmid-borne integrons have been identified as one of the crucial factors for the development of multidrug resistance in *Enterobacteriaceae* as well as many other bacterial species by harbouring and lateral gene transfer of gene cassettes (Roe *et al*., 2003; Stokes *et al*., 2001; Ochman *et al*., 2000; Bass *et al*., 1999). To date, three distinct classes of resistance integrons have been described with more than 60 different antibiotic resistance genes (Fluit and Schmitz, 2004; Heir *et al*. 2004; Collis *et al*., 2002). Most common integrons among resistant *Enterobacteriaceae* are class 1 resistance integrons, which are primarily located on elements derived from *Tn5090* such as *Tn402* and *Tn21*. They carry the site-specific tyrosine recombinases *IntI*, often contain *qacED1* and *sul1* conferring resistance to quaternary ammonium compounds and sulphonamide and harbor gene cassettes encoding resistance to β-lactams, streptomycin-spectinomycin and trimethoprim. Several investigators observed a significant correlation between

the presence of *class 1 integrons* and multiresistance in Gram-negative isolates (Leverstein-van Hall *et al*., 2003; Hansson *et al*., 2002; Bass *et al*., 1999; Martinez-Freijo *et al*., 1998).

Class 2 integrons, which possess a defective integrase gene, are less prevalent and are associated with transposons of *Tn7* family. *Class 3 integrons* are the least common (Cambray *et al*., 2010). The fourth and fifth class has been identified in *Vibrio* species (Mazel, 2006). It has been reported that multidrug resistance among *Enterobacteriaceae* is associated with the presence of *class 1 integrons* (Leverstein-van Hall *et al.*, 2003). Indeed, *class 1 integrons* are the most common in known multiresistance regions, which are modular, mosaic structures composed of different combinations of mobile components like integrons, insertion sequences, and transposons of *Tn3* and *Tn5053* families (Partridge, 2011). *Class 1* and/or class *2 integrons* have been reported in clinical isolates of the *Enterobacteriacea* family (Leverstein-van Hall *et al*., 2003, 2002a, b), in bacteria from food (Sunde, 2005) and also in aquatic environments (Roe *et al*., 2003).

5.3 Objectives

1) To analyse the occurrence of various antibiotic resistance genes in *E. coli* isolates from five different stations in Cochin estuary.

2) To analyse the occurrence of various antibiotic resistance genes in *E. coli* isolates from estuarine, seafood and clinical sources.

3) To investigate the occurrence of *class 1 integrons* in *E. coli* isolates from estuarine, seafood and clinical sources.

4) To identify the prevalence of antibiotic resistance genes and *integrons* in phylogenetic group A, B1, B2 and D *E. coli* isolates from estuarine, seafood and clinical sources.

5.4 Materials and Methods

In the present study a total of 300 *E. coli* isolates of estuarine origin, 100 *E. coli* of clinical and 100 *E. coli* of seafood origin were tested for the presence of various antibiotic resistances genes.

5.4.1 Isolation of DNA from *E. coli*

DNA from the bacterial genome was extracted as described in 2.4.6.1.

5.4.2 Isolation of plasmid DNA from *E. coli*

Plasmid DNA from the bacterial genome was extracted as per alkali lysis with SDS method: midipreparation (Sambrook and Russel, 2006). Plasmid DNA obtained by sequential phenol-chloroform and chloroform-isoamyl alcohol extractions was precipitated by adding 600 µl of isopropanol, and plasmid DNA was suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA-pH 7.5) containing 20 µg/ml of DNase-free RNase A (GeNei^{TM}, India). Plasmid DNA was checked for purity by agarose gel electrophoresis.

5.4.3 PCR detection of antibiotic resistant genes

The primers used in the detection of antibiotic resistant genes are listed in Table 5.1.

Table 5.1 Gene analysed and list of primers used in the detection of antibiotic resistant genes

Genes investigated	Primer sequence (5′ - 3′)		Reference
	Forward	reverse	
Beta-lactams			
bla$_{TEM}$	gagtattcaacattttcgt	accaatgcttaatcagtga	Marynard *et al*., (2003)
bla$_{CTX-M}$	cgatgtgcagtaccagtaa	ttagtgaccagaatcagcgg	Batchelor *et al*., (2005)
Tetracycline			
tet(A)	gtgaaacccaacatacccc	gaaggcaagcaggatgtag	Marynard *et al*., (2003)
tet(B)	ccttatcatgccagtcttgc	actgccgtttttcgcc	Marynard *et al*., (2003)
Gentamicin			
aphA2	gaacaagatggattgcacgc	gctcttcagcaatatcacgg	Marynard *et al*., (2003)
Streptomycin			
strA	cctggtgataacggcaattc	ccaatcgcagatagaaggc	Rosengren *et al*., (2009)
Trimethoprim			
dhfrIa	gtgaaactatcactaatgg	ttaacccttttgccagattt	Navia *et al*., (2003)
dhfrVII	ttgaaaatttcattgattg	ttagccttttttccaaatct	Navia *et al*., (2003)
Sulphonamide			
sul1	ttcggcattctgaatctcac	atgatctaaccctcggtctc	Marynard *et al*., (2003)
sul2	cggcatcgtcaacataacc	gtgtgcggatgaagtcag	Marynard *et al*., (2003)
Phenicol			
catI	agttgctcaatgtacctataacc	ttgtaattcattaagcattctgcc	Marynard *et al*., (2003)
Integrons 1			
int1	cctcccgcacgatgatc	tccacgcatcgtcaggc	Kraft *et al*., (1986)
Integron variable regions			
int1 CS	ggcatccaagcagcaag	aagcagacttgacctga	Levesque *et al*., (1995).

5.4.3.1 Detection of *bla*$_{TEM}$ gene

The presence of *bla*$_{TEM}$ gene was detected using the PCR method as described previously (Marynard *et al*., 2003). The optimized protocol was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 1X Taq buffer (Tris (pH 9.0) at 25 °C, KCl and Triton X-100), 2.5 mM each of dNTP

mixture, 1 pmol/µl of each of the primers, 1 U of Taq polymerase (GeNei™, India) and 1 µg of the DNA template. Amplification was performed with a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) programmed for 1 cycle of 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei™, India), and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.2 Detection of *bla*$_{CTX-M}$ gene

The optimized protocol was carried out with a PCR mix of 25 µl containing 2.5 mM $MgCl_2$, 2.5 µl of Taq buffer (Tris (pH 9.0) at 25 °C, KCl and Triton X-100), 2.5 mM each of dNTP mixture, 1 pmol/µl of each of the primers, 1 U of Taq polymerase (GeNei™, India) and 1 µl of the DNA template. Amplification was performed with a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) programmed for 1 cycle of 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 60 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C (Batchelor *et al*., 2005). PCR products were then electrophoresed using 1.5% agarose gel containing ethidium bromide (GeNei™, India), and visualized by Gel Documentation System (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.3 Detection of *tetA* and *tetB* gene

The presence of *tet* genes was detected using the PCR method described by Marynard *et al*., (2003). The optimized protocol was carried out with a PCR mix of 20 µl contained 1.5 mM $MgCl_2$, 1x Taq buffer (Tris (pH 9.0) at 25°C, KCl and Triton X-100), 2.5 mM each of dNTP mixture, 1 pmol/µl each of the primers, 1 U of Taq polymerase (GeNei™, India) and 1 µg of the DNA

template. Amplification was performed with a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) programmed for 1 cycle of 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C. Amplified PCR products were separated using 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei^{TM}, India), and visualized by Gel Documentation System (BioRad Gel Doc^{TM} EZ Imager, USA).

5.4.3.4 Detection of *sul1* and *sul2* gene

The presence of *sul1* and *sul2* genes was detected using the PCR method described by Marynard *et al*., (2003). Amplification was performed with a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) programmed for 1 cycle of 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1.5 min at 72 °C; 1 cycle of 5 min at 72 °C. PCR products were then electrophoresed using a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei^{TM}, India), and visualized by Gel Documentation system (BioRad Gel Doc^{TM} EZ Imager, USA).

5.4.3.5 Detection of *dhfrIa and dhfrVII* gene

The optimized protocol was carried out with a PCR mix of 20 µl contained 1.5 mM $MgCl_2$, 1x Taq buffer (Tris (pH 9.0) at 25 °C, KCl and Triton X-100), 2.5 mM each of dNTP mixture, 1 pmol/µl of each of the primers, 1 U of Taq polymerase (GeNei^{TM}, India) and 1 µg of the DNA template. The program consisted of 30 cycles; 1 min denaturation (94 °C), 1 min annealing (55 °C) and 1 min elongation (72 °C), plus a final extension of 10 min at 72 °C (Navia *et al*., 2003). PCR products were then electrophoresed using a 1.5% agarose gel (HiMedia, India), stained with

ethidium bromide (GeNei™, India), and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.6 Detection of *strA*

DNA amplification was carried out in a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) by using the following conditions: 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1.5 min and a final extension at 72 °C for 5 min (Rosengren *et al*., 2009). The optimized protocol was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 2.5µl of Taq buffer, 2.5mM each of dNTP mixture, 25pmol/µl of each of the primers, 1 U of Taq polymerase and 1µl of the DNA template. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei™, India), and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.7 Detection of *aphA2*

DNA amplification was carried out in a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) by using the following conditions: 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1.5 min and a final extension at 72 °C for 5 min (Marynard *et al*., 2003). The optimized protocol was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 2.5µl of Taq buffer, 2.5mM each of dNTP mixture, 25 pmol/µl of each of the primers, 1 U of Taq polymerase and 1µl of the DNA template. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei™, India), and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.8 Detection of *cat1*

DNA amplification was carried out in a Bio-Rad MJ Mini Thermal cycler (BioRad, USA) by using the following conditions: 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1.5 min and a final extension at 72 °C for 5 min (Marynard *et al*., 2003). The optimized protocol was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 2.5µl of Taq buffer, 2.5mM each of dNTP mixture, 25pmol/µl of each of the primers, 1 U of Taq polymerase and 1µl of the DNA template. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei™, India), and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.9 Detection of *integrase1*

PCR conditions for the *integrase 1 gene* included an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 55 °C and extention for 2 min at 72 °C, and a final extention at 72 °C for 10 min (Kraft *et al*., 1986). The optimized protocol was carried out with a PCR mix of 20 µl contained 1.5 mM $MgCl_2$, 1x Taq buffer (Tris (pH 9.0) at 25 °C, KCl and Triton X-100), 2.5 mM each of dNTP mixture, 1 pmol/µl of each of the primers, 1 U of Taq polymerase (GeNei™, India) and 1 µg of the DNA template. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei™, India), and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.10 Detection of *class I integron* variable regions

The presences of *class 1 integron variable regions* were detected using the PCR method described by Levesque *et al*., (1995). The optimized protocol

was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 2.5µl of Taq buffer, 2.5mM each of dNTP mixture, 1pmol/µl of each of the primers, 1 U of Taq polymerase and 1µl of the DNA template. PCR conditions for the *class I integrons* included an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 54 °C and extention for 2 min at 72 °C, and a final extention at 72 °C for 10 min. PCR products were then electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

5.4.3.11 Statistical analysis

Statistical analysis of the results was performed using SPSS software 13 (Statistical Package for the Social Science) One-Way Analysis of Variance (ANOVA, Games-Howell) was applied to test difference in the distribution of antibiotic resistance genes, among *E. coli* from different stations, different sources and different phylogenetic groups. Comparison of associations between different antibiotic resistance genes were performed using Pearson's correlation coefficient test. Significance level was set at $\alpha = 0.05$.

5.5 Results

5.5.1 Prevalence of antibiotic resistance genes among *E. coli* isolates from Cochin estuary

5.5.1.1 Overall prevalence of antibiotic resistance genes among *E. coli* isolates from Cochin estuary

Prevalence of antibiotic resistance genes among the *E. coli* isolates from Cochin estuary was analysed. Antibiotic resistance genes analysed in relation to different class of antibiotics such as β-lactams, aminoglycosides, sulphonamides,

trimethoprim, chloramphenicol, and tetracyclins. Results are presented in various Figures and Tables. In Cochin estuary, significant differences were found in the distribution of different resistance genes (ANOVA, p = 0.000). Figure 5.1 shows the distribution of antibiotic resistance genes among *E. coli* isolates from Cochin estuary.

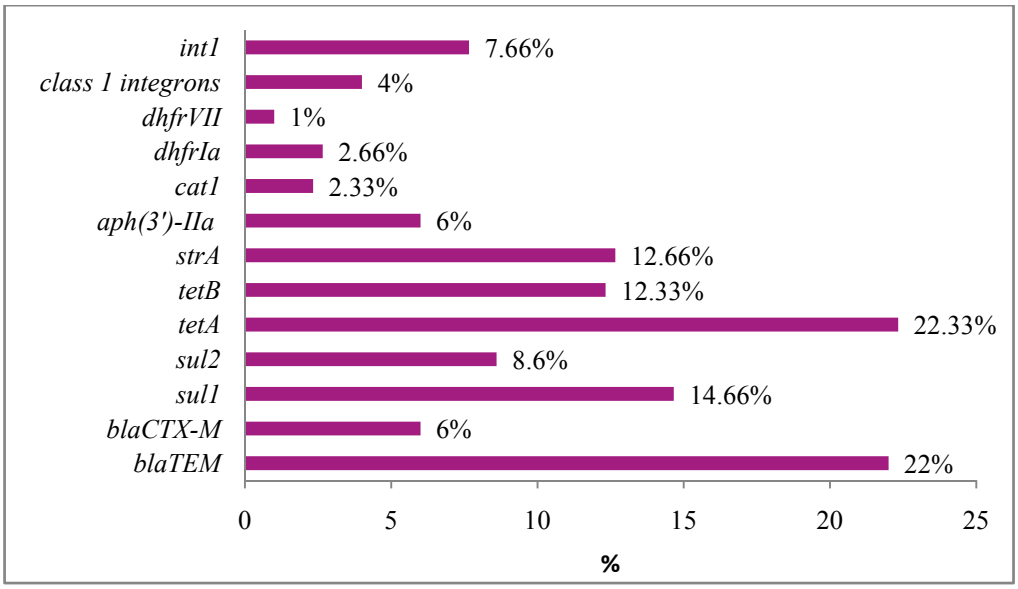


Figure 5.1 Distribution of antibiotic resistance genes among *E. coli* isolated from Cochin estuary (n = 300)

5.5.1.1.1 *bla$_{TEM}$*

Gel image of representative isolates carrying the *bla$_{TEM}$* is shown in Figure 5.2. Out of the 300 *E. coli* isolated from Cochin estuary 22% of isolates carried *bla$_{TEM}$* gene. Of the 129 ampicillin-resistant isolates, 66 (51.66%) were found to contain *bla$_{TEM}$* gene. Games-Howell analysis showed that the percentage of distribution of *bla$_{TEM}$* gene was significantly higher than *to bla$_{CTX-M}$*, *sul2*, *aphA2*, *catI*, *dhfrIa*, *dhfrVII*, *class 1 integrons* and *int1*

(p = < 0.05). Statistical analysis of the result revealed a highly significant association was found between bla_{TEM} and *sul1* gene (p = 0.25).

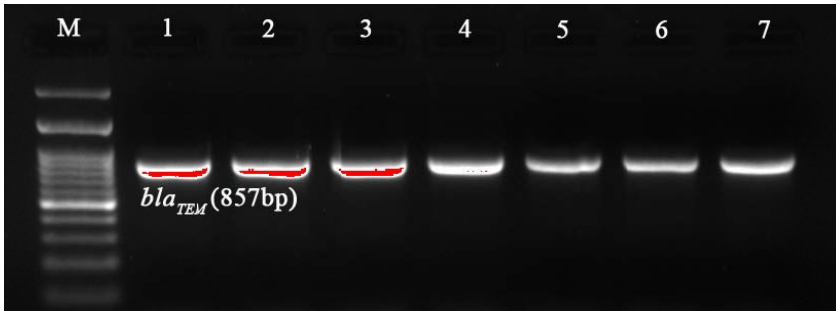


Figure 5.2 Gel image showing occurrence bla_{TEM} gene in ampicillin-resistant *E. coli* isolates
Lanes: M, 100 bp Marker. Lane 1-7, *E. coli* isolates

5.5.1.1.2 bla_{CTXM}

Compared to bla_{TEM} prevalence of bla_{CTX-M} was low among *E. coli* from Cochin estuary. Only 6% of *E. coli* isolates from Cochin estuary carried bla_{CTX-M} gene. Of 23 ESBL producing isolates, 18 (78.2%) were found to contain bla_{CTX-M} gene. Gel image of PCR products with representative isolates carrying the bla_{CTX-M} is shown in Figure 5.3.

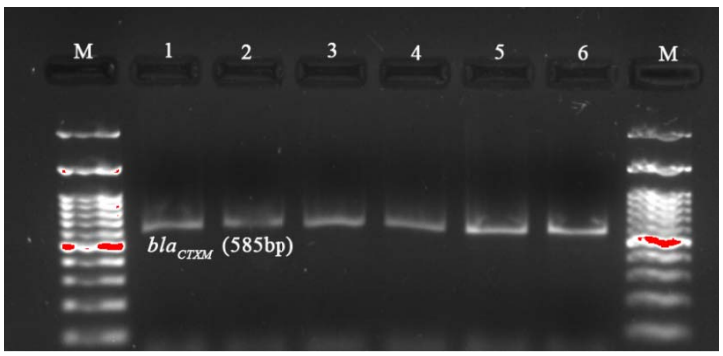


Figure 5.3 Gel image showing occurrence of bla_{CTX-M} gene in ampicillin-resistant *E. coli* isolates
Lanes: M, 100 bp Marker. Lane 1-6, *E. coli* isolates

5.5.1.1.3 Sulphonamide resistance genes (*sul1* and *sul2*)

Gel image of PCR products with representative isolates carrying the *sul1* and *sul2 genes* are shown in Figure 5.4. Out of the 300 *E. coli* isolated from Cochin estuary 14.6% and 6% of isolates carried *sul1* and *sul2* respectively. Fifty seven isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (75.43%), *sul2* (45.61%), and *sul1* + *sul2* (38.59%). Statistical analysis of the result revealed a highly significant association was found between *sul1* with *sul2* ($p = 0.20$). Furthermore, a significant association was found between sulphonamide resistance genes and *strA* and *integrase 1 gene* ($p < 0.05$).

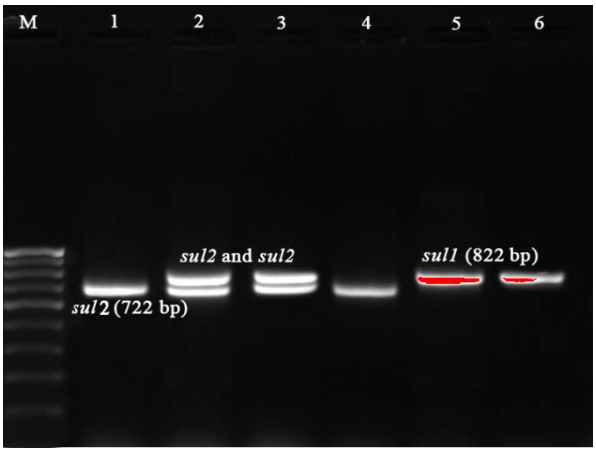


Figure 5.4 Gel image showing occurrence of *sul1 and sul2* gene in *E. coli* isolates
Lanes: M, 100 bp ladder; Lane 1-6, *E. coli* isolates

5.5.1.1.4 Trimethoprim-resistant genes (*dhfrIa* and *dhfrVII)*

Out of the 300 *E. coli* isolated from Cochin estuary 2.6% and 1% of isolates carried *dhfrIa* and *dhfrVII* respectively. Among 57 trimethoprim-resistant *E. coli* isolates, *dhfrIa* was found in 8 (14.03%) isolates, and *dhfrVII* was found in 3 (5.26%) isolates. Figure 5.5 shows the gel image of PCR products with representative isolates carrying the *dhfrIa* and *dhfrVII*.

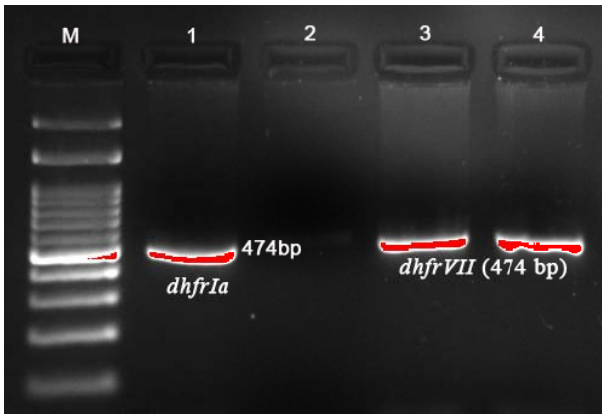


Figure 5.5 Gel image showing occurrence of *dhfrIa* and *dhfrVII* gene in *E. coli* isolates
Lanes: M, 100 bp Marker. Lane 1-4, *E. coli* isolates

5.5.1.1.5 Tetracycline resistance genes (*tetA* and *tetB*)

Of the 300 *E. coli* isolates 22.33% and 12.33% of isolates carried *tetA* and *tetB*, respectively. Regarding the 91 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 67 (73.62%), 37 (40.65%), and 33 (36.26%) isolates, respectively. Figure 5.6 shows the gel image of PCR products with representative isolates carrying the *tetA* and *tetB* genes. Significant association was found between *tetA* and *tetB* ($p = 0.016$).

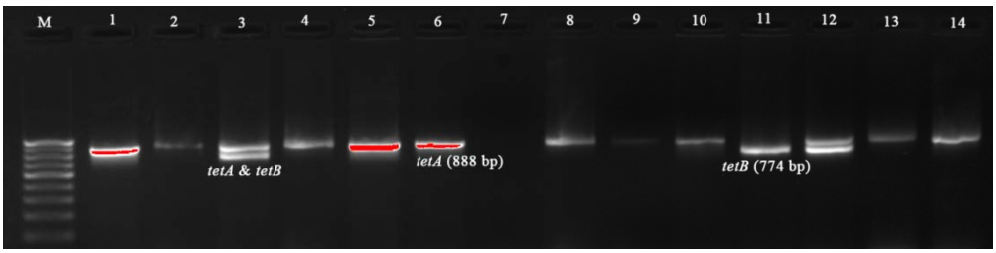


Figure 5.6 Gel image showing occurrence of t*etA* and tetB gene in *E. coli* isolates. Lanes: M, 100 bp Marker. Lane 1-14, *E. coli* strains.

5.5.1.1.6 Aminoglycoside resistance genes (*strA* and *aphA2*)

Figure 5.7 shows the gel image of PCR products with representative isolates carrying the *strA*. More than 12% of *E. coli* isolates from Cochin estuary carried *strA* gene. Of the 47 streptomycin-resistant isolates, 36 (76.59%) of them harboured the *strA* gene. There was a significant association between *strA* with *sul1*, *sul2* and *integrase 1 gene* ($p < 0.05$). Agarose gel electrophoresis of PCR products with representative isolates carrying *aph A2* is shown in Figure 5.8. Six percentage of *E. coli* isolates from Cochin estuary carried *aphA2* gene. Out of 13 gentamicin-resistant isolates, *aphA2* gene was found in 11 isolates (84.61%).

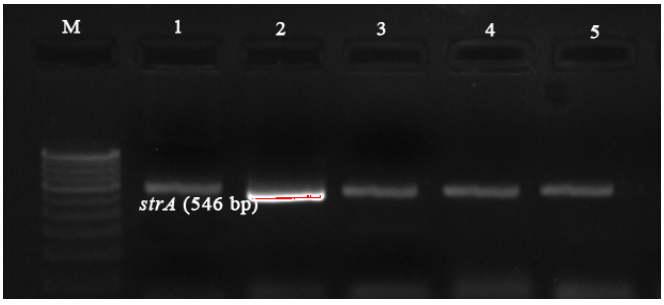


Figure 5.7 Gel image showing occurrence of *strA* gene in *E. coli* isolates. Lanes: M, 100 bp Marker. Lane 1-5, *E. coli* isolates

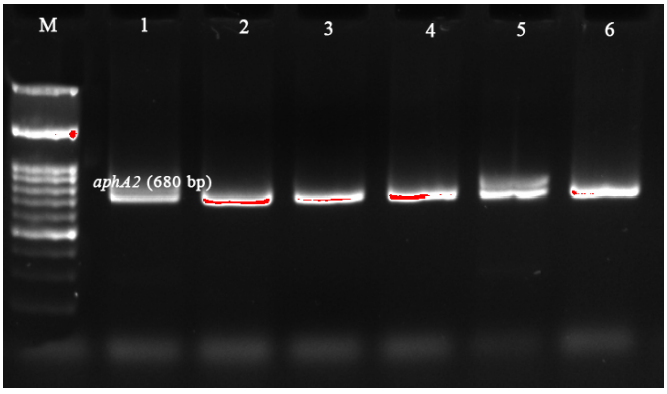


Figure 5.8 Gel image showing occurrence of *aphA2* gene in *E. coli* isolates. Lanes: M, 100 bp Marker. Lane 1-6, *E. coli* isolates

5.5.1.1.7 Chloramphenicol-resistant gene (*catI*)

Out of 300 *E. coli* isolates from Cochin estuary, 2.33% of isolates carried *catI* gene. Seven out of 12 chloramphenicol-resistant isolated harboured a *cat1* (58.33%) gene. Gel image of PCR products with representative isolates carrying *catI* is shown in Figure 5.9.

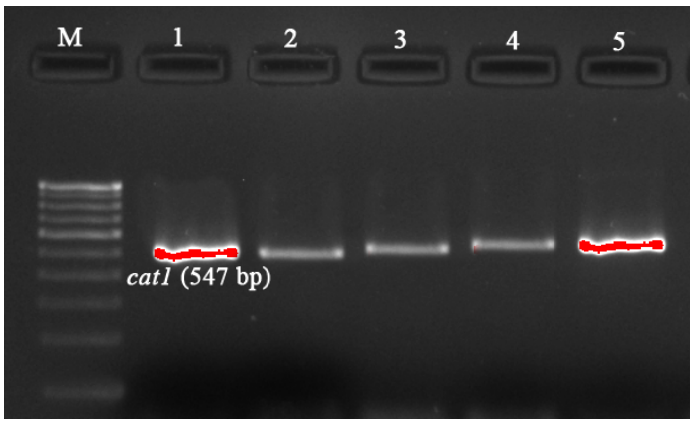


Figure 5.9 Gel image showing occurrence of *catI* gene in *E. coli* isolates. Lanes: M, 100 bp Marker. Lane 1-5, *E. coli* isolates

5.5.1.1.8 *Class 1 integrons* and *int1* gene

Class 1 integron variable regions and integrase gene (*int1*) were identified in 12 and 23 isolates, respectively. Figure 10 shows the gel image of PCR products with representative isolates carrying the *integrase 1* gene. Gel image of PCR products with representative isolates carrying *class 1 integron* variable regions is shown in Figure 5.11. There was a significant association between *class 1 intergon* variable regions and *integrase 1* gene ($p < 0.05$). Furthermore, significant association was also found between *integrase 1* gene and *sul1*, *sul2* and *strA* genes ($p < 0.05$).

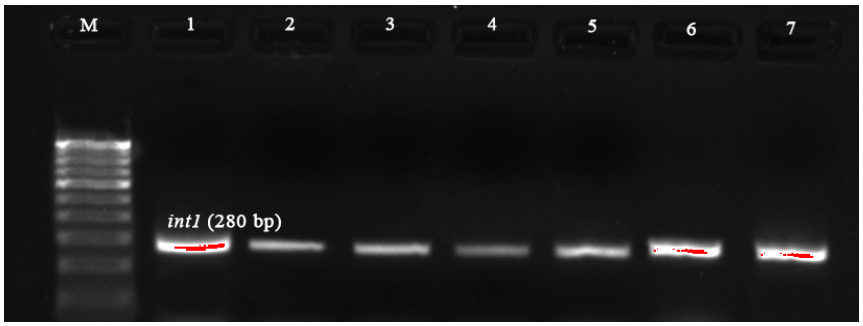


Figure 5.10 Gel image showing occurrence of *integrase1* gene in *E. coli isolates*
Lanes: M, 100 bp Marker. Lane 1-5, *E. coli* isolates

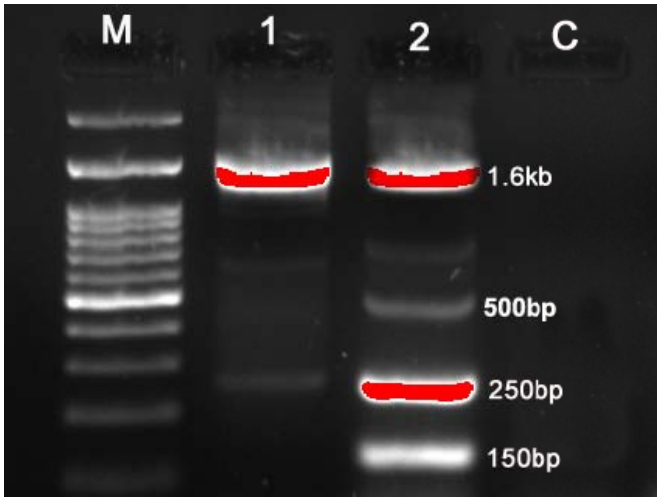
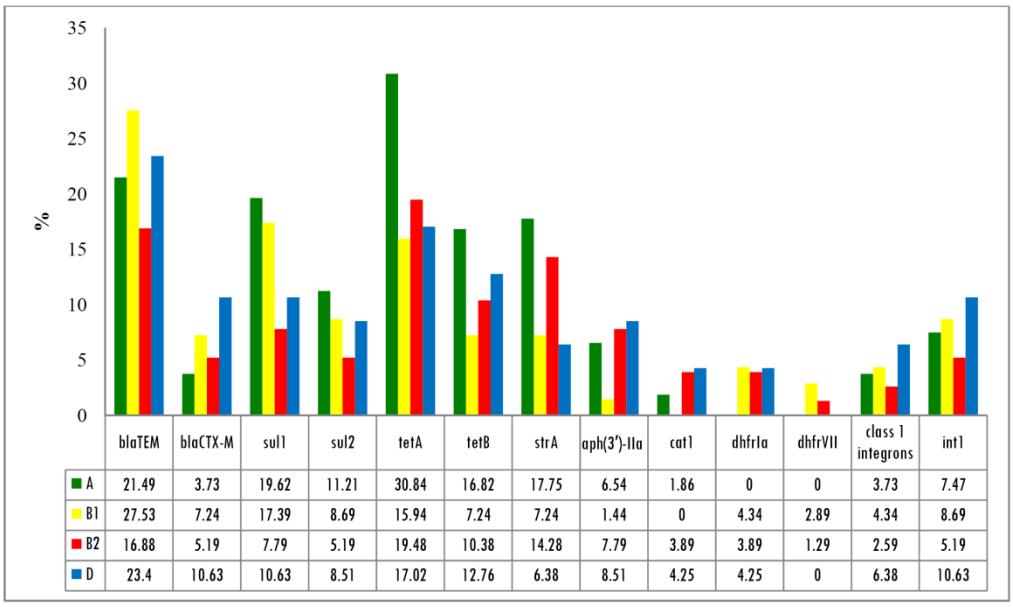


Figure 5.11 Gel image showing occurrence of *Class 1* integron variable regions in *E. coli* isolates
Lanes: M, 100 bp ladder; 1, ES44; 2, ES11; C, Negative Control

5.5.1.2 Prevalence of antibiotic resistance genes among various phylogenetic groups of *E. coli* isolates from Cochin estuary

All genes were distributed in 4 different phylogenetic groups except *dhfr* gene. Though the prevalence varied among different phylogenetic groups *dhfrIa* and *dhfrVII* was absent in group A isolates. Among *E. coli* isolates of different phylogenetic groups, group D strains showed high prevalence for $bla_{CTX\text{-}M}$, *aph(3)-IIa*, *catI*, *class 1 integrons* and *int1* (Figure 5.12). Phylogenetic group

A strains showed highest percentage of occurrence for *sul1*, *sul2*, *tetA*, *tetB*, and *strA*. Phylogenetic group B1 strains showed highest percentage of occurrence for *dhfrIa* and *dhfrVII*. Cholamphenicol resistant gene *cat1* was absent in phylogenetic group B1. No significant difference was observed in the distribution of resistance genes among different phylogenetic groups (ANOVA, p = 0.783) among the *E. coli* isolates from Cochin estuary.



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfrIa	dhfrVII	class 1 integrons	int1
A	21.49	3.73	19.62	11.21	30.84	16.82	17.75	6.54	1.86	0	0	3.73	7.47
B1	27.53	7.24	17.39	8.69	15.94	7.24	7.24	1.44	0	4.34	2.89	4.34	8.69
B2	16.88	5.19	7.79	5.19	19.48	10.38	14.28	7.79	3.89	3.89	1.29	2.59	5.19
D	23.4	10.63	10.63	8.51	17.02	12.76	6.38	8.51	4.25	4.25	0	6.38	10.63

Figure 5.12 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from Cochin estuary (n = 300)

5.5.1.3 Occurrence of antibiotic resistance genes among *E. coli* isolates from station 1 (Chitoor, n = 47)

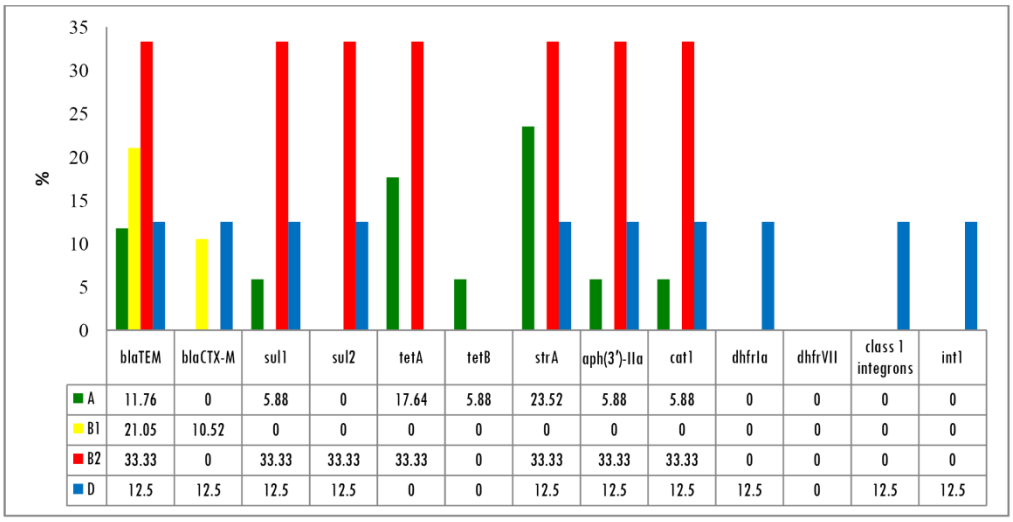
There was no significant difference in the distribution of different antibiotic resistance genes among *E. coli* from station 1 (ANOVA, p = 0.234). Out of 47 *E. coli* isolated from Chitoor, 17.02% of isolates carried bla_{TEM} gene. Of the 13 ampicillin resistant isolates, 8 (61.53%) were found to contain

bla$_{TEM}$ gene. Six percentages of *E. coli* isolates carried *bla*$_{CTX-M}$ gene. Out of 4 ESBL producing isolates, 3 (75%) were found to contain *bla*$_{CTX-M}$ gene. About 6% and 4% of isolates carried *sul1* and *sul2* gene, respectively. Three isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (3, 100%), *sul2* (2, 66.66%), and *sul1* + *sul2* (2, 66.66%). Trimethoprim resistance genes such as, *dhfrIa*, and *dhfrVII* were not detected among *E. coli* from this station. Tetracycline resistance genes (*tetA* and *tetB*) harboured isolates were 8.51% and 2.12%, respectively. Regarding the 5 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 4 (80%), 1 (20%), and 1 (20%) isolates, respectively. Nearly 13% and 7% of isolates harboured *strA* and *aphA2* genes. Five streptomycin-resistant isolates were detected; all of them harboured the *strA* gene. The *aph(3')-IIa* gene was found in all gentamicin-resistant isolates. About 2% of isolates carried *catI* gene. One out of 2 chloramphenicol-resistant isolated harboured a *cat1* (58.33%) gene. *Integrase* gene (*int1*) and *class 1 integron* variable regions and were identified only in one isolate.

5.5.1.3.1 Incidence of antibiotic resistance genes among various phylogenetic groups of *E. coli* isolates from station 1 (Chitoor)

Among *E. coli* isolates of different phylogenetic groups, group B2 strains showed highest percentage of occurrence for *bla*$_{TEM}$, *sul1*, *sul2*, *tetA*, *strA*, *aph(3)-IIa*, and *catI*. *dhfrIa*, *class 1 integrons* and *int1* were occurred only in group D isolates. *tetB* occured only in group A isolates. *bla*$_{CTX-M}$ gene was distributed in B1 and D group only (Figure 5.13). Group B1 isolates harboured *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes only. *bla*$_{TEM}$ gene was distributed among 4 differrent phylogenetic groups. In station 1, there was a significant difference in the distribution of resistance genes among different phylogenetic groups

(ANOVA, p = 0.003). Group B2 and D isolates harboured more resistance genes than group B1, with a significance of 0.037 and 0.024



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3")-IIa	cat1	dhfrIa	dhfrVII	class 1 integrons	int1
A	11.76	0	5.88	0	17.64	5.88	23.52	5.88	5.88	0	0	0	0
B1	21.05	10.52	0	0	0	0	0	0	0	0	0	0	0
B2	33.33	0	33.33	33.33	33.33	0	33.33	33.33	33.33	0	0	0	0
D	12.5	12.5	12.5	12.5	0	0	12.5	12.5	12.5	12.5	0	12.5	12.5

Figure 5.13 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from station 1 (Chitoor)

5.5.1.4 Occurrence of antibiotic resistance genes among *E. coli* isolates from station 2 (Bolgatty, n = 75))

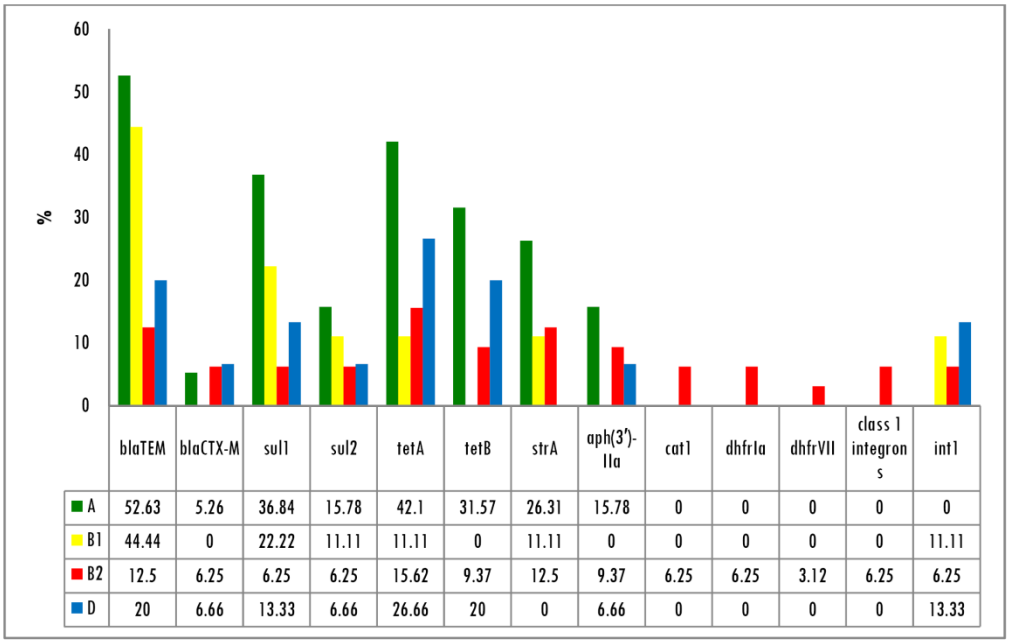
E. coli strains isolated from station 2 harboured all the 13 resistance genes tested. There was a significant difference in the distribution of different antibiotic resistance genes in station 2 (ANOVA, p = 0.000)

Out of 75 isolates, 28% of isolates carried bla_{TEM} gene. Of the 39 ampicillin-resistant isolates, 21 (53.84%) were found to contain bla_{TEM} gene. More than 5% of isolates carried $bla_{CTX\text{-}M}$ gene. Out of 5 ESBL producing isolates, 4 (80%) were found to contain $bla_{CTX\text{-}M}$ gene. More than 17% and 9% of isolates carried *sul1* and *sul2* gene, respectively. Twenty two isolates showed sulphonamide resistance phenotype which was encoded by *sul1*

(13, 59.09%), *sul2* (7, 31.81%), and *sul1* + *sul2* (5, 22.72%). Out of 75 isolates, 2.66% and 1.33% of isolates carried *dhfrIa* and *dhfrVII* gene, respectively. Among 18 trimethoprim-resistant *E. coli* isolates, *dhfrIa* was found in 2 (33.33%) isolates, and *dhfrVII* was found in 1 (5.55%) isolates. About 24% and 16% of isolates carried *tetA* and *tetB* gene, respectively. Regarding the 26 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 18 (69.23%), 12 (46.15%), and 12 (46.15%) isolates, respectively. About 13% and 9% of isolates carried *strA* and *aphA2*, respectively. Eleven streptomycin-resistant isolates were detected, 10 (90.9%) of them harboured the *strA* gene. The *aph(3)-IIa* gene was found in all the seven gentamicin-resistant isolates. Nearly 3% of isolated carried *cat1* gene. Two out of 6 chloramphenicol-resistant isolates harboured *cat1* (33.33%) gene. More than 6% and 2% of isolates harboured *class 1 integrase* gene and *class 1 integron* variable regions, respectively.

5.5.1.4.1 Incidence of antibiotic resistance genes among various phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty)

Antibiotic resistance genes, such as bla_{TEM}, *sul1*, *sul2*, and *tetA* were distributed in all 4 phylogenetic groups (Figure 5.14). Among *E. coli* isolates of different phylogenetic groups, group A strains showed highest percentage of occurrence for bla_{TEM}, *sul1*, *sul2*, *tetA*, *strA*, and *aph(3)-IIa*. *Cat1*, *dhfrIa*, *dhfrVII* *class 1 integrons* and *int1* were absent in group A isolates. Group B2 strains showed highest percentage of occurrence for *cat1*, *dhfrIa*, *dhfrVII*, and *class 1 integrons*. In station 2, there was no significant difference in the distribution of resistance genes among different phylogenetic groups (ANOVA, $p = 0.180$).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
A	52.63	5.26	36.84	15.78	42.1	31.57	26.31	15.78	0	0	0	0	0
B1	44.44	0	22.22	11.11	11.11	0	11.11	0	0	0	0	0	11.11
B2	12.5	6.25	6.25	6.25	15.62	9.37	12.5	9.37	6.25	6.25	3.12	6.25	6.25
D	20	6.66	13.33	6.66	26.66	20	0	6.66	0	0	0	0	13.33

Figure 5.14 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty)

5.5.1.5 Occurrence of antibiotic resistance genes among *E. coli* isolates from station 3 (Off Marine Science Jetty, n = 69)

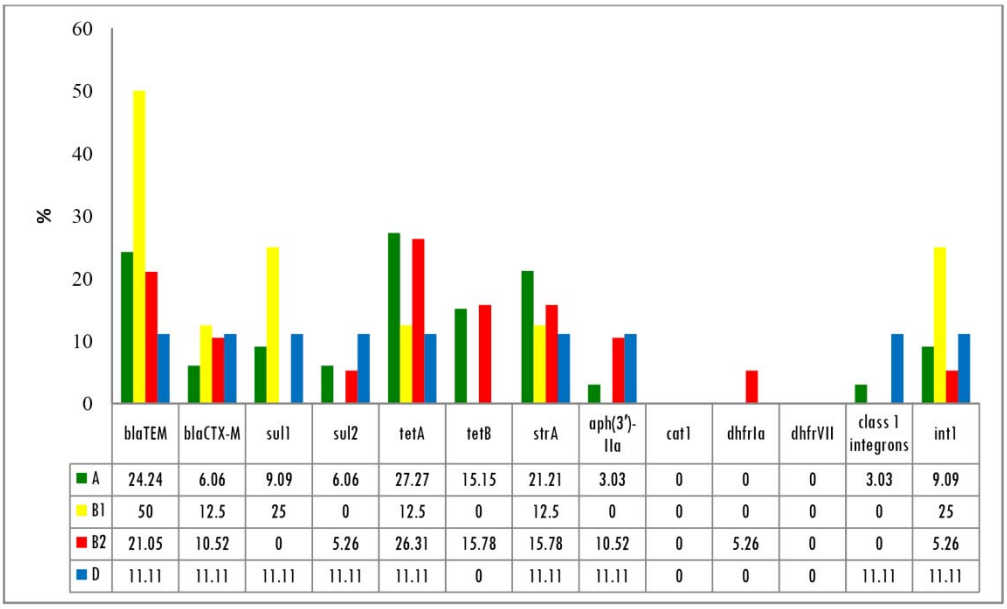
There was a significant difference in the distribution of different antibiotic resistance genes in station 3 (ANOVA, p = 0.000). Isolates from station 3, harboured significantly more $bla_{CTX\text{-}M}$ gene than other genes such as *cat1* and *dhfrVII* (ANOVA, p = 0.49).

Out of 69 isolates, 24.63% carried bla_{TEM} gene. Of the 29 ampicillin-resistant isolates, 17 (58.62%) were found to contain bla_{TEM} gene. Nearly 7% of isolates carried bla_{CTXM} gene. All six ESBL producing isolates were found to contain bla_{CTXM} gene. More than 8% and 5% of isolates harboured *sul1* and *sul2*, respectively. Seven isolates showed sulphonamide resistance phenotype

which was encoded by *sul1* (6, 85.71%), *sul2* (4, 57.14%), and *sul1* + *sul2* (2, 28.57%). Among 10 trimethoprim-resistant *E. coli* isolates, *dhfrIa* was found in 1 (10%) isolates. About 8% and 11% of *E. coli* isolates harboured *tetA* and *tetB*, respectively. Regarding the 22 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 6 (27.27%), 8 (36.36%), and 8 (36.36%) isolates, respectively. Nearly 17% and 5% of *E. coli* isolates harboured *strA* and *aphA2*, respectively. Eighteen streptomycin-resistant isolates were detected, 11 (66.66%) of them harboured the *strA* gene. The *aph(3)-IIa* gene was found in 1 isolates (33.33%) out of 3 gentamicin-resistant isolates. Choramphenicol resistant gene *catI* was not detected in *E. coli* isolated from station 3. *class 1* integron variable regions and integrase gene (*int1*) were identified in 2 and 7 isolates, respectively.

5.5.1.5.1 Incidence of antibiotic resistance genes among various phylogenetic groups of *E. coli* isolates from station 3 (Off Marine Science Jetty)

Antibiotic resistance genes, such as bla_{TEM}, bla_{CTX-M}, *strA*, and *tetA* were distributed in 4 different phylogenetic groups (Figure 5.15). Among *E. coli* isolates of different phylogenetic groups, group B1 strains showed highest percentage of occurrence for bla_{TEM}, *sul1*, and *int1*. Group A strains showed highest percentage of occurrence for *tetA*, and *strA*. Resistance genes such as, *sul2*, *tetB*, *aph(3)-IIa*, *dhfrIa*, and *class 1 integrons* were absent in group B1 isolates. *dhfrIa* occured only in group B2 isolates. *class 1 integrons* was distributed in A and D group only. In station 3, there was no significant difference in the distribution of resistance genes among different phylogenetic groups (ANOVA, p = 0.910).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3")-IIa	cat1	dhfrIa	dhfrVII	class 1 integrons	int1
A	24.24	6.06	9.09	6.06	27.27	15.15	21.21	3.03	0	0	0	3.03	9.09
B1	50	12.5	25	0	12.5	0	12.5	0	0	0	0	0	25
B2	21.05	10.52	0	5.26	26.31	15.78	15.78	10.52	0	5.26	0	0	5.26
D	11.11	11.11	11.11	11.11	11.11	0	11.11	11.11	0	0	0	11.11	11.11

Figure 5.15 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from station 3 (Off Marine Science Jetty).

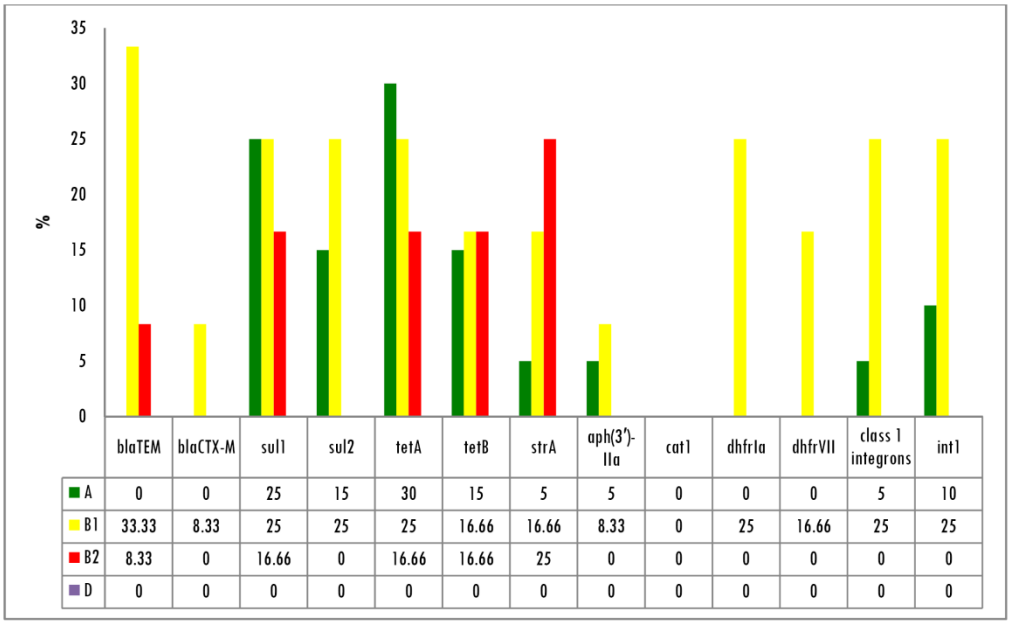
5.5.1.6 Occurrence of antibiotic resistance genes among *E. coli* isolates from station 4 (Thevara, n = 49)

There was no significant difference in the distribution of different antibiotic resistance genes in station 4 (ANOVA, p = 0. 428). In Thevara, about 10% of isolates carried bla_{TEM} gene. Of the 23 ampicillin-resistant isolates, 5 (21.73%) were found to contain bla_{TEM} gene. Only 2% of isolates carried bla_{CTXM} gene. Out of 5 ESBL producing isolates, 1 (20%) were found to contain bla_{CTXM} gene. Out of 49 isolates, 20.4% and 12.24% of isolates carried *sul1* and *sul2*, respectively. Nine isolates showed sulphonamide resistance phenotype which was encoded by *sul 1* (9, 100%), *sul2* (6, 66.66%), and *sul1* + *sul2* (38.59%). About, 6% and 4% of *E. coli* isolates carried *dhfrIa* and *dhfrVII*, respectively. Among 10 trimethoprim-resistant *E. coli* isolates,

dhfrIa was found in 3(30%) isolates, and *dhfrVII* was found in 2 (20%) isolates. Tetracycline resistance genes (*tetA* and *tetB*) harboured isolates were 18.38% and 14.28%, respectively. Regarding the 16 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 9 (56.25%), 7 (43.75%), and 6 (37.5%) isolates, respectively. More than 12% and 4% of isolates carried *strA* and *aphA2,* respectively. Seven streptomycin-resistant isolates were detected, 2 (28.57%) of them harboured the *strA* gene. The *aph(3)-IIa* gene was found all gentamicin-resistant isolates. Choramphenicol resistant gene *catI* was not detected in isolates from station 4. *Class 1 integrons* and integrase gene (*int1*) was identified in 4 and 5 isolates, respectively.

5.5.1.6.1 Incidence of antibiotic resistance genes among various phylogenetic groups of *E. coli* isolates from station 4 (Thevara)

Antibiotic resistance genes, such as *sul1*, *tetA*, *tetB*, and *strA* were distributed among A, B1 and B2 phylogenetic groups (Figure 5.16). Among *E. coli* isolates of different phylogenetic groups, group B1 strains showed highest percentage of occurrence for bla_{TEM}, $bla_{CTX\text{-}M}$, *sul2*, *aph(3)-IIa*, *dhfrIa*, *dhfrVII*, *class 1 integrons* and *int1*. Group D isolates were absent for all the resistant genes tested. Group B2 isolates showed higher percentage of occurrence for *strA* gene. Group A isolates showed higher percentage of occurrence for *tetA* gene. In station 4, there was a significant difference in the distribution of resistance genes among different phylogenetic groups (ANOVA, p = 0.000). Group A isolates harboured significantly more resistance genes than group D (ANOVA, p = 0.045). Group B1 isolates harboured more resistance genes than group A, B2 and D, with a significance of 0.042, 0.008 and 0.000 respectively.



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
A	0	0	25	15	30	15	5	5	0	0	0	5	10
B1	33.33	8.33	25	25	25	16.66	16.66	8.33	0	25	16.66	25	25
B2	8.33	0	16.66	0	16.66	16.66	25	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 5.16 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from station 4 (Thevara)

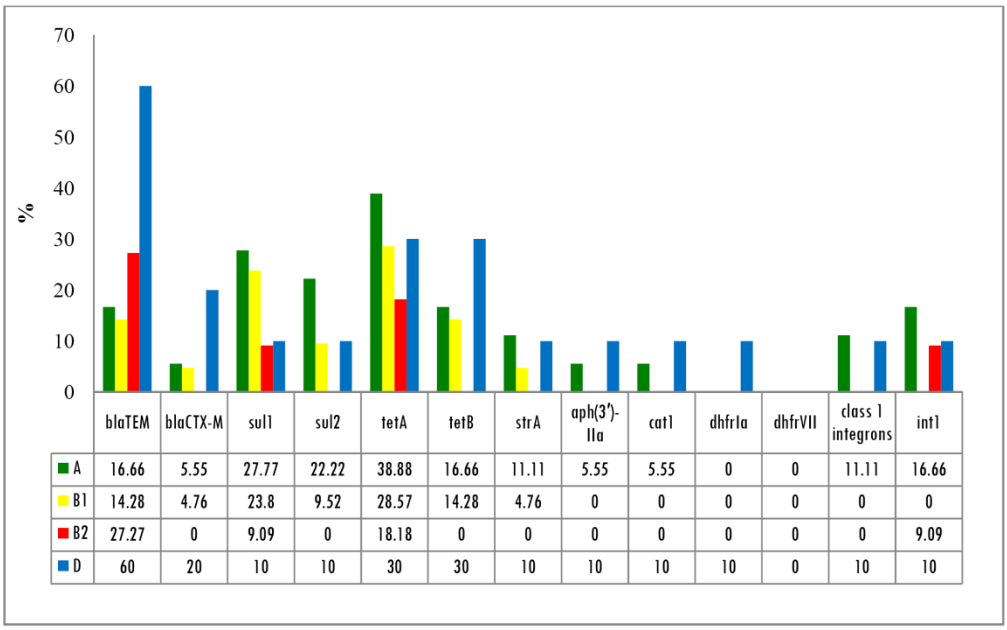
5.5.1.7 Occurrences of antibiotic resistance genes among *E. coli* isolates from station 5 (Barmouth n = 60)

There was a significant difference in the distribution of different antibiotic resistance genes in station 5 (ANOVA, p = 0.000). Isolates from station 5, harboured significantly more *tetA* gene than *dhfrVII* (ANOVA, p = 0.49). Twently five percentages of *E. coli* isolates carried bla_{TEM} gene. Of the 25 ampicillin-resistant isolates, 15 (60%) were found to contain bla_{TEM} gene. Six percentages of *E. coli* isolates carried $bla_{CTX\text{-}M}$ gene. Out of 6 ESBL producing isolates, 4 (66%) were found to contain $bla_{CTX\text{-}M}$ gene. Out of 60 isolates, 20% and 11.66% of isolates carried *sul1* and *sul2*, respectively. Sixteen isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (12, 75%), *sul2* (7, 43.75%), and *sul1* + *sul2* (7, 43.75%).

Among 16 trimethoprim-resistant *E. coli* isolates, *dhfrIa* was found in 1 (6.25%) isolates. About 30% and 15% of isolates carried *tetA* and *tetB*, respectively. Regarding the 91 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 18 (81.81%), 9 (40.9%), and 33 (9, 40.9%) isolates, respectively. About 6% and 2% of isolates carried *strA* and *aphA2*, respectively. Six streptomycin-resistant isolates were detected, 4 (66.66%) of them harboured the *strA* gene. Nearly 3% of isolates carried *cat I* gene. All chloramphenicol-resistant isolated harboured a *cat1* gene. *Class 1 integrons* and integrase gene (*int1*) were identified in 3 and 5 isolates, respectively.

5.5.1.7.1 Incidence of antibiotic resistance genes among various phylogenetic groups of *E. coli* isolates from station 5 (Barmouth)

Antibiotic resistance genes, such as bla_{TEM}, *sul1*, and *tetA* were distributed among 4 different phylogenetic groups (Figure 5.17). Among *E. coli* isolates of different phylogenetic groups, group D strains showed highest percentage of occurrence for bla_{TEM}, $bla_{CTX\text{-}M}$, *tetB*, *aph(3)-IIa*, *cat1*, and *dhfrIa.* Group A isolates showed higher percentage of occurrence for *strA* gene. Group A isolates showed higher percentage of occurrence for *sul1*, *sul2, tetA*, *class 1 integrons* and *int1*. *class 1 integrons*, *cat1* and *aph(3)-IIa*, was occurred only in A and D isolates. In station 5, there was a significant difference in the distribution of resistance genes among different phylogenetic groups (ANOVA, $p = 0.045$).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
A	16.66	5.55	27.77	22.22	38.88	16.66	11.11	5.55	5.55	0	0	11.11	16.66
B1	14.28	4.76	23.8	9.52	28.57	14.28	4.76	0	0	0	0	0	0
B2	27.27	0	9.09	0	18.18	0	0	0	0	0	0	0	9.09
D	60	20	10	10	30	30	10	10	10	10	0	10	10

Figure 5.17 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from station 5 (Barmouth)

5.5.1.8 Spatial variation in incidence of antibiotic resistance genes among *E. coli* isolates from different stations in Cochin estuary

Table 5.2 shows the prevalence of antibiotic resistance genes among *E. coli* isolates from different stations at Cochin estuary. *E. coli* isolates from station 2 showed higher prevalence for antibiotic resistance genes such as, bla_{TEM}, *tetBI* and *aphA2*. While isolates from station 3, showed higher prevalence for *strA*. Whereas *E. coli* isolates from station 4, exhibited higher prevalence for sulphonamide resistance genes, such as *sul1* and *sul2*.

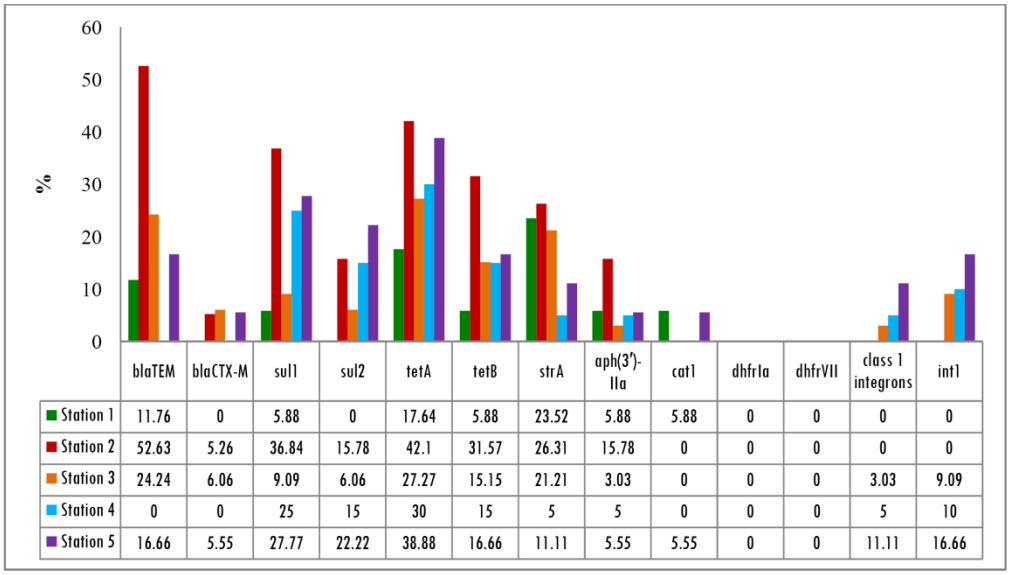
Table 5.2 Prevalence of antibiotic resistance genes among *E. coli* isolates from different stations at Cochin estuary

Antibiotic resistance genes	Station No*				
	1 (n = 47)	2 (n = 75)	3 (n = 69)	4 (n = 49)	5 (n = 60)
bla_{TEM}	17.02	28	24.63	10.2	25
bla_{CTX-M}	6.38	5.33	8.69	2.04	6.66
sul1	6.38	17.33	8.69	20.4	20
sul2	4.25	9.33	5.79	12.24	11.66
tetA	8.51	24	8.69	18.36	30
tetB	2.12	16	11.59	14.28	15
strA	12.76	13.33	17.39	12.24	6.66
aph(3')-IIa	6.38	9.33	5.79	4.08	2.89
cat1	2.12	2.66	0	0	2.89
dhfrIa	0	2.66	1.44	6.12	1.66
dhfrVII	0	1.33	0	4.08	0
class 1 integrons	2.12	2.66	2.89	8.16	5
int1	2.12	6.66	10.14	10.2	8.33

*station 1, Chitoor; station 2, Bolgatty; station3, Off Marine Science Jetty; station 4, Thevara; station 5, Barmouth.

5.5.1.8.1 Distribution of antibiotic resistance genes among *E. coli* phylogenetic group 'A' isolates from different stations at Cochin estuary

Station 1, Chitoor and station 3, Off marine Science Jetty showed moderate percentages of occurrence for bla_{TEM}, *sul1*, *tetA*, *tetB*, *strA*, and *aph(3)-IIa* (Figure 5.18). Station 2, Bolgatty showed higher percentages of occurrence for bla_{TEM}, *sul1*, *tetA*, *tetB*, *strA*, and *aph(3)-IIa* among group A isolates. Resistance genes such as bla_{TEM}, bla_{CTX-M}, *catI* , *dhfrIa,* and *dhfrVII* were absent in station 4 (Thevara). Relatively higher percentages of occurrence for *catI*, *sul2*, *class 1 integrons* and *int1* were observed in station 5, Barmouth. There was no significant difference in the distribution of resistance genes in group A isolates from different stations (ANOVA, p = 0.602).

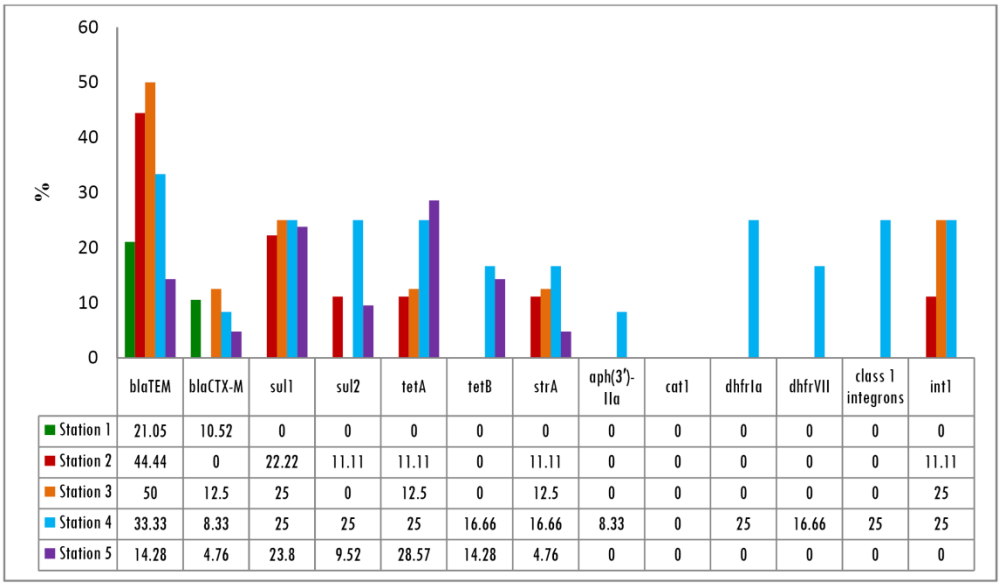


	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfrIa	dhfrVII	class 1 integrons	int1
Station 1	11.76	0	5.88	0	17.64	5.88	23.52	5.88	5.88	0	0	0	0
Station 2	52.63	5.26	36.84	15.78	42.1	31.57	26.31	15.78	0	0	0	0	0
Station 3	24.24	6.06	9.09	6.06	27.27	15.15	21.21	3.03	0	0	0	3.03	9.09
Station 4	0	0	25	15	30	15	5	5	0	0	0	5	10
Station 5	16.66	5.55	27.77	22.22	38.88	16.66	11.11	5.55	5.55	0	0	11.11	16.66

Figure 5.18 Percentage occurrence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'A' from different stations at Cochin estuary

5.5.1.8.2 Distribution of antibiotic resistance genes among *E. coli* phylogenetic group 'B1' isolates from different stations at Cochin estuary

Group B1 isolates from Chitoor showed moderate percentages of occurrence for bla_{TEM}, and $bla_{CTX\text{-}M}$ (Figure 5.19). Station 2 showed moderate percentages of occurrence for bla_{TEM}, *sul1*, *sul2*, *tetA*, *strA* and *int1*. Station 3 showed highest percentage of occurrence for bla_{TEM} and $bla_{CTX\text{-}M}$ gene. While percentage of occurrence for *sul2*, *tetB*, *strA*, *aph(3)-IIa*, *dhfrIa, dhfrVII* and *class 1 integrons* was high at station 4. Percentage of occurrence of *tetA* gene was high among *E. coli* from station 5. There was a significant difference in the distribution of resistance genes in group B1 isolates from different stations (ANOVA, $p < 0.001$). Group B1 isolates from stations 4 harboured more resistance gene compared to other stations ($p < 0.05$).



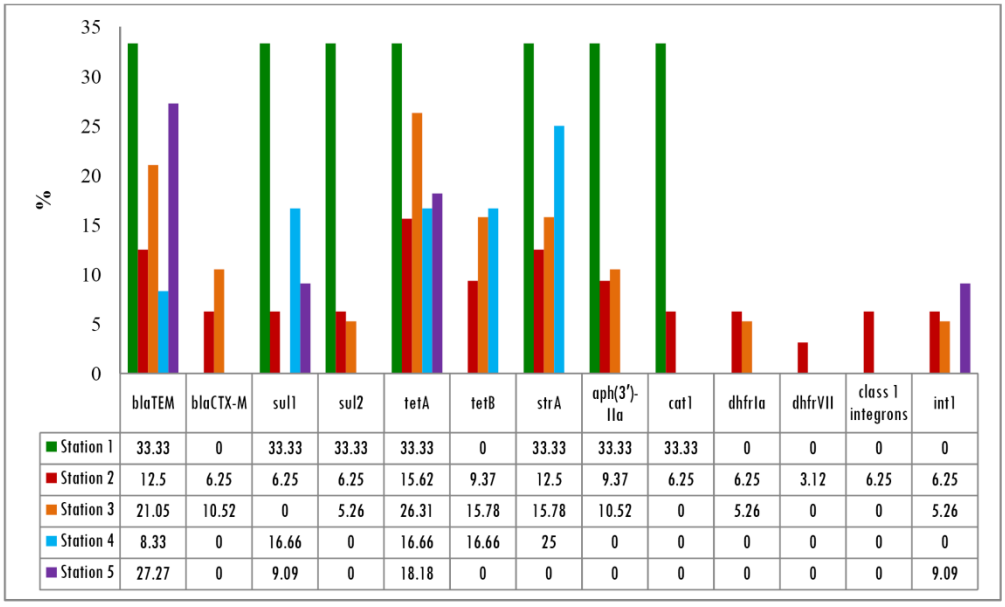
	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
Station 1	21.05	10.52	0	0	0	0	0	0	0	0	0	0	0
Station 2	44.44	0	22.22	11.11	11.11	0	11.11	0	0	0	0	0	11.11
Station 3	50	12.5	25	0	12.5	0	12.5	0	0	0	0	0	25
Station 4	33.33	8.33	25	25	25	16.66	16.66	8.33	0	25	16.66	25	25
Station 5	14.28	4.76	23.8	9.52	28.57	14.28	4.76	0	0	0	0	0	0

Figure 5.19 Percentage occurrence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'B1' from different stations at Cochin estuary

5.5.1.8.3 Distribution of antibiotic resistance genes among *E. coli* phylogenetic group 'B2' isolates from different stations at Cochin estuary

Resistance genes such as bla_{TEM}, *sul1*, *sul2*, *tetA*, *tetB*, *strA*, *aphA2*, and *catI* (Figure 5.20) were frequently encountered among group B2 isolates from station 1 (Chitoor). While station 2 showed moderate percentages of occurrence for all antibiotic resistance genes, B2 isolates from station 3 also showed similar level of occurrence for most of the antibiotic resistance genes except, *catI*, *sul1*, *dhfrVII*, and *class 1 integrons*. *E. coli* belonging to group B2 from station 4 and 5 showed moderate percentages of occurrence for bla_{TEM}, *sul1*, *tetA*, *tetB*, *strA* and bla_{TEM}, *sul1*, *tetA*, *int1* respectively. There was a significant difference in the distribution of resistance genes in group B2 isolates from different stations (ANOVA, $p < 0.001$). Phylogenetic group B2

isolates from Station 2, Bolgatty carried significantly higher resistance genes compared to stations 1, 4 and 5 ($p < 0.05$).



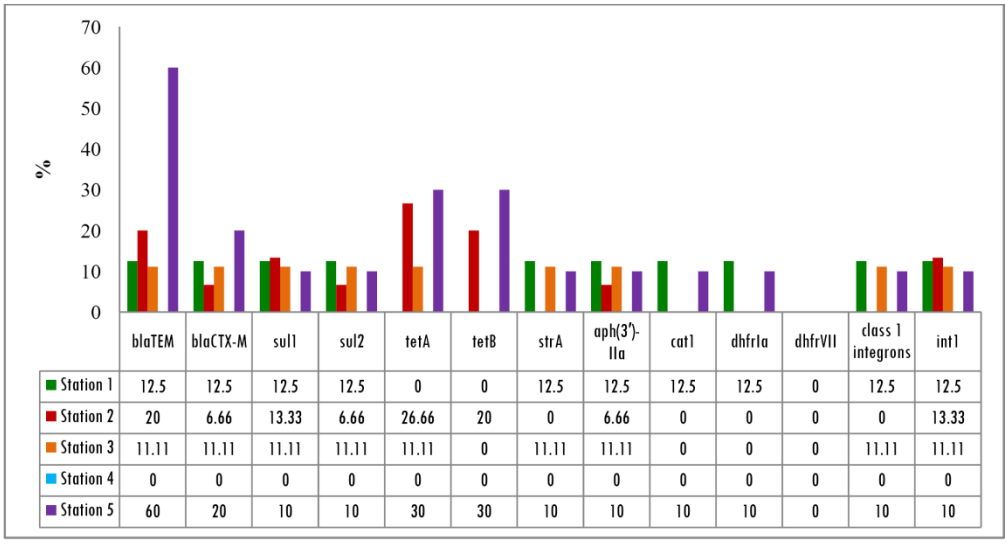
	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
Station 1	33.33	0	33.33	33.33	33.33	0	33.33	33.33	33.33	0	0	0	0
Station 2	12.5	6.25	6.25	6.25	15.62	9.37	12.5	9.37	6.25	6.25	3.12	6.25	6.25
Station 3	21.05	10.52	0	5.26	26.31	15.78	15.78	10.52	0	5.26	0	0	5.26
Station 4	8.33	0	16.66	0	16.66	16.66	25	0	0	0	0	0	0
Station 5	27.27	0	9.09	0	18.18	0	0	0	0	0	0	0	9.09

Figure 5.20 Percentage occurrence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'B2' from different stations at Cochin estuary

5.5.1.8.4 Distribution of antibiotic resistance genes among *E. coli* phylogenetic group 'D' isolates from different stations at Cochin estuary

There was a significant difference in the distribution of resistance genes in group D isolates from different stations (ANOVA, $p < 0.001$). While station 1 showed moderate percentages of occurrence for most of the antibiotic resistance genes except, *tetA*, *tetB*, and *dhfrVII* (Figure 5.21), station 2 and 3 showed moderate percentages of occurrence for bla_{TEM}, $bla_{CTX\text{-}M}$, *sul1*, *sul2*, *tetA*, *aphA2*, and *int1*. Group D isolates were absent in station 4 (Thevera). Group D isolates from station 5 (Barmouth) harboured highest percentages of resistance genes such as bla_{TEM}, $bla_{CTX\text{-}M}$, *tetA*, and *tetB*. Group D isolates

from stations such as Bolgatty and Barmouth harboured more resistance gene than station 4 ($p < 0.05$).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
Station 1	12.5	12.5	12.5	12.5	0	0	12.5	12.5	12.5	12.5	0	12.5	12.5
Station 2	20	6.66	13.33	6.66	26.66	20	0	6.66	0	0	0	0	13.33
Station 3	11.11	11.11	11.11	11.11	11.11	0	11.11	11.11	0	0	0	11.11	11.11
Station 4	0	0	0	0	0	0	0	0	0	0	0	0	0
Station 5	60	20	10	10	30	30	10	10	10	10	0	10	10

Figure 5.21 Percentage occurrence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'D' from different stations at Cochin estuary

5.5.2 Occurrence of antibiotic resistance genes among *E. coli* isolates from seafood sources

E. coli isolates from seafood showed significant differences in the distribution of different resistance genes (ANOVA, $p = 0.000$). Figure 5.22 shows the percentage occurrence of antibiotic resistance genes among *E. coli* isolated from seafood sources. Of the 51 phenotypically ampicillin resistant isolates, 21 (41.17%) were found to contain bla_{TEM} gene. Out of 7 ESBL producing isolates, 3 (42.85%) were found to contain $bla_{CTX\text{-}M}$ gene. Seven isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (4, 57.14%), *sul2* (3, 42.85%), and *sul1* + *sul2* (3, 42.85%). Statistical analysis of the result revealed a highly significant association was found between *sul1* with *sul2* ($p < 0.001$). Furthermore, a significant association was

found between sulphonamide resistance genes and *tetA*, *tetB, aphA2* and *integrase 1 gene* ($p < 0.001$).

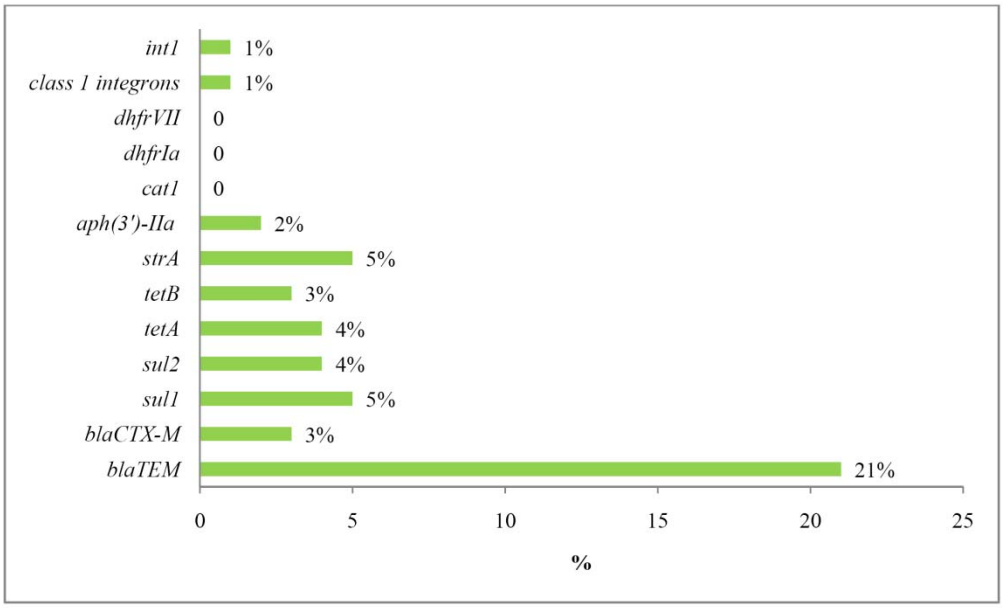


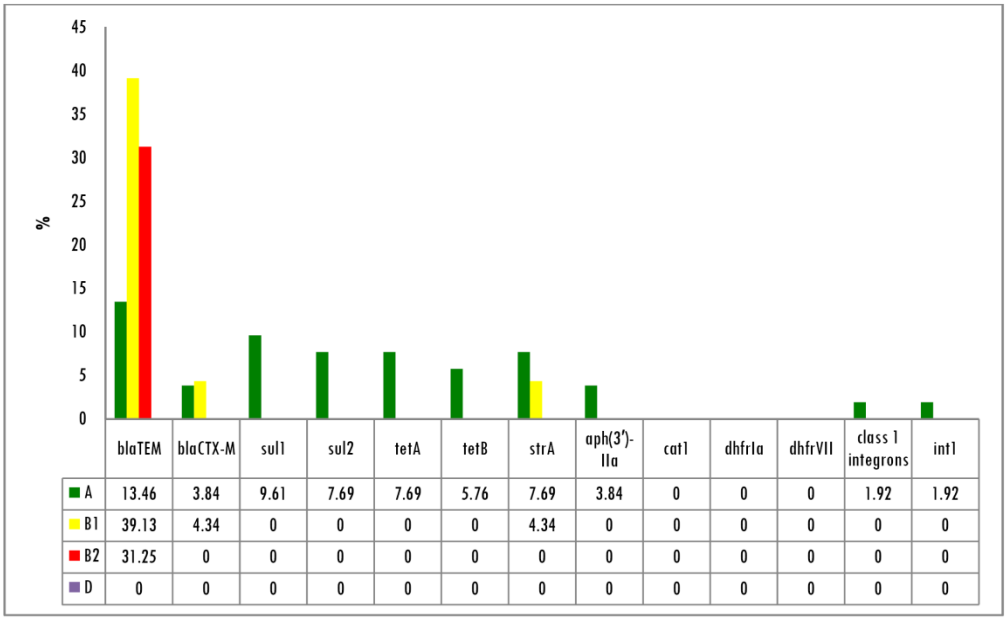
Figure 5.22 Percentage occurrence of antibiotic resistance genes among *E. coli* isolates from seafood sources (n = 100)

Trimethoprim resistance genes such as, *dhfrIa* and *dhfrVII* were not detected in seafood isolates. Regarding the 9 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 4 (33.33%), 3 (25%), and 3 (25%) isolates, respectively. Statistical analysis of the result revealed a highly significant association was found between *tetA* with *tetB* ($p < 0.001$). Furthermore, a significant association was found between tetracycline resistance genes and *sul1*, *sul2, aphA2* and *integrase 1 gene* ($p < 0.001$). Eleven streptomycin-resistant isolates were detected, 5 (45.45%) of them harboured the *strA* gene. Two out of three gentamicin-resistant isolated harboured *aph(3)-IIa* gene (66.66%). There was a significant association was found between *aphA2* and

sul1, *sul2, tetA, tetB* and *integrase 1 gene* ($p < 0.001$). Chloramphenicol-resistant gene *catI* was not detected in seafood isolates. *Class 1 integrons* and integrase gene (*int1*) were identified in only in one isolate. Statistical analysis of the result revealed a highly significant association was found between *integrase 1 gene* with *class 1 integron variable regions* ($p < 0.001$). Furthermore, a significant association was found between integrons and *sul1, sul2, tetA*, *tetB,* and *aphA2* ($p < 0.001$).

5.5.2.1 Incidence of antibiotic resistance genes in various phylogenetic groups of *E. coli* isolates from seafood sources

E. coli isolates belonged to phylogenetic group A which showed relatively high percentage of occurrence for *sul1*, *sul2*, *tetA*, *tetB*, *strA*, *aphA2*, *class 1 integrons* and *int1* (Figure 5.23). Group B1 showed the occurrence of bla_{TEM}, $bla_{CTX\text{-}M}$, and *strA*, group B2 showed the occurrence of bla_{TEM} gene only.



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
A	13.46	3.84	9.61	7.69	7.69	5.76	7.69	3.84	0	0	0	1.92	1.92
B1	39.13	4.34	0	0	0	0	4.34	0	0	0	0	0	0
B2	31.25	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 5.23 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from seafood sources (n = 100)

None of the resistance related genes was encountered in group D isolates. In Seafood isolates, there was no significant difference in the distribution of resistance genes among different phylogenetic groups (ANOVA, p = 0.364). But phylogenetic group A isolates harboured significantly more resistance genes than group D (ANOVA, p = 0.006).

5.5.3 Occurrence of antibiotic resistance genes among *E. coli* isolates from clinical sources

In clinical isolates, significant differences were found in the distribution of different resistance genes (ANOVA, p = 0.000). Figure 5.24 shows the percentage occurrence of antibiotic resistance genes among *E. coli* isolates from clinical sources.

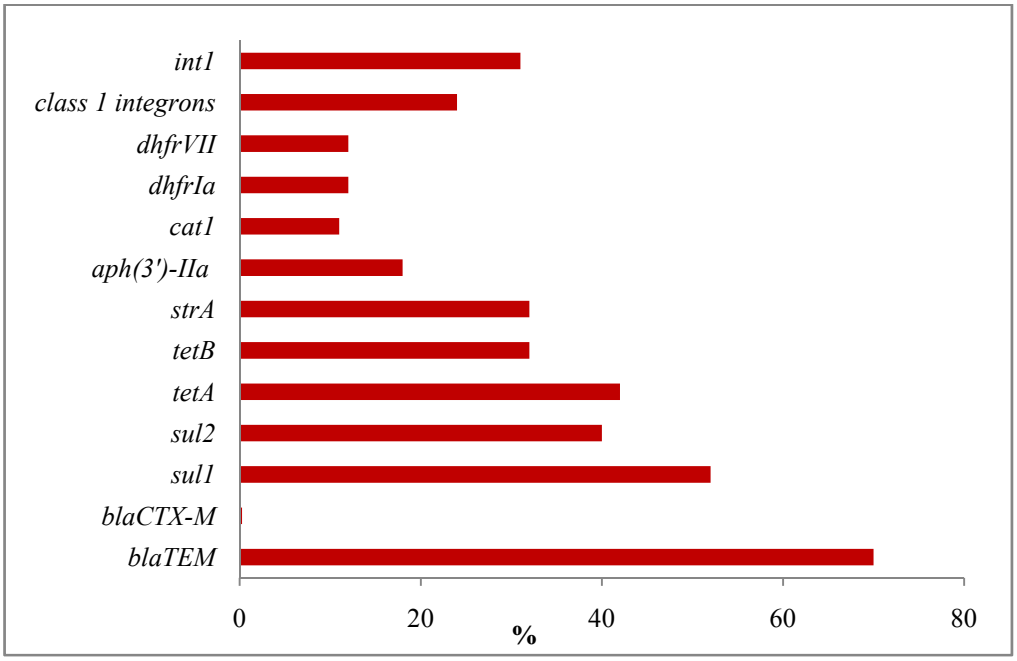


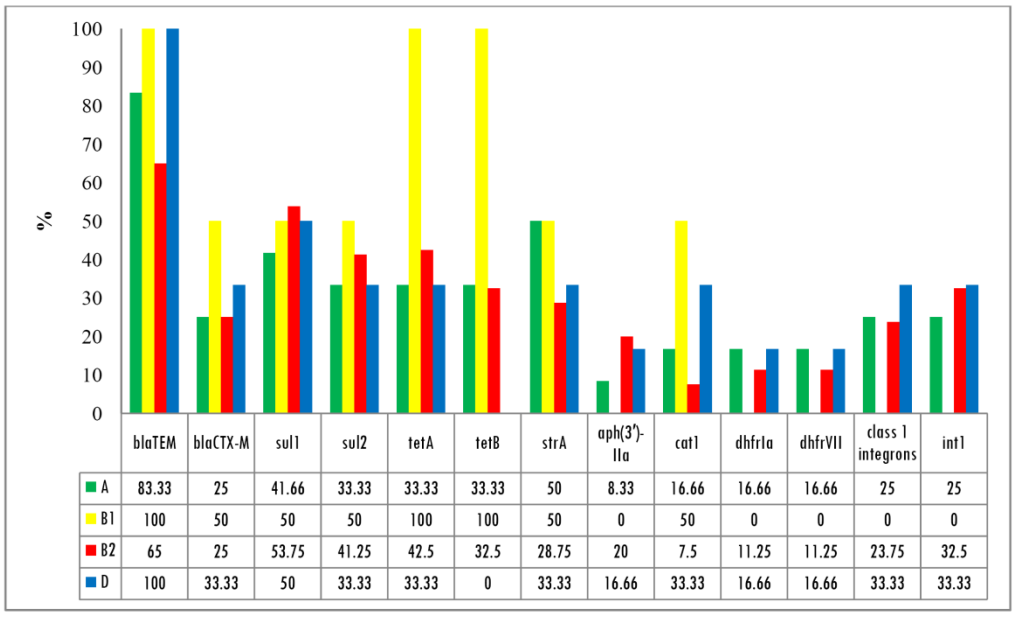
Figure 5.24 Percentage occurrence of antibiotic resistance genes among *E. coli* isolates from clinical sources (n = 100)

Games-Howell analysis showed that the percentage of distribution of bla_{TEM} gene was significantly higher than *to* bla_{CTX-M}, *aphA2*, *dhfrIa, dhfrVII*, *class 1 integrons* and *int1*($p < 0.05$). The percentage occurrence of *sul1* gene was significantly higher than to *aphA2*, *dhfrIa* and *dhfrVII*, ($p < 0.05$). Similarly occurrence of *sul2* gene was significantly higher than *to dhfrIa*, and *dhfrVII* ($p = 0.042$).

Of the 98 phenotypically ampicillin resistant isolates, 70 (71.42%) were found to contain bla_{TEM} gene. Out of 51 ESBL producing isolates, 5.26 (50.98%) were found to contain bla_{CTXM} gene. Statistical analysis of the result revealed a highly significant association was found between bla_{CTX-M} and *catI genes* ($p < 0.05$). Fifty five isolates showed sulphonamide resistance phenotype which was encoded by *sul 1* (42, 76.36%), *sul2* (30, 54.54%), and *sul1* + *sul2* (27, 49.09%). Regarding the 46 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 30 (65.21%), 24 (52.17%), and 17 (36.95%) isolates, respectively. Of the 38 streptomycin-resistant isolates detected, 30 (78.94%) of them harboured the *strA* gene. The *aph(3)-IIa* gene was found in 14 isolates (63.63%) out of 22 gentamicin-resistant isolates. Eleven out of 12 chloramphenicol-resistant isolated harboured a *cat1* (91.66%) gene. Among 54 trimethoprim-resistant *E. coli* isolates, *dhfrIa* and *dhfrVII* was found in 12 (22.22%) isolates. Statistical analysis of the result revealed a highly significant association was found between *dhfrIa* and *dhfrVII genes* ($p < 0.001$). Furthermore, a significant association was found between trimethoprim-resistant genes and *class 1 integron* variable regions ($p < 0.05$). *Class 1 integrons* and integrase gene (*int1*) were identified in 24 and 31 isolates, respectively. A significant association was found between *integrase 1* gene and *class 1 integron* variable regions ($p < 0.05$)

5.5.3.1 Incidence of antibiotic resistance genes in various phylogenetic groups of *E. coli* isolates from clinical sources

Antibiotic resistance genes such as bla_{TEM}, bla_{CTX-M}, *sul1*, *sul2*, *tetA*, *tetB*, *strA*, and *catI* were distributed in 4 different phylogenetic groups (Figure 5.25). While other genes such as *aphA2*, *dhfrIa, dhfrVII class 1 integrons* and *int1* were distributed among A, B2 and D groups. *tetB* gene was distributed among A, B1 and B2 groups. Phylogenetic group B1 strains showed highest percentage of occurrence for bla_{TEM}, bla_{CTX-M}, *sul2*, *tetA*, *tetB*, *strA* and *cat1*. Phylogenetic group B2 strains showed highest percentage of occurrence for *sul1* and *aphA2*. Phylogenetic group D strains showed highest percentage of occurrence *class 1 integrons* and *int1*. In clinical isolates, there was no significant difference in the distribution of resistance genes among different phylogenetic groups (ANOVA, p = 0.653).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfrIa	dhfrVII	class 1 integrons	int1
A	83.33	25	41.66	33.33	33.33	33.33	50	8.33	16.66	16.66	16.66	25	25
B1	100	50	50	50	100	100	50	0	50	0	0	0	0
B2	65	25	53.75	41.25	42.5	32.5	28.75	20	7.5	11.25	11.25	23.75	32.5
D	100	33.33	50	33.33	33.33	0	33.33	16.66	33.33	16.66	16.66	33.33	33.33

Figure 5.25 Percentage occurrence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from clinical sources (n = 100).

5.5.4 Relative prevalence of antibiotic resistance genes among *E. coli* isolates from different sources: a comparison

Clinical *E. coli* isolates carried significantly high prevalence of resistance genes than seafood (p = 0.000) and estuarine isolates (p = 0.002). Figure 5.26 shows the relative prevalence of antibiotic resistance genes among *E. coli* isolates from different sources.

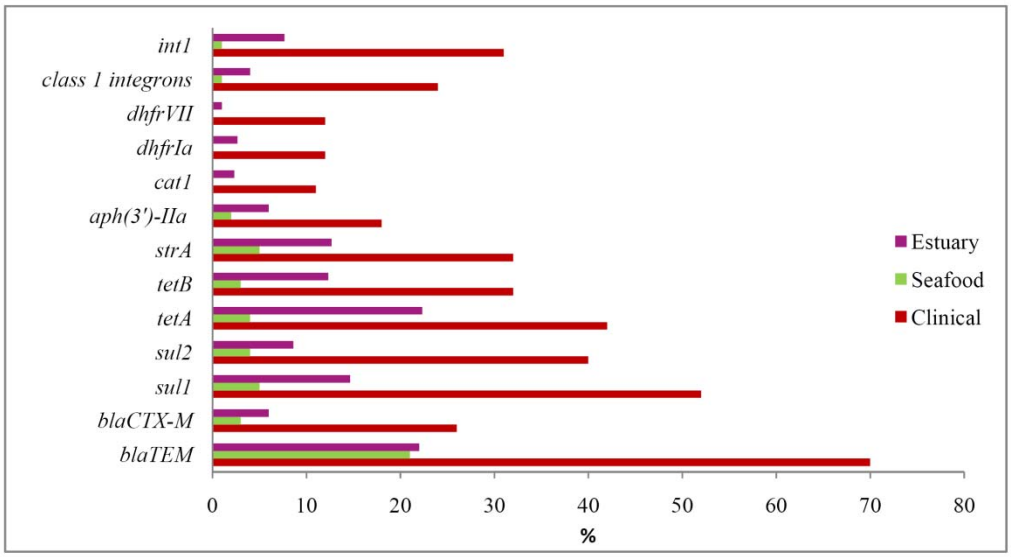
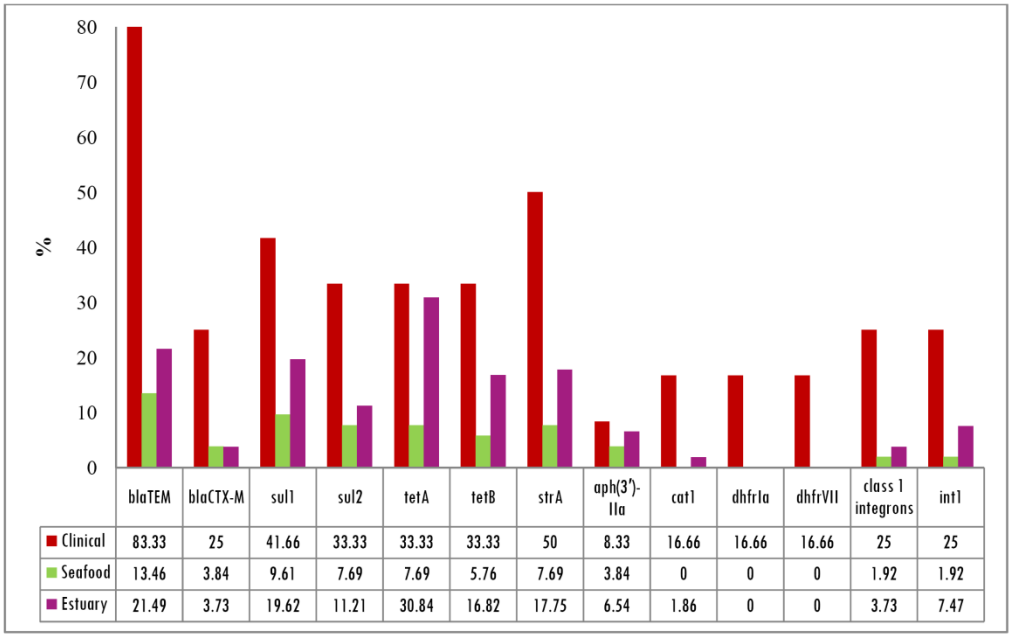


Figure 5.26 Relative prevalence of antibiotic resistance genes among *E. coli* isolates from different sources

5.5.5 Relative prevalence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from estuary, seafood and clinical Sources

Clinical isolates of *E. coli* belonging to group A phylogenetic group had high occurrence for all the genes tested (Figure 5.27). Estuarine group A isolates showed moderate percentages of occurrence for all the genes. *dhfrIa* and *dhfrVII* genes were absent in estuarine isolates. While group A seafood isolates showed least percentages of occurrence for all the genes. Genes such

as, *catI*, *dhfrIa,* and *dhfrVII* were absent in seafood isolates. There was a significant difference in the distribution of resistance genes in group A isolates from different sources (ANOVA, p = 0.000). Group A *E. coli* isolates from clinical sources carried significantly more resistance genes than seafood (p = 0.001) and estuarine isolates (p = 0.008).

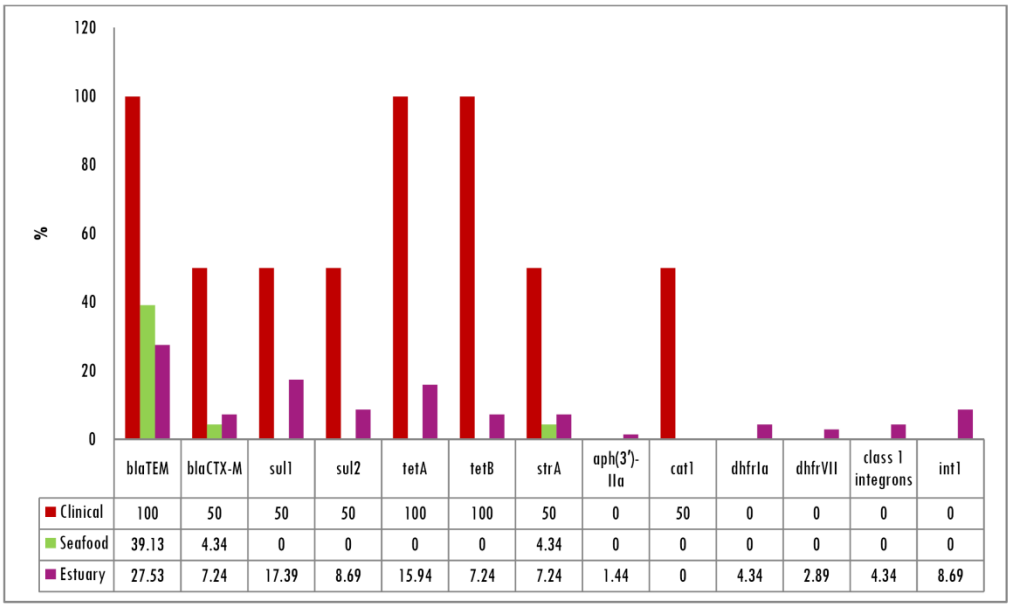


	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfrIa	dhfrVII	class 1 integrons	int1
Clinical	83.33	25	41.66	33.33	33.33	33.33	50	8.33	16.66	16.66	16.66	25	25
Seafood	13.46	3.84	9.61	7.69	7.69	5.76	7.69	3.84	0	0	0	1.92	1.92
Estuary	21.49	3.73	19.62	11.21	30.84	16.82	17.75	6.54	1.86	0	0	3.73	7.47

Figure 5.27 Prevalence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'A' from different sources

Phylogenetic group B1 isolates from clinical sources showed highest prevalence for all the genes tested (Figure 5.28), except *aphA2*, *dhfrIa*, *dhfrVII*, *class 1 integrons* and *int1* which were absent in group B1 clinical isolates. Estuarine group B1 isolates showed moderate percentages of occurrence for all the genes. While group B1 seafood isolates showed least percentages of occurrence for $bla_{CTX\text{-}M}$, and *strA*. Group B1 isolates from seafood sources carried more bla_{TEM} gene than those from estuarine isolates. There was a

significant difference in the distribution of resistance genes in group B1 isolates from different sources (ANOVA, p = 0.000). Clinical B1 isolates carried significantly more resistance genes than seafood (p = 0.012) and estuarine isolates (p = 0.027).

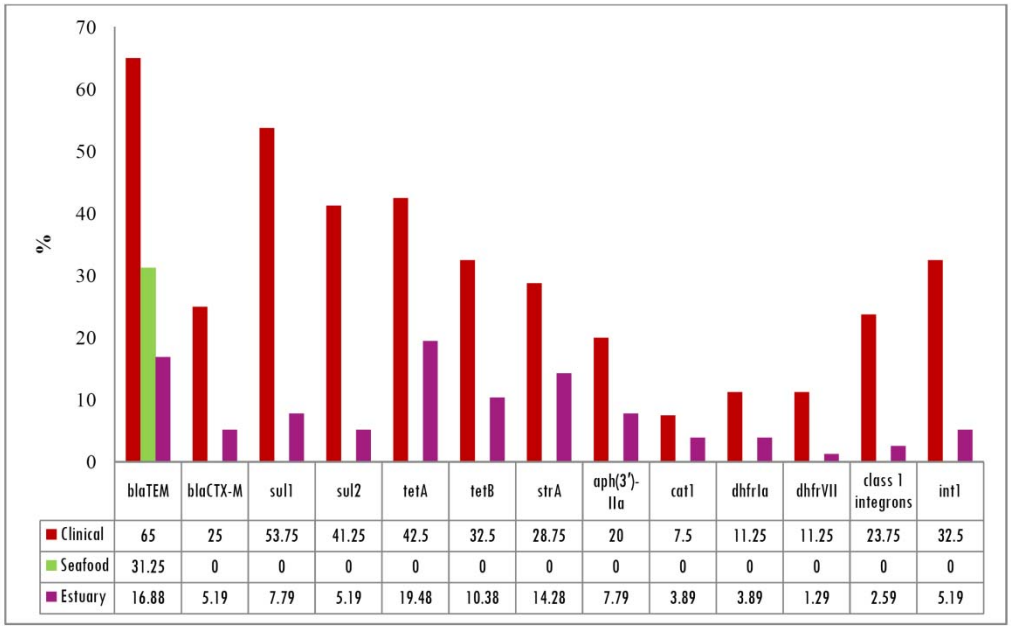


	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3")-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
Clinical	100	50	50	50	100	100	50	0	50	0	0	0	0
Seafood	39.13	4.34	0	0	0	0	4.34	0	0	0	0	0	0
Estuary	27.53	7.24	17.39	8.69	15.94	7.24	7.24	1.44	0	4.34	2.89	4.34	8.69

Figure 5.28 Prevalence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'B1' from different sources

Prevalence of all resistance genes were relatively higher in group B2 isolates from clinical sources. While group B2 isolates from estuarine showed moderate percentages of occurrence for all the genes (Figure 5.29). Group B2 isolates from seafood showed the occurrence of bla_{TEM} gene only. However, the percentage of bla_{TEM} gene was higher when compared to estuarine isolates. There was significant difference in the distribution of resistance genes in group B2 isolates from different sources (ANOVA, p = 0.000). Phylogenetic group B2 isolates from clinical sources carried significantly more resistance

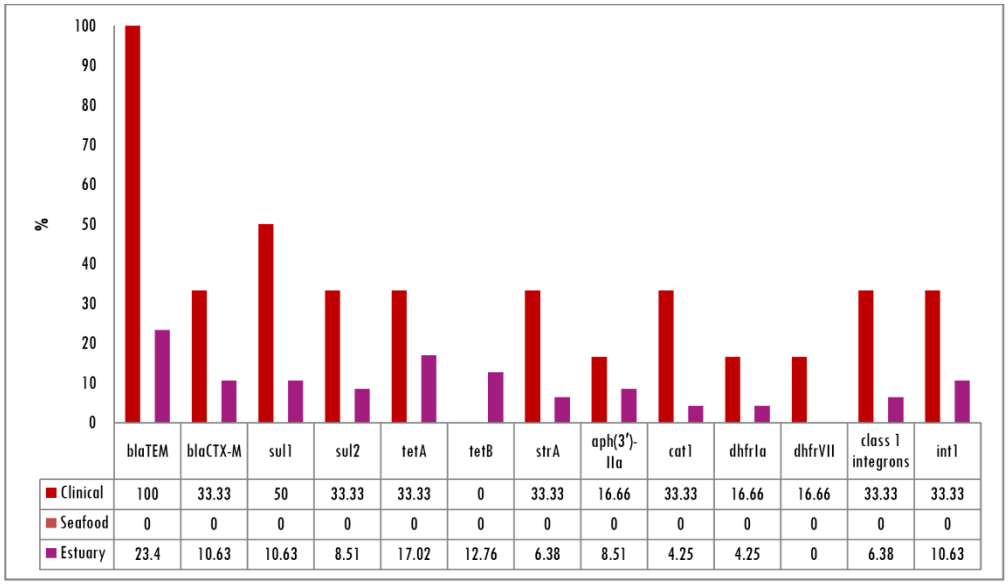
genes when compared to B2 group isolates from seafood (p = 0.000) and estuarine sources (p = 0.001).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
Clinical	65	25	53.75	41.25	42.5	32.5	28.75	20	7.5	11.25	11.25	23.75	32.5
Seafood	31.25	0	0	0	0	0	0	0	0	0	0	0	0
Estuary	16.88	5.19	7.79	5.19	19.48	10.38	14.28	7.79	3.89	3.89	1.29	2.59	5.19

Figure 5.29 Prevalence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'B2' from different sources

As in the case of other phylogenetic groups of *E. coli*, group D isolates from clinical sources showed highest prevalence for all the genes tested except *tetB* (Figure 5.30). *tetB* was absent in D isolates from clinical and seafood sources. Estuarine group D isolates showed moderate percentages of occurrence for all the genes except *dhfrVII*. *dhfrVII* was absent in estuarine D isolates. Group D isolates was absent in seafood isolates. There was a significant difference in the distribution of resistance genes in group D isolates from different sources (ANOVA, p = 0.000). Clinical D isolates carried significantly more resistance genes than seafood and estuarine isolates (p = 0.009).



	blaTEM	blaCTX-M	sul1	sul2	tetA	tetB	strA	aph(3')-IIa	cat1	dhfr1a	dhfrVII	class 1 integrons	int1
Clinical	100	33.33	50	33.33	33.33	0	33.33	16.66	33.33	16.66	16.66	33.33	33.33
Seafood	0	0	0	0	0	0	0	0	0	0	0	0	0
Estuary	23.4	10.63	10.63	8.51	17.02	12.76	6.38	8.51	4.25	4.25	0	6.38	10.63

Figure 5.30 Prevalence of antibiotic resistance genes among *E. coli* isolates belonged to phylogenetic group 'D' from different sources.

5.6 Discussion

5.6.1 Prevalence of antibiotic resistance genes among *E. coli* isolates from Cochin estuary

A total of three hundred *E. coli* were isolated from Cochin estuary and analysed the presence of antibiotic genes. In Cochin estuary, significant differences were found in the distribution of different resistance genes. The prevalence of bla_{TEM} gene was significantly higher than to $bla_{CTX\text{-}M}$, *sul2*, *aphA2*, *catI*, *dhfr Ia, dhfrVII*, *class 1 integrons* and *int1*. Among individual genes, the highest percentage of occurrence was *tetA* (22.23%), followed by bla_{TEM} (22%), *sul1* (14.66%), *strA* (12.66%) and *tetB* (12.33%). Between 5-10% occurrence was found for *sul 2* (8.6%), *int 1* (7.66%), $bla_{CTX\text{-}M}$ (6%), and *aphA2* (6%); occurrence of *class 1 integrons*, *dhfrIa*, *cat1*, and *dhfrVII* were lower, with percentages of 4%, 2.66%, 2.33%, and 1%, respectively.

Multiple genes encoding for antibiotic resistance have been frequently detected both in liquid (wastewater, surface water, groundwater and even drinking water) and solid (sludge, soil and sediment) environmental media (Munir *et al*., 2011; Storteboom *et al*., 2010; Xi *et al*., 2009; Brooks *et al*., 2007; Pei *et al*., 2006; Chee-Sanford *et al*., 2001).

5.6.1.1 *bla$_{TEM}$*

Of the 129 ampicillin-resistant isolates, 66 (51.66%) were found to contain *bla$_{TEM}$* gene. Out of 23 ESBL producing isolates, 18 (78.2%) were found to contain *bla$_{CTX-M}$* gene. Korzeniewska and Harnisz, (2013) analysed the β-lactamase producing *Enterobacteriacea*e in hospital effluents and reported that 54.9% and 11% of isolates carried *bla$_{CTX-M}$* and *bla$_{TEM}$*, respectively. The frequency of *bla$_{TEM}$* detected by Korzeniewska and Harnisz, (2013) was almost similar to our results. Hu *et al*., (2008) reported that 97% of the ampicillin (AMP) resistant mechanism in *E. coli* could be explained by the resistance gene *bla$_{TEM}$*. Our results revealed higher prevalence of *bla$_{TEM}$* gene than those obtained by Hamelin *et al*., (2006), who reported the frequency of *bla$_{TEM}$* in 4.9% of *E. coli* isolates only. Korzeniewska *et al*., (2013) analysed the antibiotic resistant *E. coli* in hospital and municipal sewage and reported a lower prevalence of *bla$_{TEM}$* (33.6%) and *bla$_{CTX-M}$* (60.6%) than our results. Henriques *et al*. (2006) detected the β-Lactamase genes in 77.8% of the *Enterobacteriaceae* and also reported that most frequently detected gene was *bla$_{TEM}$*. In a study by Alpay-Karaoglu *et al*., (2007) on the determinants of the β-lactam resistance from drinking waters showed that TEM-1 type β-lactamase genes have been wide spread in ampicillin-resistant *E. coli* strains isolated from fresh waters indicating a fecal contamination.

5.6.1.2 *bla$_{CTX-M}$*

Plasmids carrying ESBL genes frequently are conjugative and lodge determinants of resistance to non-β-lactams such as tetracyclines, quinolones or aminoglycosides. In that case, ESBL-positive strains are multiresistant and pose major public health concerns (Carattoli *et al*., 2011; Coque *et al*., 2008a). Tacao *et al*., (2012) analysed a set of cefotaxime-resistant strains isolated from river waters in Portugal and it was found that multiresistance was frequent among ESBL positive strains mostly carrying *bla$_{CTX-M}$*. They also reported the prevalence of *intI1* was 56.41% (22 out of 39) among ESBL positive strains and 27.67% (31 out of 112) among ESBL negative strains. A recent study has shown that *E. coli* from environmental sources, including public tap water from New Delhi area, India were positive for multiple classes of β-lactamase, including the NDM-1 (Walsh *et al*., 2011b).

5.6.1.3 Tetracycline resistance genes (*tetA* and *tetB*)

In this study, 67 isolates among 91 tetracycline-resistant *E. coli* isolates carried *tetA* with a frequency of 73.62%, which is much higher than that (66%) in *E. coli* isolated from natural river basin (Hu *et al*., 2008). Whereas frequency of *tetB* (40.65%) detected in our study was lower than those reported by Hu *et al*., (2008). The presented results are higher than those obtained by Hamelin *et al*., (2006), who reported the frequency of *tet*(A) and *tet*(B) in 5.2% and 2.6% of *E. coli* isolates, respectively

5.6.1.4 Aminoglycoside resistance genes (*strA* and *aphA2*)

Forty-seven streptomycin-resistant isolates were detected, 36 (76.59%) of them harboured the *strA* gene. Out of 13 gentamicin-resistant isolates, the

aphA2 gene was found in 11 isolates (84.61%). The presented results for *strA*, *aphA2* and *cat1* were higher than those obtained by Hamelin *et al*., (2006), who reported the frequency of *strA*, *aac (3)1b* and *cat1* in 4.9%, 0.3% and 1.3% of *E. coli* isolates, respectively.

5.6.1.5 Chloramphenicol-resistant gene (*catI*)

Seven out of 12 chloramphenicol-resistant isolated harboured a *cat1* (58.33%) gene. Hamelin *et al*., (2006) reported that 1.3% of *E. coli* isolates recovered from a beach carried *cat1* gene. Detection of chloramphenicol resistance determinants has received little attention, because chloramphenicol is little used for the treatment of severe infections.

5.6.1.6 Sulphonamide resistance genes (*sul1* and *sul2*)

Fifty seven isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (75.43%), *sul2* (45.61%), and *sul1* + *sul2* (38.59%). Multiple *sul* gene combinations were also observed in our study. Su *et al*., (2012) studied the *sul* resistance genes and antibiotic resistance in *E. coli* isolated from a river and reported that among the resistant isolates, *sul2* had the highest prevalence (89.2%), followed by *sul3* (42.2%) and *sul1* (40.6%). Su *et al*., (2012) also reported the combination of *sul1* and *sul2* (23.2%), which was lower than the level encountered in our results. The presented result for *sul2* was also higher than those obtained by Hamelin *et al*., (2006), who reported the frequency of *sul2* in 4.6% of *E. coli* isolates. In contrast with our results, Gao *et al*., (2012) reported that *sul2* was detected with higher frequency than *sul1* at all of the sampling sites in water and sediment. Previous studies (Frank *et al*., 2007; Blahna *et al*., 2006; Enne *et al*., 2002) showed that the *sul1* was typically associated with *class1 integrons*, while *sul2*

was mostly found on small non-conjugative plasmids (Skold, 2000) or large transmissible multi-resistant plasmids (Enne *et al*., 2001), which may lead to *sul2* widespread detection in the environment. The observed frequency for both *sul1* and *sul2* was high in this study. A similar pattern is also found in sulphonamide resistant *E. coli* isolates from a natural river basin (Hu *et al*., 2008). Hoa *et al*., (2008) reported that *sul1* was major in human-mediated environments of North Vietnam, with the frequency of 60.0%. Being different from the study by Hoa *et al*., (2008), *sul2* was found positive in 89.2% of the SXT-resistant *E. coli* isolates, followed by *sul3* (42.2%) and *sul1* (40.6%) in the present study. This indicates the diversity of *sul* genes distribution profiles among various areas and various bacterial species. Byrne-Bailey *et al*., (2009) found that *sul2* had the highest prevalence (51.4%), followed by *sul1* (48.0%) and subsequently by *sul3* (29.9%) in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom.

5.6.1.7 Trimethoprim-resistant genes (*dhfrIa* and *dhfrVII)*

Among 57 trimethoprim-resistant *E. coli* isolates, *dhfrIa* was found in 8 (14.03%) isolates, and *dhfrVII* was found in 3 (5.26%) isolates. Previous studies reported the occurrence of *dhfr* genes among food and food producing animals (Smith *et al*., 2010; Blahna *et al*., 2006; Marynard *et al*., 2003) and clinical isolates (Baudry *et al*., 2009; Frank *et al*., 2007).

5.6.1.8 *Class 1 integrons* and *int1* gene

In this study, 300 strains of *E. coli* were isolated from different stations of Cochin estuary, of which 12 (4%) were harboured *class 1 integrons*. Consistent with our findings, Hamelin *et al*., (2006) reported that many of the *E. coli* isolates that carried resistance genes had a *class 1 integron* (4% of the

isolates) and also carried two or more antibiotic resistance genes. In a study by Rosser and Young (1999) who showed that 3.6% of Gram-negative bacteria in an estuarine environment contained the *class 1 integron*. Prevalence level encountered in our result was higher than that reported by Ozgumus *et al*., (2007) who reported that *E. coli* isolates from public drinking waters harbored 2.5% of *class 1 integrons*.

All (12 of 12) integron-bearing isolates of *E. coli* were multiresistant, i.e. resistant to antibiotics of at least three different classes. Isolates with *class 1 integrons* expressed significantly broader antibiotic resistance ranges compared to those harbouring *class 2 integrons*. This may be explained by greater diversity of the gene cassette content of *class 1 integrons* (Ozgumus *et al*., 2009; Gu *et al*., 2008; Moura *et al*., 2007).

A total of 86 *E. coli* isolates from Cochin estuary were multidrug-resistant. *Class 1 integrons* were found in twelve (13.95%) out of 86 multidrug resistant *E. coli* isolates from Cochin estuary. In consistent with our study, Roe *et al*., (2003), reported that 13% of *E. coli* in irrigation water and sediments contained the *class 1 integron*. Whereas, the prevalence observed in our studies is much lower than those reported by Lin and Biyela (2005) and Park *et al*., (2003), who showed that 58% and 24% of multiresistant isolates both with aquatic environments contained the *class 1 integron*, respectively. Mokracka *et al*., (2012) analysed the integrons in *Enterobacteriaceae* isolated from municipal wastewater treatment plant and reported that the percentage of integron-harbouring strains differed between sampling places and was the highest in untreated sewage (22.5%), the lowest in aeration tank (4.9%), whereas in final effluent it was equal to 9.1%.

Our results revealed that, 86.05 percentages of multidrug-resistant isolates did not harbour *class 1 integrons*. Although multiresistance phenotypes in clinical strains have been correlated with the presence of integrons (Leverstein-van Hall *et al*., 2002b), this association has not been observed in isolates from waste water environments (Li *et al*., 2010), suggesting that multiresistance is a common trait in waste water bacteria even in the absence of integrons. In a study by Mokracka *et al*., (2012) 1832 strains of the family *Enterobacteriaceae* were isolated from different stages of a municipal wastewater treatment plant, of which 221 (12.1%) were *intI*-positive. They also observed that among them 61.5% originated from raw sewage, 12.7% from aeration tank and 25.8% from the final effluent.

In this study, 300 strains of *E. coli* were isolated from five different stations of Cochin estuary, of which 22 (7.33%) were harboured *integrase I* gene. *Integrase1* gene was found in 23 (25.58%) of 86 multidrug resistant *E. coli* isolates from Cochin estuary. The percentage of *intI*-positive strains in estuarine water is probably a reflection of frequency of strains bearing integrons in hospitals, commensal flora and animal-originating bacteria. For comparison, the occurrence of *intI*-positive strains among clinical isolates of *Enterobacteriaceae* ranges from 12.3% to 59.9% (Gu *et al*., 2008; Dakic *et al*., 2007). The frequency of strains with integrons in commensal flora reaches about 11% - 49% and in animal-originating bacteria 27% - 82% (Shaheen *et al*., 2010; Sepp *et al*., 2009; Cocchi *et al*., 2007b; Skurnik *et al*., 2005; Keyes *et al*., 2000).

The percentages of integrase-positive *E. coli* in Cochin estuary reached 7.33% of isolates and were lower to the values reported by Moura *et al*., (2007),

who have detected more than 30% of integron positive *Enterobacteriaceae* and *Aeromonas* sp. in a slaughterhouse waste water treatment plant in Portugal by using dot blot hybridization with integrase specific probes. Similar values of *int1* were observed by Moura *et al*., (2012) in *Enterobacteriaceae* isolated from urban waste water influents. They also reported that overall prevalence of *intI1* was 3.59% and was more abundant in raw waters. Ma *et al*., (2011) have examined the same families of bacteria in a municipal waste water treatment plant and recorded mean prevalence of strains harboring *class 1 integrase* equal to 30.2%. The obtained value were lower than those presented by Chen *et al*., (2011), who found 41% of *intI*-positive *E. coli* in a Minjiang River, China. Similar proportions of isolates with integrons have been found in coliform isolated from the rivers of Northern Turkey (Ozgumus *et al*., 2009) and *E. coli* in Seine River, France (Laroche *et al*., 2009). The frequency of *intI*-positive strains in *E. coli* in the research of Ferreira da Silva *et al*., (2007) was almost the same at different sites of treatment process and reached 10% in raw sewage and 9.6% in effluent. Koczura *et al*. (2012) determined the frequency of integron presence in *E. coli* isolates cultured from a river (6%), wastewater treatment plant (11%) and raw sewage (15.1%). In a study by Mokracka *et al*., (2012) 1832 strains of the family *Enterobacteriaceae* were isolated from different stages of a municipal wastewater treatment plant, of which 221 (12.1%) were *intI*-positive. They also observed that among them 61.5% originated from raw sewage, 12.7% from aeration tank and 25.8% from the final effluent.

Analysis of antibiotic resistance profiles of *intI*-positive *E. coli* showed that 95.65% of them were multiresistant. Mokracka *et al*., (2012) and Leverstein-van Hall *et al*., (2003) analysed the antibiotic resistance profiles of

intI-positive *E. coli* and reported that 99% of them were multiresistant, which is typical of bacteria harbouring integrons.

5.6.2 Prevalence of antibiotic resistance genes among *E. coli* isolates from seafood sources

In seafood isolates, significant differences were found in the distribution of different resistance genes. Marynard *et al*., (2003) reported a significant differences in the distributions of tetracycline (*tet*(A), *tet*(B), *tet*(C)), trimethoprim (*dhfrI*, *dhfrV*, *dhfrXIII*), and sulphonamide (*sulI*, *sulII*) resistance genes in *E. coli* isolated from pigs. Boerlin *et al*., (2005) observed an associations among *tetA*, *sul1*, *aadA*, and *aac(3)IV* and among *tetB*, *sul2*, and *strA*/*strB*, with a strong negative association between *tetA* and *tetB*.

5.6.2.1 bla_{TEM}

Of the 51 phenotypically ampicillin resistant isolates, 21 (41.17%) were found to contain bla_{TEM} gene. Out of 7 ESBL producing isolates, 3 (42.85%) were found to contain $bla_{CTX\text{-}M}$ gene. These findings concur with previous studies reporting that TEM-type β-lactamases were the most prevalent in ampicillin-resistant *E. coli* isolates from food and food-producing animals (Jouini *et al*., 2009; Van *et al*., 2008; Saenz *et al*., 2004; Guerra *et al*., 2003; Brinas *et al*., 2002). In consistent with our results, Ahmed *et al*., (2013) and Cocchi *et al*., (2007a) reported similar value for bla_{TEM} gene. Many studies have reported higher frequency of bla_{TEM} gene in *E. coli* isolated from cattle (Karczmarczyk *et al*., 2011; Guerra *et al*., 2003), poultry (Soufi *et al*., 2011; Brinas *et al*., 2002), pig (Smith *et al*., 2010; Marynard *et al*., 2003), and shellfish (Van *et al*., 2008). Ryu *et al*., (2012), Scaria *et al*., (2010) and Blanc *et al*., (2006) reported lower values for bla_{TEM} gene in seafood and cattle, respectively.

5.6.2.2 *bla*$_{CTX-M}$

In our study, 42% of cephalosporin resistant *E. coli* carried *bla*$_{CTX-M}$. Ahmed *et al*., (2013), Ramos *et al*., (2012) and Blanc *et al*., (2006) reported lower value for *bla*$_{CTX-M}$, than our results. Whereas Stuart *et al*., (2012), Slama *et al*., (2010) and Smet *et al*., (2008) reported higher values for *bla*$_{CTX-M.}$

5.6.2.3 Sulphonamide resistance genes (*sul1* and *sul2*)

Seven isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (4, 57.14%), *sul2* (3, 42.85%), and *sul1* + *sul2* (3, 42.85%). Srinivasan *et al*., (2007) reported that among sulfisoxazole resistance genes, *sulI* (29.6%) was found in higher frequency than *sulII* (25%) in sulfisoxazole-resistant *E. coli*. Lanz *et al*., (2003) also observed that prevalence of *sul1* than *sul2* in *E. coli* isolated from different animal species. Lanz *et al*., (2003) also detected significantly less *sul2* genes in *E. coli* from pigs (8%) than from cattle, cats/dogs, and hens. However, Marynard *et al*., (2003) reported that *sul2* was more dominant than *sulI* in *E. coli* isolated from pigs. In a previous study, transfer of *sul2* from an *E. coli* strain of pig origin to a sulphonamide sensitive *E. coli* strain of human origin in the intestine of streptomycin-treated mice was demonstrated (Sandvang *et al*., 2003). This indicates that *sul* genes of animal origin might transfer from commensal bacteria to pathogenic *E. coli* in the intestines of humans. Many studies have reported higher frequency of *sul2* gene in *E. coli* isolated from cattle (Guerra *et al*., 2003), and poultry (Soufi *et al*., 2011; Kozak *et al*., 2009). Previous studies also reported low frequency for *sul 2* in cattle (Srinivasan *et al*., 2007), food producing animals (Ho *et al*., 2009), swine (Kozak *et al*., 2009), pig (Schwaiger *et al*., 2010; Smith *et al*., 2010), and poultry (Soufi *et al*., 2011). Previous studies reported

lower frequency for *sul1* in cattle (Karczmarczyk *et al*., 2011; Srinivasan *et al*., 2007; Guerra *et al*., 2003), food producing animals (Ho *et al*., 2009), poultry (Kozak *et al*., 2009), pig (Schwaiger *et al*., 2010; Kozak *et al*., 2009) and shellfish (Van *et al*., 2008). Smith *et al*., (2010) reported high frequency for *sul1* from *E. coli* isolated from pigs with post weaning diarrhoea.

5.6.2.4 Tetracycline resistance genes (*tetA* and *tetB*)

Regarding the 91 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 4 (33.33%), 3 (25%), and 3 (25%) isolates, respectively. In consistent with our results, Schwaiger *et al*., (2010) and Costa *et al*., (2009) reported similar frequency for *tetA*. Gow *et al*., (2008) found a negative association between the presence of *tet(A)* and *tet(B)*. Their results also suggested that the horizontal transfer of *tet* genes, rather than the dissemination of a specific clonal strain, led to widespread distribution of tetracycline resistance, as suggested in another study (Sawant *et al*., 2007). Lanz *et al*., (2003) showed that the *tet*(A) gene alone is the most prevalent *tet* gene among *E. coli* isolates from pigs with diarrhea or enterotoxemia. Miles *et al*., (2006) noted that tetracycline resistance was generally associated with plasmids, and the resistance was mediated by *tet*B or *te*tD efflux genes. Previous studies has shown that *tet(A)* was detected mainly in isolates of animal origin, while *tet(B)* was predominant in human isolates (Roberts, 2005; Bryan *et al*., 2004; Helmuth *et al*., 2004; Sengelov *et al*., 2003; Chopra and Roberts, 2001; Levy *et al*., 1985). Many studies have reported higher frequency of *tet* gene in *E. coli* isolated from cattle (Karczmarczyk *et al*., 2011; Scaria *et al*., 2010; Guerra *et al*., 2003), poultry (Ahmed *et al*., 2013; Soufi *et al*., 2011), meat products (Koo and Woo, 2011) and pig (Smith *et al*.,

2010; Marynard *et al*., 2003), and seafood (Ryu *et al*., 2012). Van *et al*., (2008) and Srinivasan *et al*., (2007) reported a low frequency for *tetB* and *tetA* gene, respectively.

5.6.2.5 Aminoglycoside resistance genes (*strA* and *aphA2*)

Eleven streptomycin-resistant isolates were detected, 5 (45.45%) of them harboured the *strA* gene. Schwaiger *et al*., (2010) reported that streptomycin resistance was mostly mediated by *str(A)/str(B)* in porcine, and by *str(A)/str(B)/aad(A)* in human strains. Chiou and Jones, (1995) found that co-occurrence of *str(A)* and *str(B)* is essential for a functional streptomycin resistance. Two out of three gentamicin-resistant isolates harboured *aph(3)-IIa* gene (66.66%). Marynard *et al.*, (2003) reported that among the genes in ETEC isolate, only the aminoglycoside resistance genes revealed limited diversity, with the most prevalent genes *aph*(*3*)-*Ia* and *aph*(*3*)-*IIa*. Scaria *et al*., (2010) reported that 17% of *E. coli* isolated from cattle carried *aph 3'-(1b)*. Soufi *et al*., (2011) reported that 29% of *E. coli* isolates from poultry carried *aphA2* gene.

5.6.2.6 Chloramphenicol-resistant gene (*catI*)

In our study *cat1* gene was not detected. Marynard *et al*., (2003) reported that 79% of isolated carried *cat1* gene in *E. coli* isolated from pigs. They also reported that resistance to chloramphenicol was closely associated with the presence of the *catI* gene. In a study done by Bischoff *et al*., (2002), only 8.33% of chloramphenicol-resistant isolates harboured the *catII* gene. Van *et al*., (2008) reported that 18.4% of chloramphenicol-resistant isolates harboured the *cat1* gene. Whereas Smith *et al*., (2010) reported lower frequency for *cat1* gene in *E. coli* isolated from pigs with post weaning diarrhoea.

5.6.2.7 Trimethoprim-resistant genes (*dhfrIa* and *dhfrVII)*

In our study *dhfr1a* and *dhfrVII* genes were not detected. Among isolates obtained in Korea during 2001-2003, it was reported that *dfrA1* and *dfrA12* were predominate alleles among intestinal *E. coli* from food animals. In a multi-centre surveillance, *dfrA1* and *dfrA17* were found in 45% and 35%, respectively, of the trimethoprim-sulfamethoxazole resistant urinary *E. coli* isolates obtained from Europe and Canada (Blahna *et al*., 2006). Smith *et al*., (2010) reported lower frequency for *dhfrI* gene in *E. coli* isolated from pigs with post weaning diarrhoea. Marynard *et al*., (2003) reported that 20% of strains carried *dhfrI* genes and they also reported that *dhfr7* was absent in isolates from pig.

5.6.2.8 *Class 1 integrons* and *int1* gene

Class 1 integrons and integrase gene (*int1*) were identified in only in one isolate. Out of 13 multidrug resistant strains, 7.69% of isolates carried *class 1 integrons* and *int1 gene*. *Class 1 integrons* have been reported previously in 39.6% of APEC strains isolated from chickens suffering from colibacillosis in Korea (Kim *et al*., 2007b), and more recently in 33.0% of APEC strains isolated from intensively farmed and free-range poultry in Australia (Obeng *et al*., 2012). Moreover, *class 1 integrons* were identified in a plasmid from an APEC strain isolated in the United States (Johnson *et al*., 2005c). Previous reports have shown higher prevalence of integrons in *E. coli* collections: 64.4% in isolates from swine with diarrhoea (Kang *et al*., 2005b), 62% in intensive-care and surgical-unit isolates from hospitals in nine European countries (Martinez-Freijo *et al*., 1998), 59% in isolates from calf diarrhea cases (Du *et al*., 2005), 52% in various clinical isolates (Chang *et al*., 2000), and 45% in

urinary-tract isolates (White *et al*., 2001). Many studies have reported higher frequency of *class 1 integrons* in poultry (Ahmed *et al*., 2013, 2012; Soufi *et al*., 2011), food producing animals (Ho *et al*., 2009), cattle (Guerra *et al*., 2003), pigs (Marynard *et al*., 2003), fish and seafood (Ryu *et al*., 2012). Marchant *et al*., (2013) reported a higher frequency of integrons in pig than in chicken *E. coli* isolates. Kang *et al*., (2005a) and Lapierre *et al*., (2008) showed higher frequency of *class 1* than *class 2 integron* in *E. coli* isolates from healthy animals. Lu *et al*., (2010) reported a low frequency of *class 1 integrons* in *E. coli* from dairy cattle, which is lower than our results. Saenz *et al*., (2010) reported moderate frequency for *int1* in food and animals, with percentages of 19% and 12.5%, respectively.

5.6.3 Prevalence of antibiotic resistance genes among *E. coli* isolates from clinical sources

5.6.3.1 bla_{TEM}

Of the 98 phenotypically ampicillin resistant isolates, 70 (71.42%) were found to contain bla_{TEM} gene. The percentage of distribution of bla_{TEM} gene was significantly higher than *to* $bla_{CTX\text{-}M}$, *aphA2*, *dhfrIa*, *dhfrVII*, *class 1 integrons* and *intI*. Higher percentages of bla_{TEM} were reported in clinical *E. coli* isolates by Cooksey *et al*., (1990). Previous studies reported very low percentages of bla_{TEM} in clinical *E. coli* isolates (Hussain *et al*., 2012; Bindayna and Murtadha, 2011; Sharma *et al*., 2010; Velasco *et al*., 2007; Machado *et al*., 2005; Chanawong *et al*., 2002), when compared to our findings. Lavigne *et al*., (2007) reported lower percentages of bla_{TEM} gene in *E. coli* from urinary, blood and reparatory sources, with percentages of 42.6%, 4.3%, and 4.3%, respectively. In our study most of the bla_{TEM} harboured isolates were B2, followed by A, D and B1. Pitout *et al*., (2005) also reported that bla_{TEM} carried isolated belonged to B2, followed by D, A, and B1.

5.6.3.2 *bla$_{CTX-M}$*

Out of 51 ESBL producing isolates, 26 (50.98%) were found to contain *bla$_{CTX-M}$* gene. Higher percentages of *bla$_{CTX-M}$* were also reported in clinical *E. coli* isolates by many workers (Hoban *et al*., 2012; Hussain *et al.,* 2012; Sallem *et al*., 2012; Bindayna and Murtadha, 2011; Ode *et al*., 2009; Naas *et al*., 2007; Pitout *et al*., 2007; Velasco *et al*., 2007; Rodriguez-Bano *et al*., 2006; Machado *et al*., 2005; Chanawong *et al*., 2002). When compared to our findings, previous studies reported very low percentages of *bla$_{CTX-M}$* in clinical *E. coli* isolates (Martinez *et al*., 2012; Pitout *et al*., 2004). Lavigne *et al*., (2007) reported lower percentages of *bla$_{CTX-M}$* gene in *E. coli* from urinary, blood and reparatory sources, with percentages of 67.8%, 3.1%, and 4.6%, respectively.

The most common cause of resistance to β-lactams is the production of extended-spectrum β-lactamases (ESBLs), especially of the CTX-M type (Pitout, 2012). Chmielarczyk *et al*., (2013) and Coque *et al*., (2008a) reported that the most common *bla* genes were CTX-M-15 and CTX-M-3. Bonnedahl *et al*., (2009) reported that among 9.4% ESBL type β-lactam resistant isolates, all 10 CTXM type isolates belonged to the CTXM-1 group, specifically nine of *bla$_{CTX-M-1}$* genotype and one *bla$_{CTX-M-15}$*. Galas *et al*., (2008) and Bonnet, (2004) also reported that CTX-M-1 group was dominant in clinical isolates. Sallem *et al*., (2012) reported that 60% of CTX-M-1-producing *E. coli* strains carried integrons and, in most of the cases, these integrons lacked the *qacEΔ1* and *sul1* genes.

In our study CTX-M-producing *E. coli* isolates were predominantly B2, followed by A, D, and B1. CTX-M–producing *E. coli*, was associated with the more virulent strains belonging to the phylogenetic groups B2 and D (Coque *et al*., 2008b; Lavigne *et al*., 2007; Machado *et al*., 2005; Pitout *et al*., 2005;

Leflon-Guibout *et al*., 2004). Pitout *et al*., (2005) reported that CTX-M-14 producers (97%) were from phylogenic group D; 67% of the CTX-M-15 producers were from group B2. They also reported that a single CTX-M-14-producing strain belonged to clonal group A.

5.6.3.3 Sulphonamide resistance genes (*sul1* and *sul2*)

Fifty five isolates showed sulphonamide resistance phenotype which was encoded by *sul1* (42, 76.36%), *sul2* (30, 54.54%), and *sul1* + *sul2* (27, 49.09%) gene combination. The percentage of distribution of *sul1* gene was significantly higher than *aphA2*, *dhfrIa*, and *dhfrVII*. The percentage of distribution of *sul2* gene was significantly higher than *to dhfrIa*, and *dhfrVII*. In consistent to our results, Infante *et al*., (2005) reported a similar percentage for *sul2* gene (55%). But they reported lower values for *sul1* and *sul1* + *sul2* combination than our study. In contrast to our results, Baudry *et al*., (2009) reported that among co-trimoxazole resistant isolates, 90.9% carried *sul1* (81.8%) and *sul2* (36.4%) genes. Blahna *et al*., (2006) also reported higher values for *sul 1* and *sul2* than our results. Frank *et al*., (2007) reported that 92% and 86% of resistant *Enterobacteriaceae* strains carried *sul1* and *sul2* genes, respectively. The more frequent presence of *sul2* than *sul1* has also been reported in sulphonamide resistant *E. coli* from urinary tract infections (Grape *et al*., 2003; Kerrn *et al*., 2002). *sul2*, present in 90% of the isolates, has been reported to be the most frequent mechanism of resistance to sulphonamides in human clinical *E. coli* isolates (Kerrn *et al*., 2002; Enne *et al*., 2001). Hussain *et al.,* (2012) observed a higher frequency of *sul1* than *sul2* in multiresistant uropathogenic *E. coli*. Bartoloni *et al*., (2009) reported lower percentages for *sul1* gene (32% and 22%) and higher percentage for *sul2* gene (70% and 97%) than our results.

5.6.3.4 Tetracycline resistance genes (*tetA* and *tetB*)

Regarding the 46 tetracycline resistant isolates, resistance was mediated by *tetA*, *tetB* and *tetA* + *tetB* in 30 (65.21%), 24 (52.17%), and 17 (36.95%) isolates, respectively. Vinue *et al*., (2010) and Frank *et al*., (2007) reported lower values for *tetA* (37.5%, 49.5%) and *tetB* (35, 35.5%) in clinical isolates than our results. In consistent with our result, Karami *et al*., (2006) reported almost similar value for *tetB* (51%).

5.6.3.5 Aminoglycoside resistance genes (*strA* and *aphA2*)

Thirty-eight streptomycin-resistant isolates were detected, of which 30 (78.94%) of them harboured the *strA* gene. The *aphA2* gene was found in 14 isolates (63.63%) out of 22 gentamicin-resistant isolates. Baudry *et al*., (2009) reported that all gentamicin-resistant isolates (27.8%) carried the *aac(3')-II* gene.

5.6.3.6 Chloramphenicol-resistant gene (*catI*)

Eleven out of 12 chloramphenicol-resistant isolated harboured a *cat1* (91.66%) gene. Detection of chloramphenicol resistance by molecular techniques has not been widely studied. Detection of chloramphenicol resistance determinants has received little attention, because chloramphenicol is little used for the treatment of severe infections.

5.6.3.7 Trimethoprim-resistant genes (*dhfrIa* and *dhfrVII)*

Among 54 trimethoprim-resistant *E. coli* isolates, *dhfrIa* and *dhfrVII* was found in 12 (22.22%) isolates. Yu *et al*., (2004) reported that *class 1 integrons* was found to be significantly higher in trimethoprim-resistant isolates (69%) than in trimethoprim-susceptible isolates (19%). They also reported that among the trimethoprim-resistant isolates, the frequency of *dhfr* genes

associated with class 1 integrons increased sharply from 10% of the isolates during 1980–1985 to 53% during 1996–1997 and to 46% during 2001–2002. Baudry *et al*., (2009) reported that *dfrA17 (*72.7%) was the predominant *dhfr* gene in resistant isolates, followed by *dfrA1* (9%), *dfrA12* (9%), and *dfrA14* (9%). Frank *et al*., (2007) reported higher prevalence for *dhfrA7* (49%) while lower prevalence for *dhfrA1* (17%), than our results.

5.6.3.8 *Class 1 integrons* and *int1* gene

Class 1 integrons and integrase gene (*int1*) were identified in 24 and 31 isolates, respectively. Out of 24 multidrug resistance isolates, 35.29% and 45.58% of isolates carried *class 1 integrons* and *int1* respectively. Kang *et al*., (2005a) reported a lower frequency for *class 1 integrons*, than our study. *Class 1 integrons* have been detected in 22 to 77% of Gram-negative clinical isolates (Gu *et al*., 2008) and have been also reported in Gram-positive bacteria (Xu *et al*., 2011; Shi *et al*., 2006). In our study *class 1 integrons* were distributed among B2 (79.16%), A (12.5%) and D (8.3%). Machado *et al*., (2005) reported that *class 1 integrons* were found among all four *E. coli* phylogenetic groups at similar rates (40 to 56%). Pitout *et al*., (2007) noticed the highest frequency of *intI*-positive isolates (56%) among clinical *E. coli*. Vinue *et al*., (2010) have found 40% of integron-positive strains among *E. coli* of blood origin in Spain. On the other hand, as much as 77% of integron-positive clinical *E. coli* isolates have been cultured from patients of four hospitals in China (Gu *et al*., 2008). High frequencies have been also reported for other *Enterobacteriaceae* of clinical origin, including 54% of clinical *Enterobacter cloacae* complex strains (Mokracka *et al*., 2012) and 33–34% of *K. pneumoniae* (Chang *et al*., 2009). The high prevalence of integrons among

isolates of hospital origin may reflect strong pressure of hospital environment for selection of strains with integrons (Skurnik *et al*., 2009). Machado *et al*., (2005) reported that *class 1 integrons* were more common among clinical-ESBL (67%) than among clinical-non ESBL (40%) or non ESBL from healthy volunteers (26%). They also observed the prevalence of *class 1 integrons* in clinical-ESBL isolates dramatically increased along the studied period from 30% during the 1988 to 1995 period to 87% during 1996 to 1998 and reached 70% in 1999 to 2000. Among clinical-non ESBL isolates, these elements increased from 36% (1996 to 1998) to 41% (1999 to 2000). Frank *et al*., (2007) reported higher frequency of *class 1 integrons* in *Enterobacteriacea*.

The present study shows the importance of monitoring samples collected from environment for the presence of multidrug resistant *E. coli*. The findings of the present study reflect the importance of surface water as a reservoir and as a medium for the dissemination of antibiotic resistance genes in the environment. Intensive human activities, especially wastewater discharge into estuary could aggravate antibiotic resistance in the environment. We observed that co-occurrence of antibiotic resistance genes is a common mechanism, indicating that further spread of these genes is possible. Our results show a wide variety of resistance genes in multiresistant *E. coli* strains from estuary, clinical (human), and fish samples. Therefore, this normal flora may play a key role as an acceptor and donor of transmissible antibiotic resistance mechanisms. The inclusion of some resistance genes inside integrons constitutes an effective means to spread antibiotic resistance among bacteria from different ecosystems. Emergence of resistance in the food chain is an important public health concern.

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Chapter 6

Prevalence of extraintestinal virulence factor genes in *Escherichia coli* isolates from estuarine, seafood and clinical sources

6.1 Introduction

E. coli strains of biological significance to humans can be broadly categorized as (1) commensal strains, (2) intestinal pathogenic (i.e. enteric or diarrheagenic) strains, and (3) extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). The available studies of *E. coli* from infections of the urinary tract, blood stream, central nervous system, respiratory tract, and peritoneum demonstrate that the majority of such isolates, as indicated by their functionally similar virulence profiles and phylogenetic background, are quite distinct from commensal and intestinal pathogenic *E. coli* (Johnson *et al*., 2003b; Johnson and Russo, 2002; Russo and Johnson, 2000). It recently has been proposed that these strains of *E. coli* be collectively termed ExPEC,

rather than split up as uropathogenic *E. coli* (UPEC), sepsis associated *E. coli* (SEPEC), neonatal meningitis-associated *E. coli* (NEMEC), etc., to reflect their shared ability to overcome or subvert host defences and cause disease at multiple anatomical sites in humans and animals (Russo and Johnson, 2000).

6.2 Review of literature

6.2.1 Extraintestinal pathogenic *E. coli* (ExPEC)

E. coli strains isolated from infections outside of the intestinal tract, e.g., *uropathogenic E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC) have been grouped as ExPEC by many workers (Smith *et al*., 2007; Johnson and Russo, 2002, 2005; Russo and Johnson, 2000). ExPEC are part of the intestinal microflora of a fraction of the healthy population and normally asymptomatically colonize the gut. Once they get access to niches outside of the gut, they are, however, able to efficiently colonize these niches and cause disease in man, i.e. urinary tract infection (UTI), septicemia or meningitis in newborns, as well as UTI or systemic disease in many animals. Human and animal pathogenic strains share common genetic backgrounds (Clermont *et al*., 2011; Johnson *et al*., 2008a, b). Although several important ExPEC virulence factors and their role during pathogenesis have been described (Henriques *et al*., 2014; Mellata *et al*., 2010; Erjavec *et al*., 2007; Smith *et al*., 2007; Mokady *et al*., 2005; Kaper *et al*., 2004), many ExPEC cannot be unambiguously distinguished from commensal *E. coli* based on a set of discriminatory virulence factors as ExPEC can use multiple virulence factors in a mix-and-match fashion. Nevertheless, ExPEC classification has been proposed on the basis of isolation site and the detection of two virulence-associated genes (VAG) typical of the specific pathotype

(Johnson and Russo, 2005), respectively two VAGs for non-host samples like food samples (Johnson *et al*., 2005a). ExPEC adhesins, siderophore systems, and toxins can be correlated with successful gut colonization in humans (Diard *et al*., 2010; Johnson *et al*., 2008b; Nowrouzian *et al*., 2006; Wold *et al*., 1992). Additionally, lipopolysaccharide (LPS) and other ExPEC virulence factors can protect bacteria against predation in the environment (Diard *et al*., 2007; Alsam *et al*., 2006). Consequently, extraintestinal virulence has been interpreted as a coincidental by-product of commensalism (Tenaillon *et al*., 2010; Le Gall *et al*., 2007). Presumably, ExPEC virulence factors have not been evolved in order to increase the ability to cause extraintestinal infections. There is no strong selective pressure to become a perfect ExPEC as the vast majority of intestinal *E. coli* variants carrying "ExPEC virulence genes" never cause disease, but live as commensals. In contrast, the finding that strains of phylogroup B2 or with high ExPEC virulence gene content represent members of the intestinal *E. coli* flora only in a subgroup of the human population does not support the idea that "ExPEC virulence factors" confer a fitness advantage in the intestine and promote commensalism.

ExPEC strains, like commensal *E. coli* (but in contrast to intestinal pathogenic *E. coli*), are often found in the normal intestinal flora and do not cause gastroenteritis in humans. Although acquisition of an ExPEC strain by the host is a prerequisite to subsequent ExPEC infection, it is not the rate-limiting step, which instead is entry of a colonizing ExPEC strain from its site of colonization (e.g. the colon, vagina, or oropharynx) into a normally sterile extraintestinal site (e.g. the urinary tract, peritoneal cavity, or lungs). ExPEC strains have acquired genes encoding diverse extraintestinal virulence factors that enable them to cause infections outside of the gastrointestinal tract, in

both normal and compromised hosts (Johnson and Stell, 2000; Picard *et al*., 1999). These virulence genes are for the most part distinct from those that enable intestinal pathogenic strains to cause intestinal disease. From a pathogenesis perspective, this is a logical evolutionary development, since host environment and associated defence mechanisms differ substantially within versus outside of the gastrointestinal tract. Characteristic virulence traits that are present in most ExPEC include various adhesins (e.g. P and type I fimbriae), factors to avoid or subvert host defense systems (e.g. capsule, lipopolysaccharide), mechanisms for nutrient acquisition (e.g. siderophores), and toxins (e.g. hemolysin, cytotoxic necrotizing factor 1).

The urinary tract is the most frequent extraintestinal site infected by *E. coli*. Urinary tract infection (UTI) is a common infection in ambulatory patients, accounting for 1% of ambulatory care visits in the US, and is second only to lower respiratory tract infection among infections responsible for hospitalization (Hooton and Stamm, 1997; Stamm and Hooton, 1993). The abdomen/pelvis is the second most frequent extraintestinal site for infections due to *E. coli*. A large variety of clinical syndromes occur in this location, including acute peritonitis secondary to faecal contamination, spontaneous bacterial peritonitis, dialysis-associated peritonitis, diverticulitis, appendicitis, intraperitoneal or visceral abscesses (hepatic, pancreatic, splenic), infected pancreatic pseudocysts, septic cholangitis and/or cholecystitis, and pelvic inflammatory disease. *E. coli* can be isolated either alone or, as often occurs, with other facultative and/or anaerobic members of the intestinal flora.

E. coli isolates from the urine (or blood) of patients with urinary tract infection (UTI), and from the blood, cerebrospinal fluid, etc., of patients with

diverse other extraintestinal infections, typically exhibit a greater prevalence of specific molecular markers than do faecal isolates from uninfected hosts (Mitsumori *et al*., 1999; Bingen *et al*., 1997; Johanson *et al*., 1993; Maslow *et al*., 1993; Arthur *et al*., 1989). Such markers are commonly regarded as 'VFs', although this must be understood as signifying 'factors associated with', not necessarily 'contributing to', virulence, since epidemiological associations do not guarantee causality. These VFs can be grouped by functional category; for example, adhesins (Mitsumori *et al*., 1998; Connell *et al*., 1996; Marklund *et al*., 1992; Nowicki *et al*., 1990), siderophore systems (Russo *et al*., 2002, 2001; Torres *et al*., 2001; Schubert *et al*., 1998; Opal *et al*., 1990), toxins (Rippere-Lampe *et al*., 2001; Guyer *et al*., 2000; Scott and Kaper, 1994; Welch *et al*., 1981), surface polysaccharides (Burns and Hull, 1999; Russo *et al*., 1996), invasins (Huang *et al*., 2001, 1999), serum resistance-associated traits (Wooley *et al*., 1994; Kanukollu *et al*., 1985), and traits of miscellaneous or unknown function (Johnson, 2003; Kurazono *et al*., 2000). Clinical isolates often contain multiple VFs from a particular functional category. Conversely, many seemingly virulent strains lack known representatives of one or more of these functional categories (Johnson *et al*., 2002c, 2001b). The biological implications of these apparent redundancies and deficits are poorly understood.

Certain VFs commonly occur together in patterns suggesting either co-selection or direct genetic linkage (Kanamura *et al*., 2003; Johnson and Stell, 2000). Genetic linkage of VFs has been demonstrated within pathogenicity-associated islands (PAIs) and on plasmids (Dobrindt *et al*., 2001; Hacker and Kaper, 2000; Guyer *et al*., 1998; Swenson *et al*., 1996; Le Bouguenec *et al*., 1993; Valvano *et al*., 1986; Low *et al*., 1984; Nilius and Savage, 1984). ExPEC strains often contain multiple PAIs, each with a distinctive combination of VFs,

which sometimes results in a strain having multiple copies of a particular VF, for example, *pap* (Welch *et al*., 2002; Dobrindt *et al*., 2001; Bloch and Rode, 1996). This co-occurrence of VFs results in overlapping statistical associations of VFs with clinical variables, leading to uncertainty regarding which VF is primary. Additionally, sequence analysis of PAIs, virulence plasmids, and genomes that contain known VFs invariably reveals novel genes of unknown function, some with homology to established VFs from other species (Welch *et al*., 2002; Dobrindt *et al*., 2001; Swenson *et al*., 1996). Thus, statistical associations of known VFs with virulence may be mediated through certain of these as-yet-uncharacterized putative VFs.

6.2.2 Phylogenetic background and virulence factor genes

The overlapping associations of virulence factor genes and phylogenetic background create uncertainty as to which characteristic primarily determines virulence. In several studies, although virulence factor genes were statistically more closely associated with clinical virulence than was phylogenetic group (Johnson *et al*., 2003c, 2000; Picard *et al*., 1999), phylogenetic group nonetheless exhibited an association with virulence after known virulence factor genes were accounted for (Johnson *et al*., 2000), consistent with the existence of as-yet undefined virulence factor genes that are both phylogenetically distributed and incompletely linked with known virulence factor genes. Extraintestinal pathogenic *E. coli* (ExPEC) isolates have been found to share a characteristic distribution within the widely used *E. coli* phylogenetic classification, A, B1, B2, and D, proposed by Clermont *et al*., (2000). Most virulent extraintestinal strains belong to group B2 or, less frequently, group D, whereas most commensal strains of *E. coli* belong to

groups A and B1 (Johnson and Stell, 2000). The phylogenetic group B2 strains evolved to become virulent by acquisition of numerous pathogenetic determinants (Lecointre *et al.*, 1998).

6.2.3 Antibiotic resistance and virulence

Older data suggest that among *E. coli* isolates from patients with urosepsis, resistance to antibiotic agents such as ampicillin, sulphonamides, tetracycline, and streptomycin is negatively associated with virulence and a group B2 phylogenetic background, but is positively associated with host compromise (Johnson *et al*., 1991). This suggests that for infections in compromised hosts, who have weakened defences but frequent exposure to antibiotics, resistance may provide a greater fitness advantage to *E. coli* strains than do traditional VFs or a group B2 background. More recent data demonstrate similar negative associations between fluoroquinolone resistance and VFs or a B2 phylogenetic background (Johnson *et al*., 2003d, 2002d; Vila *et al*., 2002; Velasco *et al*., 2001). This has been interpreted as suggesting that VFs may be lost concomitant with mutation to resistance (Vila *et al*., 2002). However, this hypothesis does not account for the phylogenetic shift (away from group B2) observed among resistant isolates, which suggest instead that resistant isolates derive primarily from distinct, less virulent bacterial populations (Johnson *et al*., 2003d, 2002d). However, antibiotic resistance clearly does not equate with reduced virulence in all circumstances.

Among faecal *E. coli* from diseased cattle and swine, most of which organisms probably are not pathogens, resistance to extended-spectrum cephalosporins or fluoroquinolones is associated with minimal shifts in VF profile or phylogenetic group (Johnson *et al*., 2003d). Likewise, the recently described

E. coli 'clonal group A', although exhibiting multidrug-resistance and accounting for 33-50% of recent trimethoprim-sulphamethoxazole- resistant *E. coli* among women with acute uncomplicated cystitis or pyelonephritis in some US centers, is replete with VFs, which presumably contribute to the success of this clonal group as a pathogen among otherwise healthy hosts (Johnson *et al*., 2002b; Manges *et al*., 2001). Recently, Sana *et al*., (2014) reported a higher prevalence of plasmid mediated quinolones resistance among uropathogenic *E. coli*.

6.2.4 Virulence versus colonization factors

Certain molecular epidemiological data suggest that some of what traditionally have been regarded as VFs in ExPEC, particularly for UTI, may also promote intestinal colonization (Nowrouzian *et al*., 2003, 2001; Wold *et al*., 1992). This hypothesis provides a more plausible mechanism for the evolution of these traits than does the associated enhanced pathogenicity, since the ability to cause self-limited or even fatal disease provides no obvious evolutionary advantage to *E. coli*. However, the colonization factor hypothesis does not explain why ExPEC are not the dominant clone(s) within the intestinal tract in most humans, as would be expected if ExPEC truly have a fitness advantage in this niche (Kanamura *et al*., 2003; Siitonen, 1992). Elucidation of this required more research on virulence factors of ExPEC.

6.3 Objectives

1) To analyse the occurrence of extraintestinal virulence factor genes in *E. coli* isolates from estuarine, seafood and clinical sources.

2) To identify the phylogenetic group distribution in ExPEC isolates from estuarine, seafood and clinical sources.

3) To investigate antibiotic resistance profiles of ExPEC isolates from estuarine, seafood and clinical sources.

4) To investigate distribution of the major antibiotic resistance genes in ExPEC isolates from estuarine, seafood and clinical sources.

6.4 Materials and Methods

In the present study a total of 300 *E. coli* isolates of estuarine origin, 100 *E. coli* of clinical and 100 *E. coli* of seafood origin were tested for the presence of various extraintestinal virulence factors genes.

6.4.1 Isolation of DNA from *E. coli*

DNA from the bacterial genome was extracted as described in 2.4.6.1.

6.4.2 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was extracted as described in 5.4.2.

6.4.3 Phylogenetic analysis

The phylogenetic group was determined as described in 5.4.2.

6.4.4 Antibiotic susceptibility testing

Antibiotic susceptibility of *E. coli* isolates was determined as described in 4.4.1.

6.4.5 Detection of antibiotic resistant genes

Detection of antibiotic resistant genes was done as described in 5.4.3.

6.4.6 Detection of virulence factor genes

All the isolates were screened by PCR for five key virulence factor genes of extraintestinal pathogenic *E. coli* (ExPEC) as previously described

(Johnson and Stell, 2000): the virulence factor genes selected included *papAH* and *papC* (P fimbriae major structural subunit and assembly), *sfa/focDE* (S and F1C fimbriae), *iutA* (aerobactin receptor) and *kpsMT II* (group II capsule). The primers used in this study are listed in Table 6.1. Each reaction mixture consisted of 4 mM $MgCl_2$, 1µl of 25 pmol of each primer (*papAH, papC, sfa/focDE, iutA, kpsMT II*), 2 µl of 2 mM dNTPs and 4 µl of 1X Taq buffer, 1 U of Taq DNA polymerase (GeNei™, India) in a total volume of 20 µl, including 1 µl DNA template. The cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C, 30 sec), annealing (64 °C, 30 sec), extension (68 °C, 3 min) and final extension (72 °C, 10 min). PCR products were then electrophoresed on 1.5% agarose gel containing ethidium bromide (GeNei™, India), and visualized by Gel Documentation System (BioRad Gel Doc™ EZ Imager, USA). *E. coli* isolates with two or more virulence factor genes were considered as Extraintestinal pathogenic *E. coli* (ExPEC).

Table 6.1 Gene analysed, list of primers and amplicon size of virulence factor genes used in this study

Genes investigated	Primer sequence (5′- 3′)		Amplicon size (bp)
	Forward	Reverse	
papAH	atggcagtggtgtcttttggtg	cgtcccaccatacgtgctcttc	720
papC	gtggcagtatgagtaatgaccgtta	atatcctttctgcagggatgcaata	200
sfa/focDE	ctccggagaactgggtgcatcttac	cggaggagtaattacaaacctggca	410
iutA	ggctggacatcatgggaactgg	cgtcgggaacgggtagaatcg	300
kpsMT II	gcgcatttgctgatactgttg	catccagacgataagcatgagca	272

6.4.7 Statistical analysis

Statistical analysis in this study was performed using SPSS software 13 (Statistical Package for the Social Science). One-Way Analysis of Variance (ANOVA, Games-Howell) was applied to test difference in the distribution of virulence factor genes, among *E. coli* from different stations, different sources and different phylogenetic groups. Pearson correlation coefficient was used to analyze the correlation between virulence factor genes with antibiotic resistance and antibiotic resistance genes.

6.5 Results

6.5.1 ExPEC isolates from cochin estuary

6.5.1.1 ExPEC and distribution of virulence factor genes in cochin estuary

Multiplex PCR analysis of the 300 isolates from cochin estuary revealed 52 (17.33%) isolates contained at least one virulence factor genes and 48 (16%) isolates carried two or more virulence factor genes. *E. coli* isolates with two or more *E. coli* isolates with two or more virulence factor genes were considered as extraintestinal pathogenic *E. coli* (ExPEC).

6.5.1.1.1 ExPEC and phylogenetic groups

The distribution of the 300 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups, revealed 48 of them as ExPEC isolates. Among the isolates which are confirmed as ExPEC, 28 isolates belonged to phylogenetic group B2, 12 isolates to group D, 4 isolates each to group A and B1 (Figure 6.1).

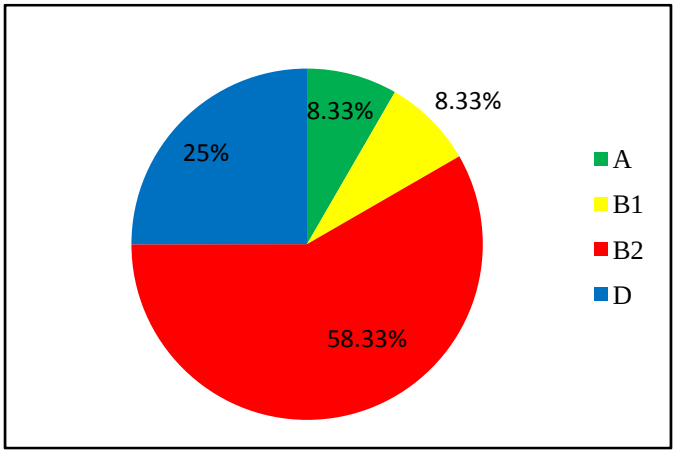


Figure 6.1 Distribution of various phylogenetic groups among ExPEC (n = 48) isolates from Cochin estuary

6.5.1.1.2 Virulence factor genes

Gel image of PCR products with representative isolates carrying the various virulence factor genes is shown in Figure 6.2. The virulence score used to classify the ExPEC isolates was calculated using the total number of virulence factor genes. Figure 3 shows the distribution of different virulence factor genes in ExPEC isolates from Cochin estuary. The *iutA* (aerobactin acquisition), *papC* (P fimbriae) and *kpsMT II* (group 2 capsule synthesis) genes were frequently encountered, whereas *sfa/focDE* (S and F1C fimbriae) and *papAH* (P fimbriae) were detected rarely. No significant difference in the distribution of virulence genes was observed in estuarine isolates. Statistical analysis of the result revealed a highly significant association between *iutA* and *kpsMT II* ($p = 0.046$) and *iutA* and *papC* ($p = 0.033$). Furthermore, a significant association was found between *papAH* and *sfa/focDE* ($p = 0.000$).

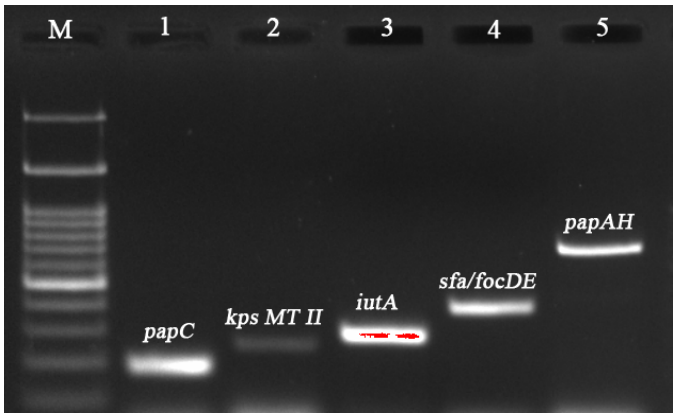


Figure 6.2 Gel image of PCR products with representative isolates carrying the various virulence factor genes.
Lane M: 100 bp ladder, lane 1: *papC*, lane 2: *kpsMT II*, lane 3: *iutA*, lane 4: *sfa/focDE*, lane 5: *papAH*

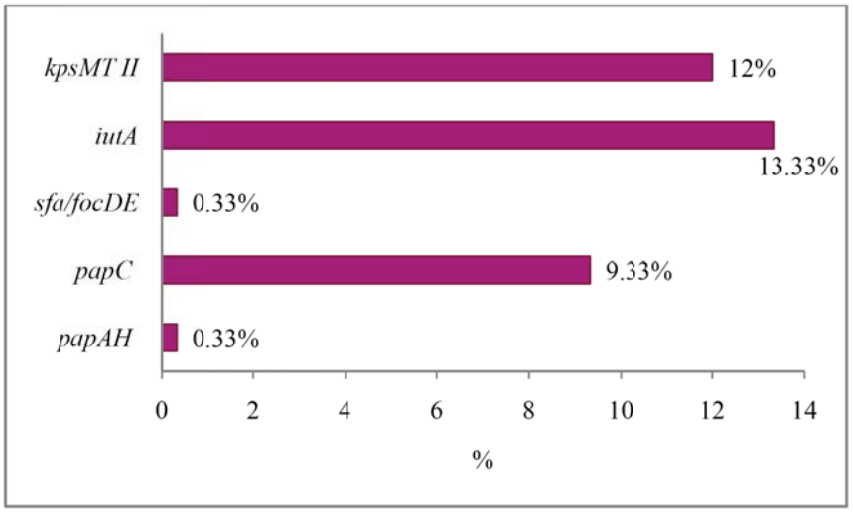
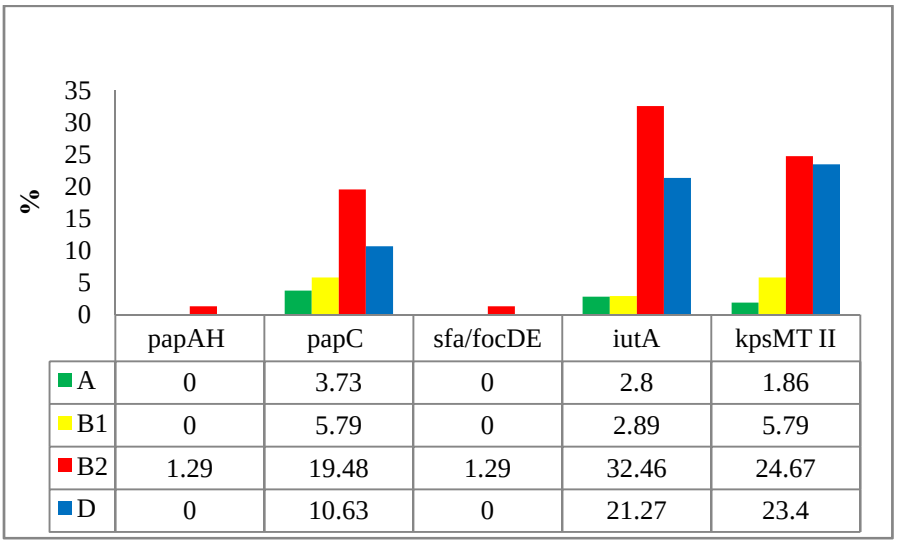


Figure 6.3 Distribution of different virulence factor genes in *E. coli* isolates from Cochin estuary (n = 300)

6.5.1.1.3 Relative prevalence of various virulence factor genes among different phylogenetic groups of *E. coli* isolates from Cochin estuary

The frequency of distribution of the virulence factor genes varied among the different groups. *E. coli* belonged to phylogenetic group B2 and D harboured

more virulence factor genes than non pathogenic groups A and B1 (Figure 6.4). Virulence genes such as, *sfa/focDE* and *papAH* were detected in B2 only. In Cochin estuary, *papC* genes were mainly seen in B2 (53.57%), followed by D (17.85%), A and B1 (14.28% each). Sixty two percentages of B2 isolates had *iutA* gene, followed by D (25%), A (7.5%) and B1 (5%). *E. coli* isolates with *kpsMT II* gene, were distributed in B2 (52.77%), D (30.55%), B1 (11.11%), and A (5.55%). In our study, *E. coli* isolates with three or more than virulence factor gene combinations also detected. Interestingly, one isolate belonging to group B2 was found to contain *papAH* + *papC* + *sfa/focDE* + *iutA* + *kpsMT II* (Table 6.2). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from Cochin estuary are listed in Table 6. 2. Distribution of virulence factor genes varied significantly among different phylogenetic groups. ExPEC isolates belonged to group B2, carried more virulence genes than those from group A, B1 and D ($p < 0.001$).



	papAH	papC	sfa/focDE	iutA	kpsMT II
A	0	3.73	0	2.8	1.86
B1	0	5.79	0	2.89	5.79
B2	1.29	19.48	1.29	32.46	24.67
D	0	10.63	0	21.27	23.4

Figure 6.4 Relative prevalence of virulence factor genes among different phylogenetic groups of *E. coli* isolates from Cochin estuary (n = 300)

Table 6.2 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from Cochin estuary (n = 300)

Distribution of virulence factor genes	A	B1	B2	D
iutA + papC	1.86	0	12.98	4.25
iutA + kpsMT	0.93	0	16.88	14.89
papC + kpsMT II	0.93	2.89	3.89	6.38
iutA + papC + kpsMT II	0	2.89	1.29	0
papAH + iutA + kpsMT II + papC + sfa/focDE	0	0	1.29	0

6.5.1.1.4 Prevalence of antibiotic resistance among ExPEC isolates from Cochin estuary

Concerning the antibiotic resistance, the results showed that 45.83% of the ExPEC isolates were resistant to at least one antibiotic; and that 29.2% were multiresistant. Resistance to ampicillin, nalidixic acid, sulphonamides and cefoxitin were relatively frequent among ExPEC from Cochin estuary. Very few ExPEC isolates from Cochin estuary were resistant to ceftazidime and cefuroxime (Figure 6.5).

The aerobactin receptor gene (*iutA*) was positively associated with resistance to cefotaxime ($p = 0.010$) and cepfodoxime ($p = 0.036$). Group 2 capsule synthesis gene *(kpsMT II)* on the other hand showed an association with resistance to ciprofloxacin, cefoxitin, cepfodoxime, and cefotaxime ($p < 0.05$). Whereas adhesion related genes, (*papAH*, and *papC*) and S/F1C fimbriae gene (*sfa/focDE*) did not showed any positive association with any antibiotic resistance traits.

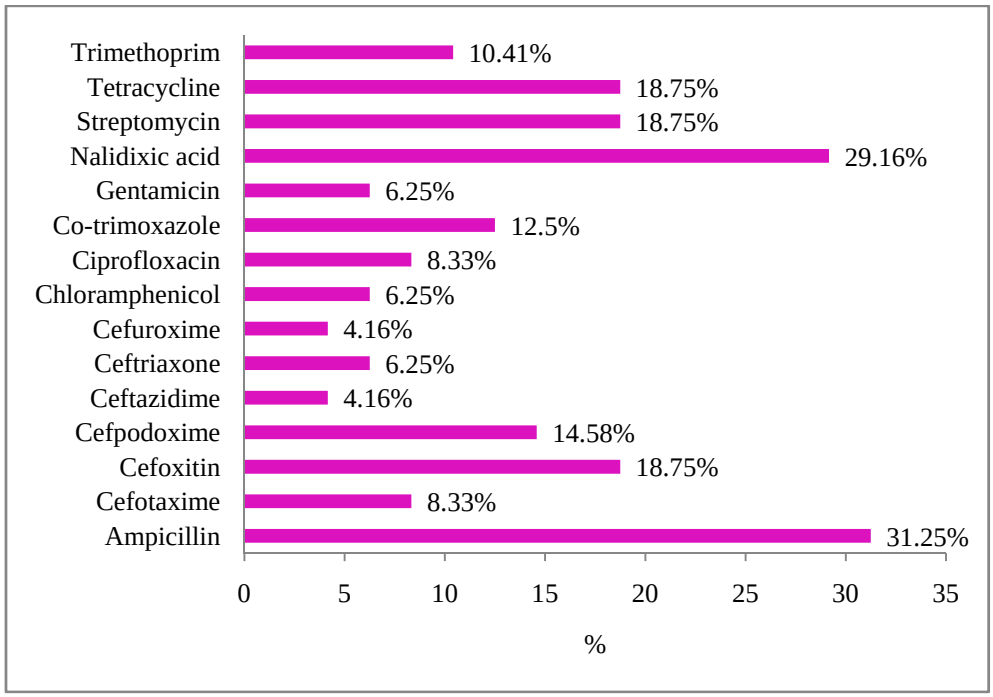


Figure 6.5 Percentage of antibiotic resistance of ExPEC isolates from Cochin estuary (n = 300)

Of 14 multidrug-resistant ExPEC isolates, 8 isolates (57.14%) belonged to phylogenetic group B2 and 4 isolates (28.575) to group D. Therefore, to assess whether the absence of these virulence genes is also related to antibiotic resistance, we evaluated the frequency of each virulence factor considered (*papAH*, *papC*, *sfa/focDE*, *iutA*, *kpsMT II*) in susceptible and resistant *E. coli* isolates within each phylogenetic group (A, B1, B2 and D) (Table 6.3). In susceptible *E. coli* B2 strains, the incidence of *papC*, *iutA*, and *kpsMT II* was, respectively, 56.25%, 93.75%, and 56.25%. Whereas in resistant B2 isolates, the incidence was 75% for *kpsMT II*, 50% for *papC* and 83.33% for *iutA*. Prevalence of *sfa/focDE* and *papAH* were seen in group B2 resistant (8.33%) isolates only. The results also indicated that within A phylogenetic

group, *papC* and *iutA* were more prevalent in resistant isolates. While *kpsMT II* was present in group A susceptible isolates only. In phylogenetic group B1, *papC* and *kpsMT II* were equally prevalent in susceptible and resistant isolates. But *iutA* was more prevalent in susceptible isolates. The results also showed that within D phylogenetic group, *iutA* and *kpsMT II* were more prevalent in resistant isolates, whereas *papC* was more prevalent in susceptible isolates.

Table 6.3 Distribution of virulence factor genes among sensitive and resistant isolates belonging to various phylogenetic groups of ExPEC

VFGs	Phylogenetic group A (n = 4)		Phylogenetic group B1 (n = 4)		Phylogenetic group B2 (n = 28)		Phylogenetic group D (n = 12)	
	S (n = 2)	R (n = 2)	S (n = 1)	R (n = 3)	S (n = 16)	R (n = 12)	S (n = 7)	R (n = 5)
papAH	0	0	0	0	0	8.33%	0	0
papC	50%	100%	100%	100%	56.25%	50	57.14%	20%
Sfa/focDE	0	0	0	0	0	8.33%	0	0
iutA	50%	100%	100%	33.33%	93.75%	83.33%	71.42%	80%
kpsMT II	100%	0	100%	100%	56.25%	75%	71.42%	100%

Group B2 ExPEC isolates showed relatively higher resistance to cefotaxime, and cefpodoxime. Whereas group D isolates were relatively more resistant to cefoxitin, gentamicin, chloramphenicol and ciprofloxacin. Whilst group A and B1 ExPEC isolates were sensitive to most of the antibiotics tested (Table 6.4).

Table 6.4 Prevalence of antibiotic resistance among ExPEC isolates belonging to various phylogenetic groups (n = 48)

Antibiotics	A (n = 4)	B1 (n = 4)	B2 (n = 28)	D (n = 12)
Ampicillin	0	50%	32.14%	33.33%
Cefotaxime	0	0	10.71%	8.33%
Cefoxitin	0	0	21.42%	25%
Cefpodoxime	0	0	17.85%	16.66%
Ceftazidime	0	0	3.57%	8.33%
Ceftriaxone	0	0	3.57%	16.66%
Cefuroxime	0	0	3.57%	8.33%
Chloramphenicol	0	0	10.71%	16.66%
Ciprofloxacin	0	0	14.28%	16.66%
Co-trimoxazole	25	0	17.85%	16.66%
Gentamicin	0	0	10.71%	16.66%
Nalidixic acid	0	50%	32.14%	25%
Streptomycin	50	0	21.42%	8.33%
Tetracycline	25	0	25%	8.33%
Trimethoprim	25	0	17.85%	0

6.5.1.1.5 Prevalence of antibiotic resistance genes among ExPEC isolates from Cochin estuary

Between 10 – 19% of ExPEC carried antibiotic resistance genes such as *strA*, bla_{TEM}, *tetA*, *sul2*, *tetB*, and *aphA2* (Figure 6.6). One of the adhesion related genes, such as *papAH*, showed significant correlation with *cat1*, *dhfrIa*, and *dhfrVII* ($p < 0.001$), whereas *papC* showed a positive association with *int 1* gene. The S/F1C fimbriae gene (*sfa/focDE*) also showed an association with *cat1*, *dhfrIa*, and *dhfrVII* ($p < 0.001$). Group 2 capsule synthesis gene *(kpsMT II)* was positively associated with bla_{CTX-M}. While aerobactin receptor gene (*iutA*) was positively associated with bla_{CTX-M} and *int 1* gene.

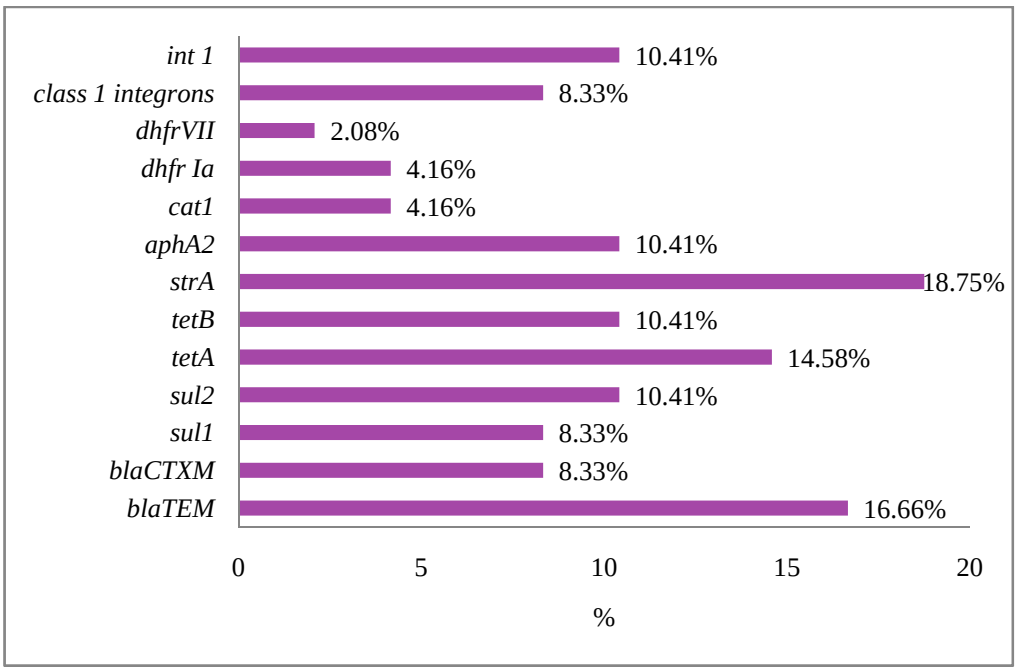


Figure 6.6 Prevalence of various antibiotic resistant genes among ExPEC isolates from Cochin estuary (n = 300)

Among the 28 ExPEC isolates in group B2, the commonly detected antibiotic resistance genes were *bla*$_{TEM}$, *strA*, *tetA*, *tetB* and *aphA2* (Table 6.5). Contrary to findings in group B2, most ExPEC strains in group B1 did not contain the antibiotic resistance genes investigated except *bla*$_{TEM}$. Only one isolate in group D was found to contain antibiotic resistance genes. In group A, two isolates were found to carry resistant genes, in which one isolate was sensitive.

Table 6.5 Prevalence of antibiotic resistance genes among ExPEC isolates belonging to various phylogenetic groups (n = 48)

Antibiotic resistance genes	A (n = 4)	B1 (n = 4)	B2 (n = 28)	D (n = 12)
bla$_{TEM}$	0	25%	21.42%	8.33%
bla$_{CTX-M}$	0	0	10.71%	8.33%
sul1	25%	0	7.14%	8.33%
sul2	50%	0	7.14%	8.33%
tetA	25%	0	17.85%	8.33%
tetB	25%	0	14.28%	0
strA	50%	0	21.42%	8.33%
aphA2	25%	0	14.28%	0
cat1	0	0	7.14%	0
dhfrIa	0	0	7.14%	0
dhfrVII	0	0	3.57%	0
class 1integrons	25%	0	7.14%	8.33%
int 1	0	0	14.28%	8.33%

6.5.1.2 ExPEC and distribution of virulence factor genes in station 1 (Chitoor)

Multiplex PCR analysis of the 47 isolates from station 1 (Chitoor) revealed 5 (10.63%) isolates contained at least one virulence factor genes and 4 (8.51%) isolates carried two or more virulence factor genes.

6.5.1.2.1 ExPEC and phylogenetic groups

The distribution of the 47 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups, revealed that out of the 4 ExPEC isolates, 3 isolates belonged to phylogenetic group D, and 1 isolate to group B2 (Figure 6.7).

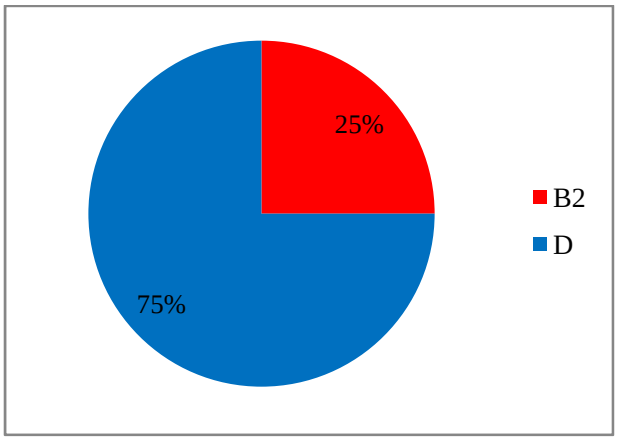


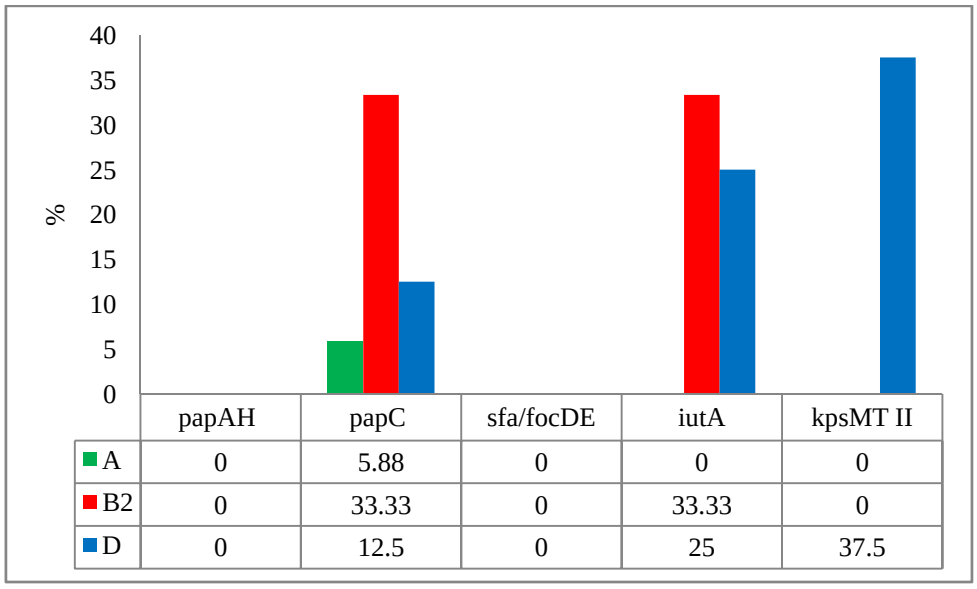
Figure 6.7 Distribution of various phylogenetic groups among ExPEC isolates (n = 4) from station 1 (Chitoor)

6.5.1.2.2 Virulence factor genes

The *iutA* (aerobactin acquisition), *papC* (P fimbriae) and *kpsMT II* (group 2 capsule synthesis) genes were frequently encountered among ExPEC from this station, whereas *sfa/focDE* (S and F1C fimbriae) and *papAH* (P fimbriae) were not detected. More than 6% of isolates carried *iutA*, *papC* and *kpsMT II*.

6.5.1.2.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 1 (Chitoor)

More than 33% of B2 and D isolates carried *papC* and *iutA* gene. *E. coli* isolates with *papC* gene were equally distributed in group B2 (0.33%), D (0.33%) and A (0.33%) (Figure 6.8). Whereas isolates with *iutA* were belonged to D (0.66%) and B2 (0.33%). While *E. coli* isolates with *kpsMT II* gene were belonged to group D only. Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from station 1 are listed in Table 6.6.



	papAH	papC	sfa/focDE	iutA	kpsMT II
A	0	5.88	0	0	0
B2	0	33.33	0	33.33	0
D	0	12.5	0	25	37.5

Figure 6.8 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from station 1 (Chitoor, n = 47)

Table 6.6 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 1 (Chitoor)

Combination of virulence factor genes	A (n = 17)	B1 (n = 19)	B2 (n =3)	D (n = 8)
iutA + papC	0	0	33.33%	0
iutA + kpsMT II	0	0	0	25%
papC + kpsMT II	0	0	0	12.5%

6.5.1.3 ExPEC and distribution of virulence factor genes in station 2 (Bolgatty)

Multiplex PCR analysis of the 74 isolates from station 2 (Bolgatty) revealed 24 (32%) isolates carried two or more virulence factor genes and identified as ExPEC.

6.5.1.3.1 ExPEC and phylogenetic groups

The distribution of the 75 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups, revealed that out of the 24 ExPEC isolates, 18 isolates belonged to phylogenetic group B2, followed by D (3 isolates), A (2 isolates) and B1 (1 isolate) (Figure 6.9).

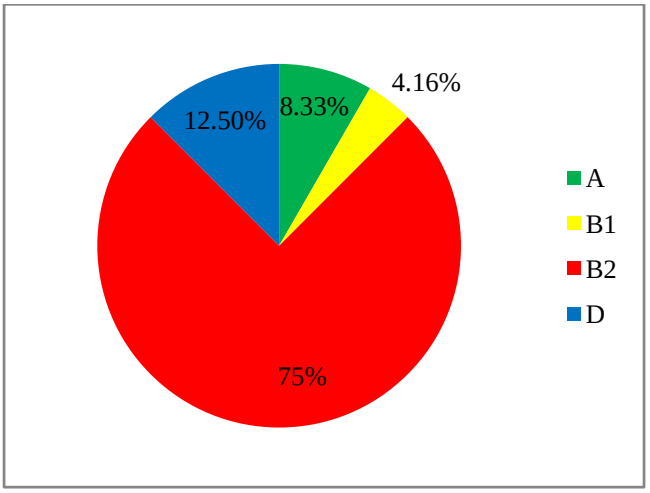


Figure 6.9 Distribution of various phylogenetic groups of ExPEC isolates (n = 24) from station 2 (Bolgatty)

6.5.1.3.2 Virulence factor genes

More than 13% of *E. coli* isolates carried *iutA* gene, while prevalence of *kpsMT II* (12%) and *papC* (9.33%) were slightly lower. *papAH* and *sfa/focDE* were detected 0.33% only.

6.5.1.3.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty)

Among isolates from station 2 (Bolgatty), group B2 isolates showed highest prevalence for all the virulence factor genes (Figure 6.10). Isolates with *papC* belonged to B2 (72.72%), followed by D, A and B1 (9.09% each).

iutA carried isolates were also belonged to B2 (83.33%), followed by D (11.11%), and A (5.55%). *E. coli* isolates with *kpsMT II* gene, were distributed in B2 (70%), D (15%), A (10%), and B1 (5%). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from station 2 are listed in Table 6.7.

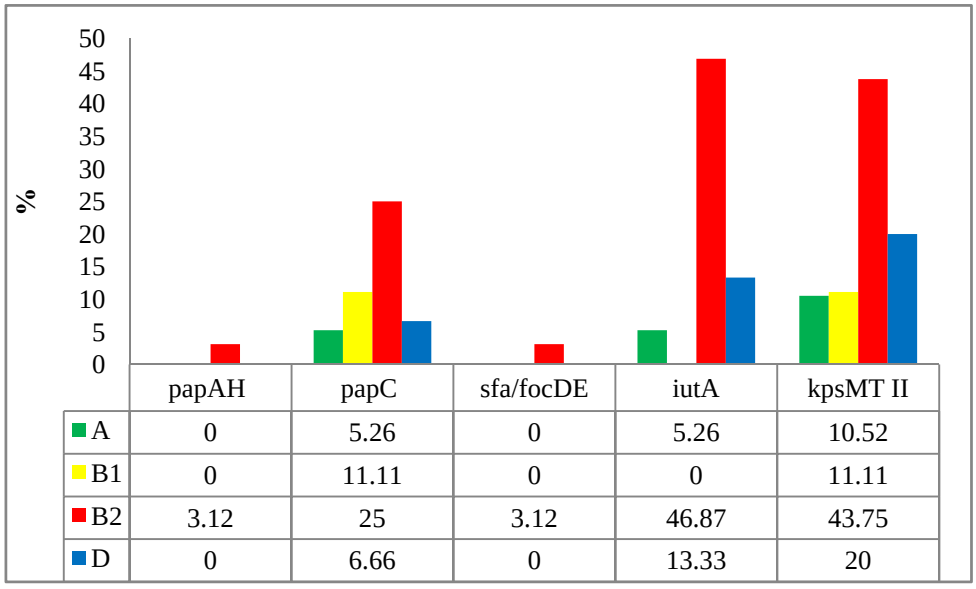


Figure 6.10 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty, n = 75)

Table 6.7 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 2 (Bolgatty)

Combination of virulence factor genes	**A (n = 19)**	**B1 (n = 9)**	**B2 (n=32)**	**D (n = 15)**
papC + kpsMT II	5.26%	11.11%	9.37%	6.66%
iutA + kpsMT II	5.26%	0	31.25%	13.33%
iutA +papC	0	0	12.5%	0
papAH + sfa/focDE + iutA + kpsMT II + papC	0	0	3.12%	0

6.5.1.4 ExPEC and distribution of virulence factor genes in station 3 (Off Marine Science Jetty)

Multiplex PCR analysis of the 69 isolates from station 3 (Off Marine Science Jetty) revealed 11 (15.94%) isolates contained at least one virulence factor genes and 10 (14.5%) isolates carried two or more virulence factor genes.

6.5.1.4.1 ExPEC and phylogenetic groups

The distribution of the 69 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups, revealed that out of the 10 ExPEC isolates, 5 isolates belonged to phylogenetic group B2, followed by D and A (2 isolates each) and B1 (1 isolate) (Figure 6.11).

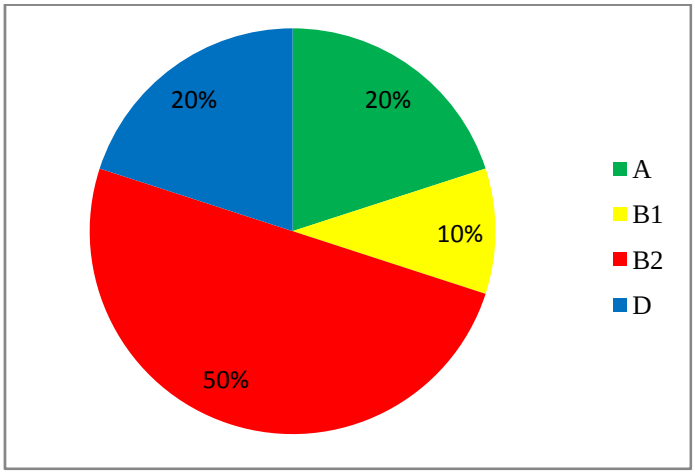


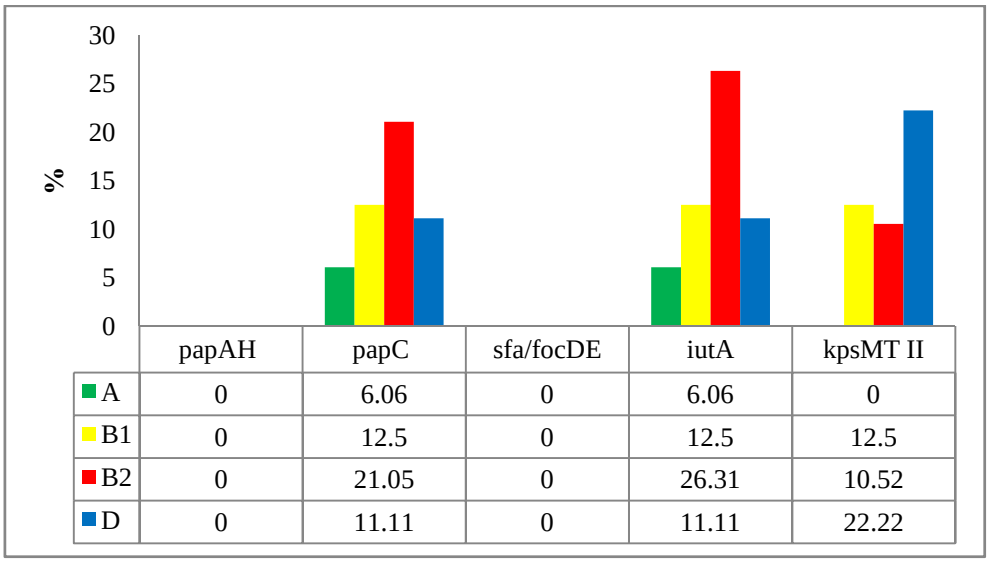
Figure 6.11 Distribution of various phylogenetic groups of ExPEC isolates (n = 10) from station 3 (Off Marine Science Jetty)

6.5.1.4.2 Virulence factor genes

E. coli isolates carried *papC* dominated in station 3. More than 13% of *E. coli* isolates carried *iutA* gene, followed by *kpsMT II* (7.04%). *papAH* and *sfa/focDE* were not detected.

6.5.1.4.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 3 (Off Marine Science Jetty)

Phylogenetic group B2 isolates showed highest occurrence of *papC* and *iutA* gene (Figure 6.12). Whereas group D isolates, showed highest occurrence for *kpsMT II* gene. *E. coli* isolates carried *papC* belonged to B2 (50%), followed by A (25%), and B1, D (12.5% each). *E. coli* isolates carried *iutA* also showed a similar distribution pattern with B2 (55.55%), A (22.22%) B1, D (11.11% each). Whereas *E. coli* carried *kpsMT II* gene were belonged to B2, D (40% each) and B1 (20%). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from station 3 are listed in Table 6.8.



	papAH	papC	sfa/focDE	iutA	kpsMT II
A	0	6.06	0	6.06	0
B1	0	12.5	0	12.5	12.5
B2	0	21.05	0	26.31	10.52
D	0	11.11	0	11.11	22.22

Figure 6.12 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from station 3 (Off marine Science Jetty, n = 69).

Table 6.8 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 3 (Off Marine Science Jetty)

Combination of virulence factor genes	A (n=33)	B1 (n = 8)	B2 (n =19)	D (n = 9)
iutA + papC	6.06%	0	21.05%	0
iutA + kpsMT II	0	0	5.26%	11.11%
papC + kpsMT II	0	0	0	11.11%
iutA + papC + kpsMT II	0	12.5%	0	0

6.5.1.5 ExPEC and distribution of virulence factor genes in station 4 (Thevara)

Multiplex PCR analysis of the 49 isolates from station 4 (Thevara) revealed 8 (16.32%) isolates contained at least one virulence factor genes and 7 (14.28%) isolates carried two or more virulence factor genes.

6.5.1.5.1 ExPEC and phylogenetic groups

The distribution of the 49 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups, revealed that out of the 8 ExPEC isolates, 3 isolates belonged to phylogenetic group D, 2 isolates each to B1 and B2 (Figure 6.13).

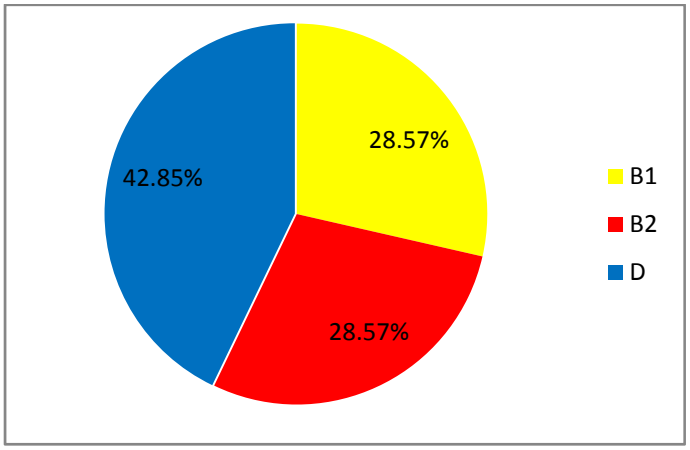


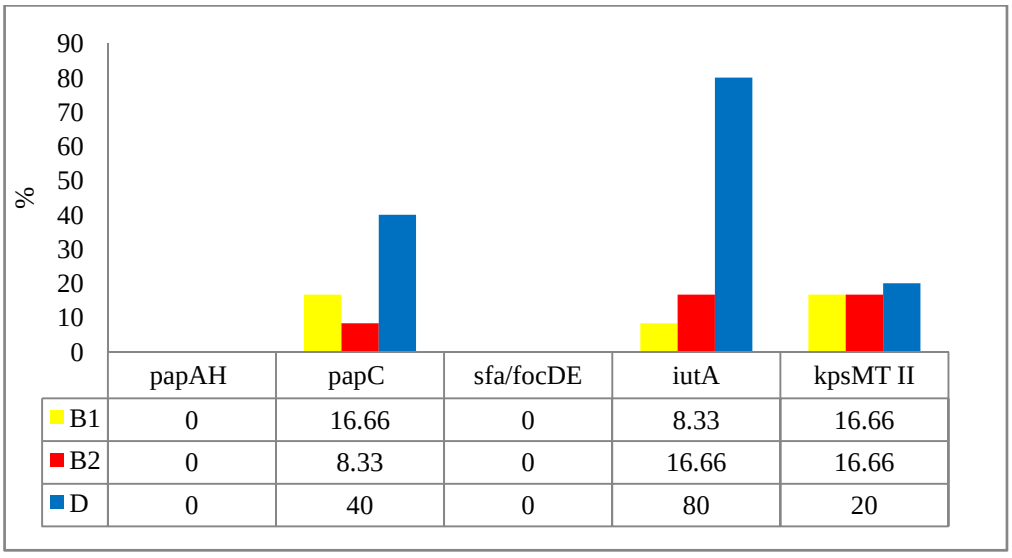
Figure 6.13 Distribution of various phylogenetic groups of ExPEC (n = 7) isolates from station 4 (Thevara)

6.5.1.5.2 Virulence factor genes

E. coli isolates carried *iutA* (14.28%) dominated in station 4. More than 10% of *E. coli* isolates carried *papC* and *kpsMT II*. *papAH* and *sfa/focDE* were not detected.

6.5.1.5.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 4 (Thevara)

Phylogenetic group D isolates showed highest occurrence of *papC*, *iutA* and *kpsMT II* gene (Figure 6.14). *E. coli* isolates carried *iutA* belonged to D (57.14%), B2 (28.57%) and B1 (14.28%). Whereas *E. coli* isolates carried *papC* belonged to D, B1 (40% each) and B2 (20%). *E. coli* carried *kpsMT II* gene were belonged to B1, B2 (40% each) and D (20%). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from station 4 are listed in Table 6.9.



	papAH	papC	sfa/focDE	iutA	kpsMT II
B1	0	16.66	0	8.33	16.66
B2	0	8.33	0	16.66	16.66
D	0	40	0	80	20

Figure 6.14 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from station 4 (Thevara, n = 49).

Table 6.9 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 4 (Thevara).

Combination of virulence factor genes	A (n = 20)	B1 (n = 12)	B2 (n = 12)	D (n = 5)
papC + kpsMT II	0	8.33%	0	0
iutA + papC	0	0	0	40%
iutA + kpsMT II	0	0	8.33%	20%
iutA + papC + kpsMT II	0	0	8.33%	0

6.5.1.6 ExPEC and distribution of virulence factor genes in station 5 (Barmouth)

Multiplex PCR analysis of the 60 isolates from station 5 (Barmouth) revealed 4 (6.66%) isolates contained at least one virulence factor genes and 3 (5%) isolates carried two or more virulence factor genes.

6.5.1.6.1 ExPEC and phylogenetic groups

The distribution of the 60 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups revealed that out of the 3 ExPEC isolates, 2 isolates belonged to phylogenetic group B2, 1 isolate to D (Figure 6.15).

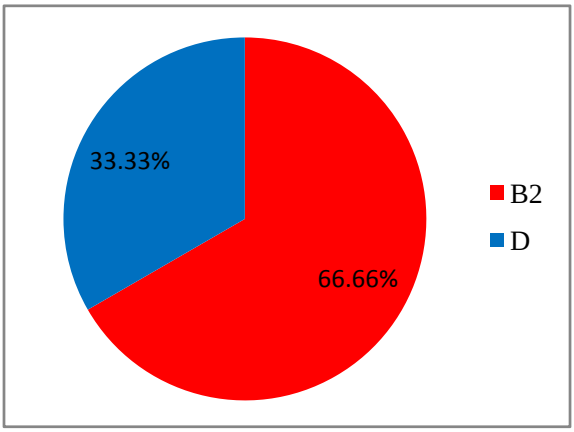


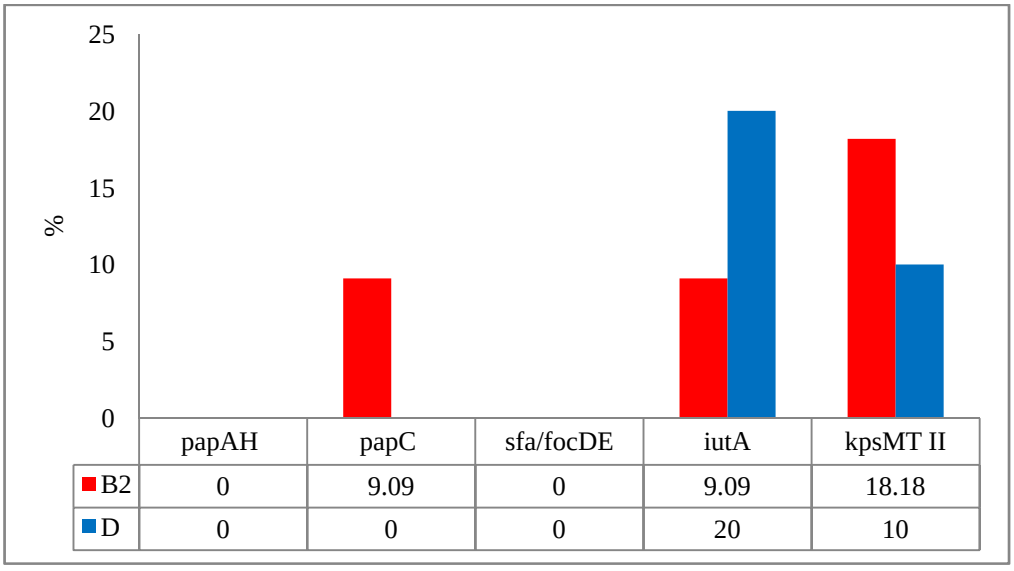
Figure 6.15 Distribution of various phylogenetic groups of ExPEC isolates (n = 3) from station 5 (Barmouth)

6.5.1.6.2 Virulence factor genes

About 5% of *E. coli* isolates carried *kpsMT II* and *iutA* gene. *E. coli* isolates carried *papC* were 1.66% only. *papAH* and *sfa/focDE* were not detected.

6.5.1.6.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 5 (Barmouth)

Phylogenetic group D isolated showed highest occurrence of *iutA* gene (Figure 6.16). Whereas group B2 isolates showed highest occurrence of *kpsMT II* and *papC* gene. *E. coli* isolates carried *kpsMT II* belonged to B2 (66.66%) and D (33.33%). Whereas *E. coli* isolates carried *iutA* belonged to D (33.33%) and B2 (66.66%). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from station 5 are listed in Table 6.10.



	papAH	papC	sfa/focDE	iutA	kpsMT II
B2	0	9.09	0	9.09	18.18
D	0	0	0	20	10

Figure 6.16 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from station 5 (Barmouth, n = 60)

Table 6.10 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from station 5 (Barmouth)

Combination of virulence factor genes	A (n = 18)	B1 (n = 21)	B2 (n = 11)	D (n =10)
iutA + papC	0	0	9.09%	0
iutA + kpsMT II	0	0	9.09%	10%

6.5.1.7 Relative prevalence of virulence factor genes in *E. coli* isolates from different stations in Cochin estuary

There is a significant diffrence in the distribution of virulence genes in group B2 isolates among different stations ($p = 0.000$) (Table 6.11). Group B2 isolates with virulence genes was significantly high in station 2 (Bolgatty) than station 1, 4 ($p < 0.001$) and station 3 ($p < 0.05$). In Bolgatty group B2 isolates carried more virulence genes than group A, B1 and D ($p < 0.001$). Whereas station 3 (Off Marine Science Jetty) showed a significant difference in the distribution of virulence genes ($p < 0.009$).

Table 6.11 Distribution of virulence factor genes in *E. coli* isolates from different stations set at Cochin estuary

Virulence factor genes	Station 1	Station 2	Station 3	Station 4	Station 5
papAH	0	1.33%	0	0	0
papC	6.38%	14.66%	11.59%	10.2%	1.66%
sfa/focDE	0	1.33%	0	0	0
iutA	6.38%	24%	13.04%	14.28%	5%
kpsMT II	6.38%	26.66%	7.24%	10.2%	5%

6.5.2 ExPEC and distribution of virulence factor genes in *E. coli* isolates from seafood sources

Multiplex PCR analysis of the 100 isolates from seafood sources revealed 3% of isolates as ExPEC.

6.5.2.1 ExPEC and phylogenetic groups

All three ExPEC isolates of seafood origin were belonged to group D (Figure 6.17)

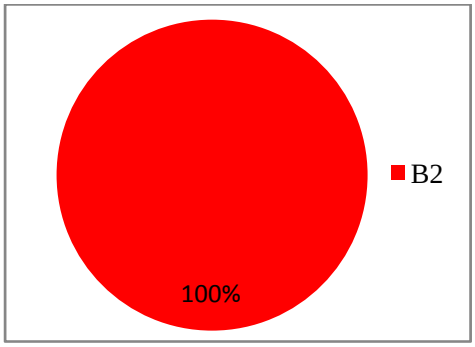


Figure 6.17 Distribution of various phylogenetic groups of ExPEC isolates (n = 3) from seafood sources

6.5.2.2 Virulence factor genes

In seafood isolates only 3% of isolates carried *papC* (Figure 6.18). *papAH* and *sfa/focDE* genes were not detected. No significant difference in the distribution of virulence genes in seafood isolates.

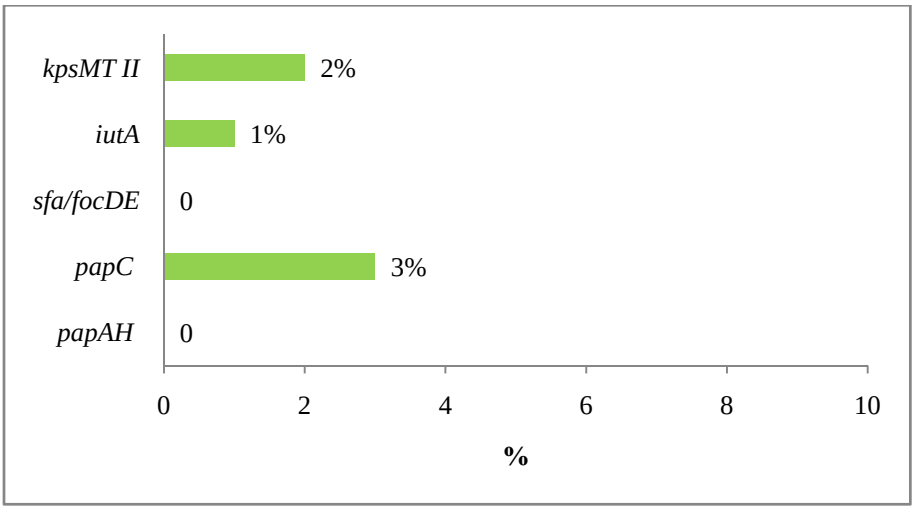


Figure 6.18 Distribution of different virulence factor genes in *E. coli* isolates from seafood sources (n = 100)

6.5.2.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from seafood isolates

In seafood isolates virulence factors carried isolates were belonged to B2 group (Figure 6.19). Distribution of virulence factor genes significantly varied among different phylogenetic groups (p = 0.006). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from seafood are listed in Table 6.12.

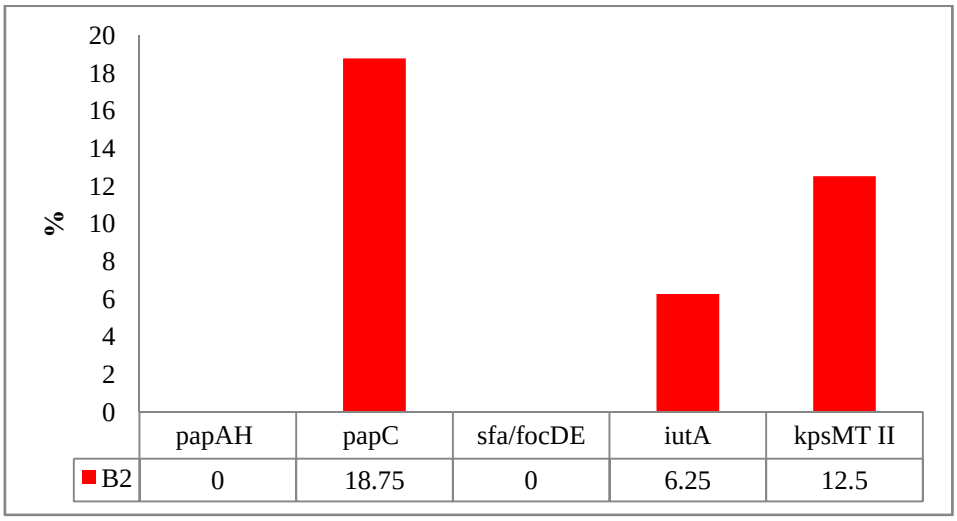


Figure 6.19 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from seafood sources (n = 100)

Table 6.12 Co-occurrence of virulence factor genes various phylogenetic groups of *E. coli* isolates from seafood sources

Combination of virulence factor genes	A (n = 52)	B1 (n = 23)	B2 (n = 16)	D (n = 9)
kpsMT II + papC	0	0	12.5%	0
PapC +iutA	0	0	6.25%	0

6.5.2.4 ExPEC and antibiotic resistance

Out of 3 ExPEC, 2 isolates showed resistance to one antibiotic. One isolate showed resistance to ampicillin and carried bla_{TEM} gene. While another isolate showed resistance to streptomycin.

6.5.3 ExPEC and distribution of virulence factor genes in *E. coli* isolates from clinical sources

Multiplex PCR analysis of the 49 isolates from clinical sources revealed 84% of isolates contained at least one virulence factor genes and 79% of isolates carried two or more virulence factor genes.

6.5.3.1 ExPEC and phylogenetic groups

The distribution of the 100 *E. coli* isolates in relation to virulence factor genes and phylogenetic groups, revealed that out of the 79 ExPEC isolates, 60 isolates belonged to phylogenetic group B2, 11 isolates to A, 6 isolates to D and 2 isolates to B1 (Figure 6.20).

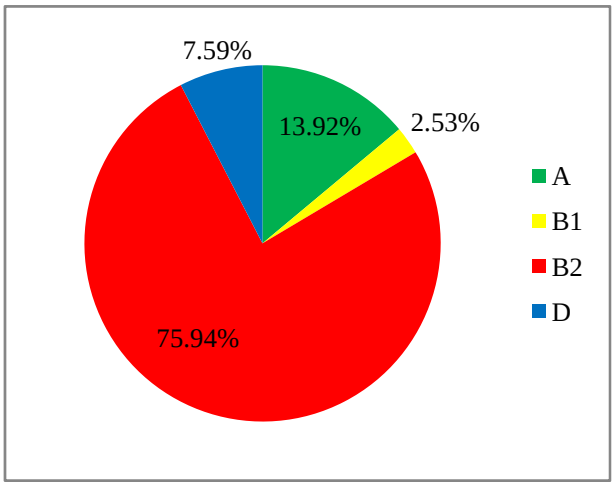


Figure 6.20 Distribution of various phylogenetic groups of ExPEC isolates (n = 79) from clinical sources

6.5.3.2 Virulence factor genes

In clinical isolates *iutA* (67%) gene was frequently encountered. *sfa/foc DE* was least detected virulence factor gene in clinical isolates (Figure 6.21). There was a significant difference in the distribution of virulence genes in clinical isolates ($p = 0.001$). *E. coli* from clinical sources carried more *iutA* gene than *papAH* and *sfa/focDE* ($p < 0.05$).

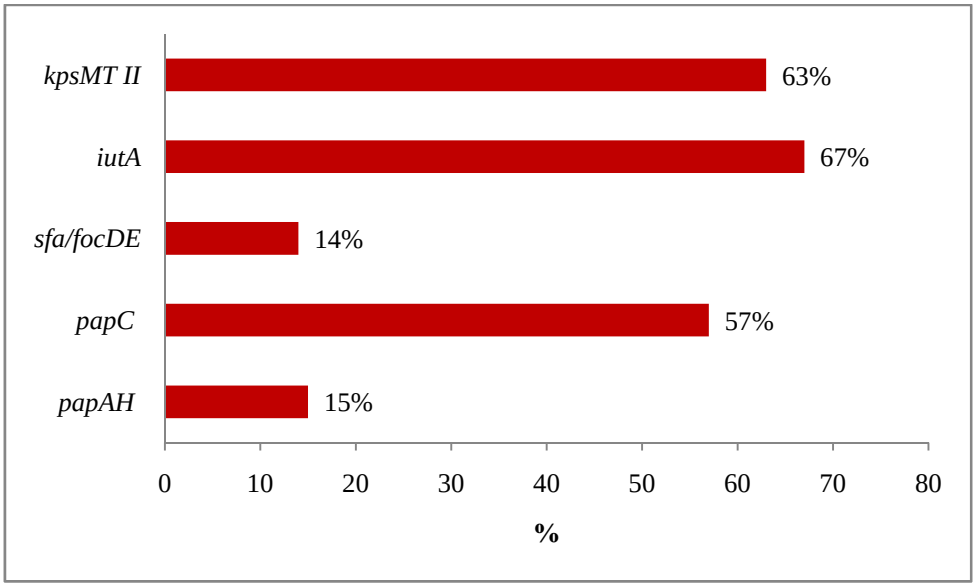
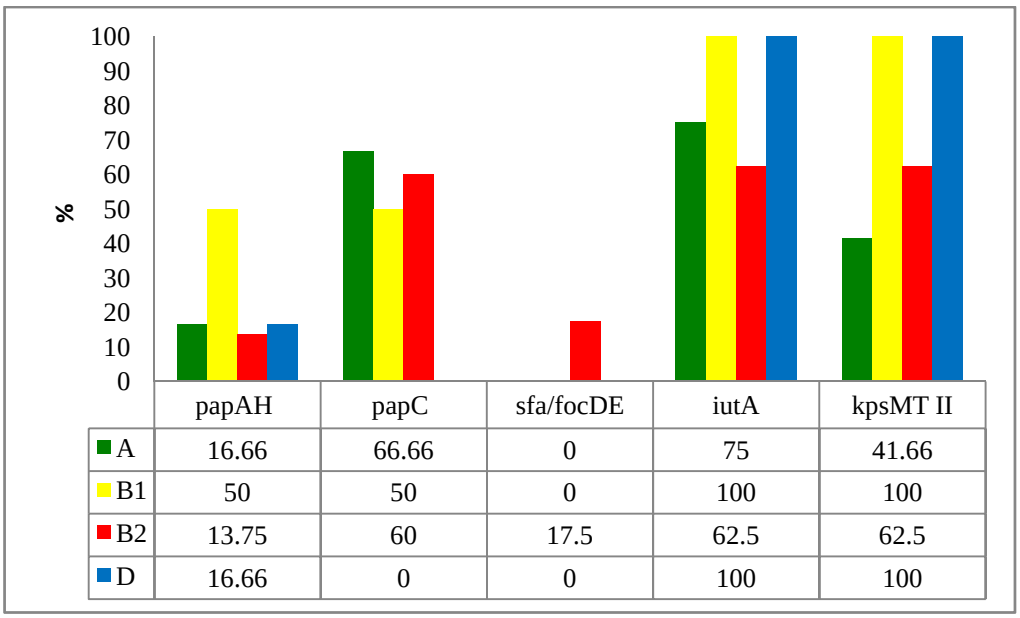


Figure 6.21 Distribution of different virulence factor genes in *E. coli* isolates from clinical sources (n = 100)

6.5.3.3 Incidence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from clinical sources

All isolates belonged to group B1 and D carried *iutA* and *kpsMT II* gene. About 60% of B2 isolates carried *papC*, *iutA*, and *kpsMT II*. Between 65-75% of A group isolated carried *papC* and *iutA*. *E. coli* isolates harboured *papAH* belonged to B2 (73.33%), A (13.33%), B1 and D (6.66% each). Wheras *papC*

carried isolates were grouped into B2 (84.21%), A (14.03%) and B1 (1.75%). *iutA* harboured isolates were belonged to B2 (74.62%), A (13.43%), D (8.95%), and B1(2.98%). *E. coli* isolates carried *kpsMT II* were belonged to B2 (79.36%), D (9.52%), A (7.93%) and B1 (3.17%). Distribution of virulence factor genes significantly varied among different phylogenetic groups (Figure 6.22). *E. coli* isolates belonged to group B2, carried more virulence genes than group A, B1 and D ($p < 0.001$). Co-occurrence of virulence factor genes in groups A, B1, B2 and D in *E. coli* isolates from clinical sources are listed in Table 6.13.



	papAH	papC	sfa/focDE	iutA	kpsMT II
A	16.66	66.66	0	75	41.66
B1	50	50	0	100	100
B2	13.75	60	17.5	62.5	62.5
D	16.66	0	0	100	100

Figure 6.22 Percentage of virulence factor genes among different phylogenetic groups of *E. coli* isolates from clinical sources (n = 100)

Table 6.13 Co-occurrence of virulence factor genes among various phylogenetic groups of *E. coli* isolates from clinical sources

Combination of virulence factor genes	A (n = 12)	B1 (n = 2)	B2 (n = 80)	D (n = 6)
iutA + papC	33.33%	0	6.25%	0
iutA + kpsMT II	25%	50%	17.5%	83.33%
iutA + sfa/focDE	0	0	1.25%	0
papC + papAH	16.66%	0	0	0
papC + sfa/focDE	0	0	1.25%	0
papC + kpsMT II	0	0	11.25%	0
iutA + kpsMT II + papAH	0	0	0	16.66%
iutA + kpsMT II + papC	16.66%	0	11.25%	0
iutA + papC + sfa/focDE	0	0	3.75%	0
iutA + kpsMT II + sfa/focDE	0	0	2.5%	0
iutA + kpsMT II + papC + sfa/focDE	0	0	6.25%	0
iutA + kpsMT II + papC + papAH	0	50	11.25%	0
iutA + kpsMT II + papC + papAH + sfa/focDE	16.66%	0	2.5%	0

6.5.3.4 Prevalence of antibiotic resistance among ExPEC isolates from clinical sources

Out of 79 ExPEC, 98.73% of isolates were resistant to at least one antibiotic and 97.4% of isolates were resistant to more than two antibiotics. Among various antibiotics tested resistance to ampicillin was highest and least resistance was observed against chloramphenicol (Figure 6.23). One of the adhesion related genes, such as *papAH*, showed significant association with ceftriaxone, and tetracycline resistance ($p < 0.05$). While another adhesion related gene *papC* showed an association with ampicillin resistance ($p = 0.033$). Whereas *sfa/focDE* and *iutA* did not show any positive association with any antibiotic resistance traits. Group 2 capsule synthesis gene *(kpsMT II)* was positively associated with ceftazidime resistance ($p = 0.008$).

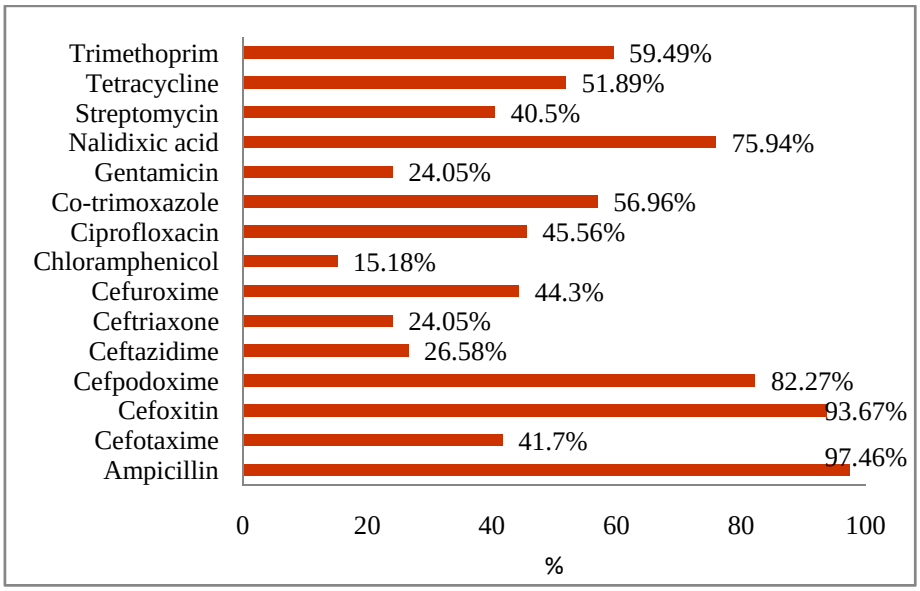


Figure 6.23 Prevalence of antibiotic resistance among ExPEC isolates from clinical sources (n = 100)

Table 6.14 Distribution of antibiotic resistance among various phylogenetic groups of ExPEC isolates from clinical sources (n = 79)

Antibiotics	A (n = 11)	B1 (n = 2)	B2 (n = 60)	D (n = 6)
Ampicillin	100%	100%	98.33%	83.33%
Cefotaxime	27.27%	50%	43.33%	50%
Cefoxitin	100%	100%	93.33%	83.33%
Cefpodoxime	100%	100%	78.33%	83.33%
Ceftazidime	18.18%	50%	25%	50%
Ceftriaxone	18.18%	50%	25%	16.66%
Cefuroxime	27.27%	50%	46.66%	50%
Chloramphenicol	18.18%	50%	11.66%	33.33%
Ciprofloxacin	63.63%	100%	43.33%	16.66%
Co-trimoxazole	45.45%	50%	60%	50%
Gentamicin	9.09%	50%	26.66%	16.66%
Nalidixic acid	63.63%	100%	78.33%	66.66%
Streptomycin	54.54%	50%	38.33%	33.33%
Tetracycline	45.45%	100%	51.66%	50%
Trimethoprim	36.36%	50%	63.33%	66.66%

Group A and B1 ExPEC isolates showed relatively higher resistance to ampicillin, cefoxitin and cefpodoxime, whereas group D isolates, showed higher resistance to trimethoprim. Whilst group B2 isolates, showed higher resistance to co-trimoxazole (Table 6.14).

6.5.3.5 Prevalence of antibiotic resistance genes among ExPEC isolates from clinical sources

Antibiotic resistance genes such as, *bla*$_{TEM}$, *sul1*, *sul2*, *tetA* and *strA* were detected more frequently among clinical ExPEC isolates (Figure 6.24). P fimbriae gene *papAH*, showed significant association with *tetA*, *dhfrIa*, and *dhfrVII* ($p < 0.001$). Aerobactin receptor gene (*iutA*), was positively associated with *bla*$_{TEM}$ ($p < 0.05$), whereas *papC* and *sfa/focDE* genes did not show any positive association with any antibiotic resistance genes.

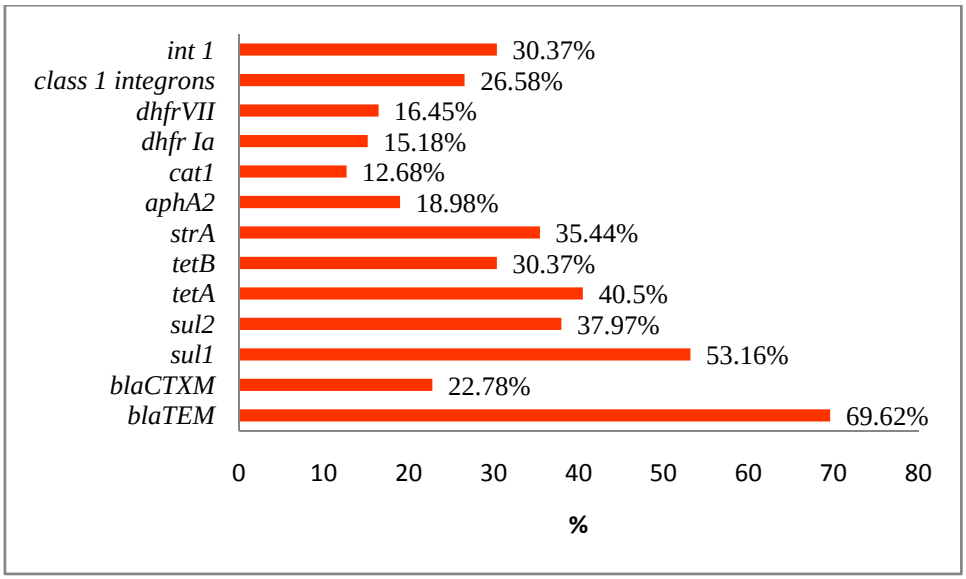


Figure 6.24 Occurrence of antibiotic resistance genes among ExPEC isolates from clinical sources (n = 100)

Among the 79 ExPEC isolates, group A and B1 carried more resistance genes than group B2 and D. Antibiotic resistance genes such as *bla*$_{TEM}$, *bla*$_{CTX\text{-}M}$, *sul2*, *tetA*, *tetB*, *strA*, *cat1*, *dhfrIa*, *dhfrVII* were highly prevalent in group B1 isolates (Table 6.15).

Table 6.15 Distribution of antibiotic resistance genes among various phylogenetic groups of ExPEC isolates from clinical sources (n = 79)

Antibiotic resistant genes	A (n = 11)	B1 (n = 2)	B2 (n = 60)	D (n = 6)
bla$_{TEM}$	81.81%	100%	63.33%	100%
bla$_{CTXM}$	18.18%	50%	21.66%	33.33%
sul1	45.45%	50%	55%	50%
sul2	36.36%	50%	38.33%	33.33%
tetA	36.36%	100%	40%	33.33%
tetB	36.36%	100%	30%	0
strA	45.45%	50%	33.33%	33.33%
aphA2	9.09%	0	21.66%	16.66%
cat1	18.18%	50%	8.33%	33.33%
dhfrIa	18.18%	50%	13.33%	16.66%
dhfrVII	18.18%	50%	15%	16.66%
class 1 integrons	18.18%	50%	26.66%	33.33%
int 1	18.18%	50%	31.66%	33.33%

6.5.4 Relative prevalence of virulence factor genes in *E. coli* isolates from different sources

There is a significant diffrence in the distribution of virulence factor genes in *E. coli* isolates among seafood, clinical and estuarine isolates ($p < 0.05$). *E. coli* isolates with virulence genes was significantly high in clinical isolates than seafood and estuarine isolates (Figure 6. 25).

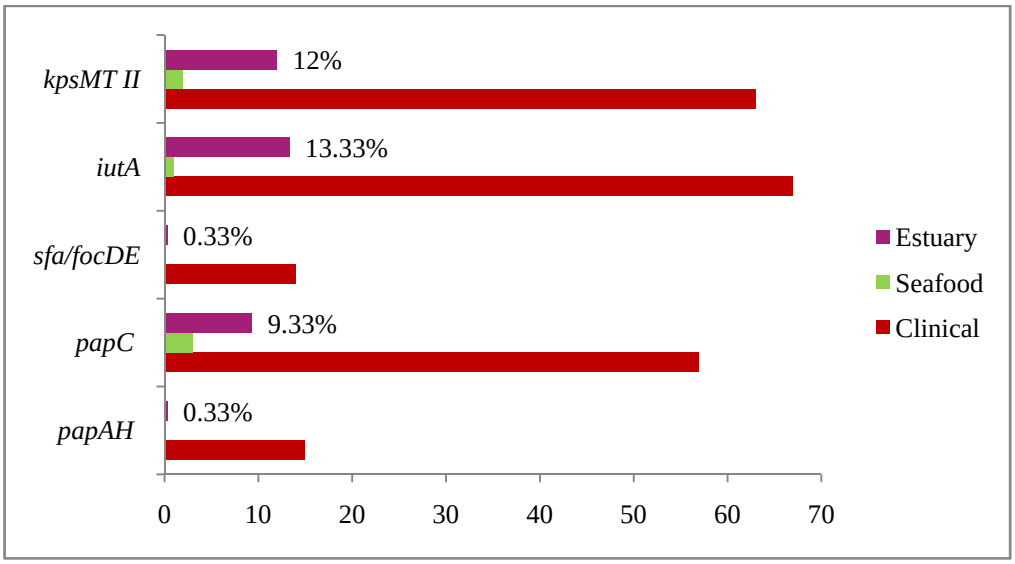


Figure 6.25 Percentage of virulence factor genes among *E. coli* isolates from different sources

6.6 Discussion

6.6.1 ExPEC isolates from Cochin estuary

6.6.1.1 Virulence factor genes

Our results revealed the prevalence of ExPEC in Cochin estuary. Ahmed *et al.*, (2011) studied extraintestinal virulence genes in *E. coli* isolates from rainwater tanks and reported that 42% of isolates were carried a single virulence genes, 37.5% carried two virulence genes, which is lower than our result. They also reported that 18% of isolates carried three virulence genes, and 3% of isolates had four or more virulence genes, which is higher than our results. In our results only 1.3% of isolates carried three virulence genes and 0.33% isolates carried four or more virulence genes.

Diallo *et al.*, (2013) reported that the prevalence of ExPEC was significantly higher in city wastewater (8.4%), compared to slaughterhouse

wastewater (1.2%). Hamelin *et al*., (2007) reported that the ExPEC pathotypes were found to occur across all aquatic ecosystems investigated, including riverine, estuarine, and offshore lake locations. Luna *et al*., (2010) studied the extraintestinal *E. coli* carrying virulence genes in coastal marine sediments and showed that 90% of B2 and 65% of D isolates were positive for at least one of the virulence genes. In a study by Aslam *et al*., (2014) reported that there was no significant difference for the presence of virulence gene number between poultry and human ExPEC isolates.

Diallo *et al*., (2013) reported that the prevalence of virulence genes was lower than 7%, and *papEF*, *kpsMT K1*, *sfa/focDE* were the most identified genes. In our study except for *papAH* and *sfa/focDE*, the prevalence for virulence genes was between 9% - 13%. In consistent with our study, Luna *et al*., (2010) reported that *sfa/focDE* gene was belonged to B2 only. Ahmed *et al*., (2011) reported that among ExPEC virulence factors, *kpsMT* allele III was the most prevalent (17.5%), followed by *papAH* (13.5%). In our study *iutA* was prevalent (13%) followed by *kpsMT* (12%), whereas *papAH* was least prevalent (0.33%).

Our results revealed that, most of the ExPEC isolates belonged to group B2 (58%) and D (25%). Predominance of either group D (56%) or B2 (35%) among ExPEC has been reported by Ahmed *et al*., (2011). Similar to our findings, Diallo *et al*., (2013) also reported that ExPEC isolates mainly belonged to phylogroup B2 (82.0%), and to a lesser extent, to phylogroup D (13.1%) and A (4.9%).

Diallo *et al*., (2013) reported that 45.8% of the ExPEC isolates were resistant to at least one antibiotic and that 31.1% as multiresistant. Contrary to

our results, they reported that ExPEC isolates were mainly resistant to ampicillin (44.2%), sulphonamide (36.1%), streptomycin (26.2%) and tetracycline (26.2%) and 4.9% were resistant to ciprofloxacin. In our study resistance was lower than those reported by Diallo *et al*., (2013) except for ciprofloxacin (8.33%).

6.6.1.2 ExPEC and phylogenetic groups

In agreement with other studies, our estuarine isolates primarily belonged to one of two virulence groups (group B2 or D) (Obeng *et al.*, 2012; Ahmed *et al*., 2011; Martinez-media *et al*., 2009b; Marynard *et al.*, 2004). There was a significant diffrence in the distribution of virulence genes in group B2 isolates among different stations. Group B2 isolates with virulence genes was significantly high in station 2 (Bolgatty). In Bolgatty group B2 isolates carried more virulence genes than group A, B1 and D. Projection of virulence factors traits onto the phylogenetic background of the isolates revealed, as expected, an association of most virulence factors with B2 and D phylogenetic group and to a lesser extend to group A and B1. This agrees with most other studies concerning phylogenetic groups in uropathogenic (Moreno *et al*., 2008; Johnson *et al*., 2005b) and bacteraemic *E. coli* (Moreno *et al*., 2005; Sannes *et al*., 2004; Johnson *et al*., 2002a). Our results also confirmed certain findings reported by others (Hamelin *et al*., 2007; Bingen *et al*., 1998), such as the presence of the *sfa-foc* operon only in the phylogenetic group B2. Strains belonging to group B2 harboured a greater number of virulence factors compared to strains from other phylogenetic groups, suggesting a putative association between virulence factors and pathogenic potential (Escobar-Paramo *et al*., 2004a).

We found that among all phylogenetic groups, B2 harboured significantly higher proportion of virulence factors genes. *PapAH* was detected only in phylogenetic group B2. This is in accordance with the report of Nowrouzian *et al*., (2006) who found that most B2 strains carried genes for P-fimbriae. Our result also supported some previous reports indicating greater association of traditionally recognized uropathogenic virulence factors (e.g. *pap* and *sfa)* with groups D and B2 as compared with A and B1(Johnson *et al*., 2001c; Boyd and Hartl, 1998). Most ExPEC, including those with most robust virulence factors and those which are best able to infect non-compromised hosts, are derived from phylogenetic group B2 (Johnson, 2002).

We have observed that group D contains the second highest number of ExPEC; extraintestinal isolates from this group typically have somewhat fewer virulence factors and a different mix of virulence factors than group B2 isolates. *E. coli* strains belonging to groups A and B1 do not frequently cause extraintestinal infection. These strains that are not highly virulent cause disease only in immunocompromised hosts, and could be pathogenic in healthy hosts only if they could acquire sufficient extraintestinal factors (Johnson, 2002).

6.6.1.3 ExPEC and antibiotic resistance

In the present study, the results showed that 45.83% of the ExPEC isolates were resistant to at least one antibiotic; and that 29.2% were multiresistant. Overall, this study reveals that resistance to antibiotics is significantly associated with presence of virulence factors. The aerobactin receptor gene (*iutA*) was positively associated with resistance to cefotaxime and cepfodoxime. Group 2 capsule synthesis gene *(kpsMT II)* on the other hand showed an association

with ciprofloxacin, cefoxitin, cepfodoxime, and cefotaxime. In contrast to our study, previous data suggest that among *E. coli* isolates from patients with urosepsis, resistance to antibiotic agents such as ampicillin, sulphonamides, tetracycline, and streptomycin is negatively associated with virulence and a group B2 phylogenetic background (Johnson *et al*., 1991). More recent data demonstrate similar negative association between antibiotic resistance and virulence factors or a B2 phylogenetic background (Diallo *et al*., 2013, Obeng *et al.*, 2012; Skjot-Rasmussen *et al*., 2012). Whereas adhesion related genes, (*papAH*, and *papC*) and S/F1C fimbriae gene (*sfa/focDE*) did not showed a positive association with any antibiotic resistance traits. This has been interpreted as suggesting that virulence factors may be lost concomitant with mutation to resistance (Vila *et al*., 2002). However, this hypothesis does not account for the phylogenetic shifts (away from group B2) observed among resistant isolates, which suggest instead that resistant isolates derive primarily from distinct, less virulent bacterial populations (Johnson *et al*., 2003d, 2002d).

One of the adhesion related genes, such as *papAH*, showed significant association with antibiotic resistance genes *cat1*, *dhfrIa*, and *dhfrVII* whereas *papC* showed a positive association with *int1* gene. The S/F1C fimbriae gene (*sfa/focDE*) also showed an association with *cat1*, *dhfrIa*, and *dhfrVII* . Group 2 capsule synthesis gene *(kpsMT II)* was positively associated with $bla_{CTX\text{-}M}$. While aerobactin receptor gene (*iutA*) was positively associated with $bla_{CTX\text{-}M}$ and *int1* gene. Biswal *et al*., (2014) reported the co-occurrence of virulence genes and antibiotic resistance genes in *E. coli* from waste water. Piatti *et al*., (2008) reported that quinolone resistant isolates were significantly associated with low incidence of *papAH*, *papC*, *sfa/focDE*, *iutA* and *kpsMT II*.

It is striking finding from the present study that ExPEC isolates belonged to group B2 showed higher resistance against cefotaxime, and cefpodoxime. Whereas group D isolates showed higher resistance against cefoxitin, gentamicin, chloramphenicol and ciprofloxacin. whilst group A and B1 ExPEC isolates were sensitive to most of the antibiotics tested. This may be because individual strains have undergone the necessary and appropriate adaptation for survival in the changing antibiotic environment. Furthermore, it may also due to the co-selection of resistance genes by the use of different antibiotics, as reported elsewhere (Ahmed *et al*., 2007, O' Connor *et al*., 2002).

6.6.2 ExPEC isolates from seafood sources

6.6.2.1 Virulence factor genes

The prevalence of ExPEC in seafood samples was very low (3%) compared to estuary. Johnson *et al*., (2003a) reported that 21% of isolates carried more than two virulence genes in retail chicken products, which is higher than what we have seen in seafood. Tramuta *et al*., (2011) reported that *papC, hlyA, cdt*, and *afa* were found in phylogenetic group B2 but were absent in the remaining groups and *sfa* and *cnf1* were predominantly detected in group B2. Johnson *et al*., (2005a) reported that ExPEC isolates in four food-sources (from pea pods, turkey parts, ground pork, and vegetable dip) closely resembled selected human clinical isolates by O antigen and genomic profile. They also suggested that retail foods may be an important vehicle for community-wide dissemination of antibiotic resistant *E. coli* and ExPEC, which may represent a newly recognized group of medically significant foodborne pathogens.

Johnson *et al*., (2003a) reported that among ExPEC virulence factors *iutA* was the most prevalent (57%) followed by *kpsMT II* (14%), *papC* (14%) and *sfa/focDE* (1%). Our results revealed that *papC* was prevalent (3%) followed by *kpsMT II* (2%), and *iutA* (1%), which is higher than our rates. Marynard *et al*., (2004) reported that *papC* (49%) was more prevalent in animals than *sfa/focDE* (26%). Tan *et al*., (2012) reported that among ExPEC, *iutA* was the most prevalent (66.7%), followed by *kpsMT II* (56.8%), *sfa/focDE* (46.7%), *papAH* (43.8%) and *papC* (38.4%), which is also higher than the prevalence level of these genes seen in our results. Tramuta *et al*., (2011) reported that among ExPEC isolated from cats and dogs, *sfa/focDE* was the most prevalent (57.5%), followed by *papC* and *iutA* (37.5%).

6.6.2.2 ExPEC and phylogenetic groups

In concurrence with several authors (Diallo *et al*., 2013; Obeng *et al*., 2012; Luna *et al*., 2010), we observed a link between phylogenetic groups and extraintestinal strains because the majority of the strains belong predominantly to phylogenetic group B2 and, to a lesser extent, to D, whereas they were sparsely represented within groups A and B1. In contrast to our results, Tan *et al*., (2012) reported that most porcine ExPEC belonged to phylogenetic groups A (30.8%) and B1 (29.2%). In our study all the ExPEC were belonged to group B2. Guo *et al*., (2013) reported that group B2 and D isolates contained the highest number of virulence genes. Tan *et al*., (2012) reported that forty percent of isolates in phylogenetic groups B2 and D were highly virulent porcine ExPEC strains. Obeng *et al*., (2012) reported that 10.0% of isolates carried two or more virulence genes typical of ExPEC and, 44.0% were found to belong to group D, while 24.0% were group B2. Johnson *et al*.,

(2003a) also reported that *papC* was associated negatively with phylogenetic group B1, *iutA* was associated negatively with groups A and B1 and positively with group D, and *kpsMT* II was associated negatively with groups A and B1 and positively with groups B2 and D. Marynard *et al*., (2004) reported that most animal isolates (71%) belonging to group B2 contained the virulence genes *sfaDE*, *papC*, and *hlyA*; among the human isolates the proportion was only 39%.

6.6.2.3 ExPEC and antibiotic resistance

Seafood ExPEC isolates showed least antibiotic resistance when compared to those from estuary and clinical samples. In contrast to our results, multi-resistance has been reported for ExPEC isolated from humans, chicken, dogs, cats and swine, with the majority of isolates exhibiting resistance to at least three antibiotic agents (Osugui *et al*., 2014; Tang *et al*., 2011; Smith *et al*., 2007; Zhao *et al*., 2005). Lima-Filho *et al*., (2013) reported that the majority of ExPEC isolated from poultry were resistant to at least four antibiotics from different classes. The most prevalent phenotypes were resistant to levofloxacin, amoxicillin/clavulanic acid, ampicillin, cefalothin, tetracycline and streptomycin. They also reported that the overall multidrug-resistance varied from 4 to 11 antibiotics and reached 92.6% of *E. coli* strains. Marynard *et al*., (2004) reported that 51% of the animal isolates were resistant to at least four antibiotics; and 43 percent had a profile of resistance to ampicillin, tetracycline, trimethoprim, and sulphonamides. Obeng *et al*., (2012) reported that among the 11 ExPEC isolates in group D, the commonly detected antibiotic resistance genes were *aadA2*, *sulI*, *tet(A),* and *tet(C).* They also reported that a few isolates were found to harbour genes for neomycin

resistance (*aph*(*30*)-*Ia*(*aphA1*)), sulphonamide resistance (*sulII*), and *class 1 integron*, and three of the isolates were found to be negative for resistant genes and/integrons. In our study only one strain carried antibiotic resistance gene (bla_{TEM}). The seafood samples which we collected from wild and the issues related to antibiotic usage in farm raised fish are not seen in this environment. This might be the most probable reason for a very low level of antibiotic resistance among ExPEC isolates from this source.

6.6.3 ExPEC isolates from clinical sources

6.6.3.1 Virulence factor genes

Our results revealed that 14% of clinical isolates carried *sfa/focDE* gene which is lower than the finding by Bashir *et al*., (2012) who reported that the prevalence *hlyA* (37%) was highest followed by *sfa/focDE* (27%), *papC* (24%), *cnf1* (20%), *eaeA* (19%) and *afaBC3* (14%). Hilali *et al*., (2000) reported that in blood isolates, 37% were positive for *papC* and in faecal isolates it was only 17%. Martinez-Medina *et al*., (2009b) reported that the *papGII* and *papGIII* alleles were the most frequent alleles found among ExPEC strains. In our study, *iutA* (67%) was dominated followed by *kpsMT II* (63%). Jaureguy *et al*., (2007) reported that *papAH* coding for P fimbriae was found to be associated with community-acquired (CA) rather than hospital-acquired (HA) isolates. Skjot-Rasmussen *et al*., (2012) reported that *papAH* associated with community-acquired (CA) than hospital-acquired (HA) isolates, and *kpsM II* and *hlyD* associated with HA rather than CA isolates. They also reported that *papAH* and *iss* were associated with females, and iroN with males. Sannes *et al*., (2004) reported that when group B2 was compared with all other phylogenetic groups combined, significant differences in

prevalence favouring group B2 were seen for most of the adhesins (multiple *pap* elements, *sfa/focDE, sfaS* [S fimbrial adhesin], *focG*, and *iha*), all of the toxins (*hlyD, cnf1,* and *cdtB*), all of the iron-acquisition factors (*iroN*, *fyuA*, *ireA*,and *iutA*), 2 capsule markers (*kpsM* II and K1 [K1 *kpsM* II variant]), and 4 miscellaneous VFs (*ibeA* [invasion of brain endothelium A], *ompT, malX*, and H7 *fliC* [flagellar antigen H7]).

Tarchouna *et al*., (2013) reported that the prevalence of genes coding for fimbrial adhesive systems was 68% for *fimH*, 41% for *pap*, and 34% for *sfa/focDE*. They also reported that the strains isolated from hospitalized patients displayed a great diversity of gene associations compared to those isolated from ambulatory patients. Our results revealed that 15% and 57% of isolates carried *papAH* and *papC* respectively. Among patients with acute pyelonephritis and cystitis, approximately 80% and 30%, respectively, possess P fimbriae (Donnenberg and Welch, 1996; Johnson, 1991). Moreover, an important role of *pap* adhesion genes in the pathophysiology of pyelonephritis caused by *E. coli* has been reported in several studies (Shohreh and Fatemeh, 2009; Westerbund *et al*., 1989). Johnson *et al*., (2005b) reported that among *E. coli* urine isolates from patients with cystitis, pyelonephritis, and prostatitis, the various virulence factors ranged in prevalence from 3% (*kpsMT* III [group 3 capsule]) to 95% (*fimH* [type 1 fimbriae]), with all except *papG* allele I being detected in one isolate each.

6.6.3.2 ExPEC and phylogenetic groups

Our results revealed that, most of the isolates belonged to B2, followed by A, D and B1. Predominance of B2 group among ExPEC has been reported (Martinez-Medina *et al*., 2009b; Moulin-Schouleur *et al*., 2007). Clinical

isolates of ExPEC typically belong to phylogenetic groups B2 and, at a lesser extend D (Picard *et al*. 1999). Johnson *et al*., (2003b) reported that in patients with invasive extra urinary infections (septic arthritis/pyomyositis, non-traumatic meningitis/hematogenous osteomyelitis, and pneumonia) had a single high-virulence phylogenetic group B2 strain resembling typical isolates causing urinary infection and/or sepsis. Chmielarczyk *et al*., (2013) reported that the mean number of genes was statistically significantly higher in the B2 group than in group D and A. They also reported that genes such as *ireA*, *sfa*, *fyuA*, *fepA*, *fhuA*, and *fimH* occurred more often in B2.

6.6.3.3 ExPEC and antibiotic resistance

Unlike the patterns seen in ExPEC from Cochin estuary and seafood, 98.73% of ExPEC isolates from clinical sources showed resistance to at least one antibiotic and 97.4% of isolates were resistant to more than two antibiotics. The level of antibiotic resistance encountered in our studies were much higher when compared to Skjot-Rasmussen *et al*., (2010) who reported that in *E. coli* isolates from patients with bacteraemia of urinary tract origin, 45% were fully antibiotic susceptible. The highest resistance rates were detected among community-acquired isolates, while the hospital-acquired isolates were the most susceptible. Soto *et al*., (2006) reported that uropathogenic *E. coli* strains exposed to sub-inhibitory concentrations of quinolones showed partial or total loss of PAIs (pathogenicity islands) containing virulence factor genes. The report by Diard *et al*., (2010) that ExPEC PAIs play an important role in intestinal colonisation and demonstration by Moreno *et al*., (2009) that group B2 and D tend to dominate faecal *E. coli* populations prior to extraintestinal infections, suggest the

presence of shared specific bacterial factors that contribute to fitness within the intestine. Virulence factor genes, such as *papAH*, *papC, kpsMT II* showed significant association with antibiotic resistance traits. Whereas *sfa/focDE* and *iutA* did not show any positive association with any antibiotic resistance traits. Antibiotic resistance genes such as, bla_{TEM}, *sul1*, *sul2*, *tetA* and *strA* were detected more frequently among clinical ExPEC isolates.

In conclusion, this study reports the presence of ExPEC strains in Cochin estuary. The presence of multiple virulence factor genes in *E. coli* strains may pose a health risk mainly to users who use water for recreational purpose. The significance of these strains in terms of health implications assessed by comparing strains from clinical and seafood isolates. The prevalence of ExPEC was significantly higher in clinical isolates than estuarine and seafood isolates. In addition, we analysed the prevalence of antibiotic resistance genes among ExPEC isolated from estuarine, seafood and clinical samples. Multidrug-resistant ExPEC, discharged into the estuary might persist in the environment and could be transmitted to humans and animals. There was considerable variation in the frequencies of virulence and antibiotic resistance genes in *E. coli* isolates from the different stations. Prevalence of virulence genes was significantly high in station 2 (Bolgatty), which is located close to Cochin city. In conclusion, the frequencies of multidrug-resistant ExPEC isolates found in estuarine water have become disturbingly high.

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Chapter 7

Genotyping of *Escherichia coli* isolates from estuarine, seafood and clinical sources using ERIC-PCR, RAPD and RFLP

7.1 Introduction

Genotyping is the process of determining differences in the genetic make-up of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence. Genotyping techniques are also becoming more useful for investigating sources of infection and routes of transmission in animals and humans. Enterobacterial repetitive intergenic consensus (ERIC) PCR relies on amplification of genomic DNA fragment using sets of primers complimentary to the short repetitive sequences. On the other hand, arbitrary developed primers can be used to amplify random DNA products under low-stringency PCR conditions. Typically, randomly designed 10-mer primers are used under conditions that allow some mismatches to increase the number of primed sites. PCR products are

produced when primer sites are situated within the amplification distance (less than 5 kb) and with the correct opposite orientation. The lengths of these products and the efficiency of annealing and thus amplification vary with the sites primed. Because RAPD reveals considerable polymorphism in genomic DNA, it has been extensively used as a genetic marker for estimating genetic, taxonomic and phylogenetic relationship in plants and animals. Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the PCR products with specific restriction endonucleases.

7.2 Review of literature

7.2.1 ERIC-PCR

Enterobacterial repetitive intergenic consensus (ERIC) sequences are the repetitive elements present in the family *Enterobacteriacae*. ERIC sequences are dispersed throughout the genome of *Enterobacteriacae* in different orientations. The discovery that prokaryotic genomes contain repeated sequences such as the enterobacterial repetitive intergenic consensus (ERIC) sequence (Hulton *et al*., 1991) has expanded the molecular biology tools that are available to assess the clonal variability of many bacterial isolates including *E. coli* (Chansiripornchai *et al*., 2001; Dalla-Costa *et al*., 1998; Versalovic *et al*., 1991). Several workers have reported the efficiency of ERIC-PCR for the genotyping of *E. coli* (Rodriguez *et al*., 2012; Tramuta *et al*., 2011; Wan *et al*., 2011; Prabu *et al*., 2010; Duan *et al*., 2009; Nicolas-Chanoine *et al*., 2008; Sabate *et al*., 2008; Casarez *et al*., 2007; da Silveira *et al*., 2002; Dalla-Costa *et al*., 1998; Lipmann *et al*., 1995) and other bacteria (Dijkman *et al*., 2012; Haitao *et al*., 2011; Nath *et al*., 2010; Chmielewski *et al*., 2002; Saxena *et al*., 2002; Rafiee *et al*., 2000; Zaher and Cimolai, 1998).

7.2.2 RAPD

Randomly amplified polymorphic DNA (RAPD) analysis (Williams *et al*., 1990) can be used to identify small sequence variations, which allows clones or strains of the same bacterial species to be differentiated (van Looveren *et al*., 1999). Under certain conditions, the RAPD assay can be used to detect DNA damage and mutations (Atienzar and Jha, 2006; Atienza *et al*., 2002). The RAPD method produces distinctive sets of DNA fragments when genomic DNA is subjected to PCR primed by short arbitrary oligonucleotide primers (Welsh and McClelland, 1990; Williams *et al*., 1990). Although RAPD has been strongly criticized for lack of reproducibility and its sensitivity to reaction conditions, it has been intensively used in the study of genomic diversity among plants (Shanmugam *et al*., 2005), animals (Spiridonava *et al*., 2005), parasites (Morozova *et al*., 2002), and bacterial species (Ertas and Seker, 2005; Byun *et al*., 2001). In addition, RAPD markers have also contributed to mapping projects and gene isolation (Wu *et al*., 2004; Atienza *et al*., 2002). It still has a major role to play when other techniques are not available (Williams *et al*., 1990). Previous workers have reported the use of RAPD for the genotyping of *E. coli* (Guo *et al*., 2013; Boczek *et al*., 2007; Haryani *et al*., 2007; Lin and Lin, 2007; Gomes *et al*., 2005; Leflon-Guibout *et al*., 2004; Chansiripornchai *et al*., 2001; Radu *et al*., 2001). RAPD-PCR methods can be used for the differentiation and characterization of *Salmonella* strains (Nath *et al*., 2010; Albufera *et al*., 2009; Lofstrom *et al*., 2006; Khoodoo *et al*., 2002; Mare *et al*., 2001; Shangkuan and Lin, 1998) and other bacteria (Adzitey *et al*., 2012; Venturi *et al*., 2012; Martinez *et al*., 2003; Gravesen *et al*., 2000; Shannon *et al*., 1998; Stephan, 1996; Lam *et al*., 1995).

7.2.3 RFLP

Restriction fragment length polymorphism (RFLP) has been used for years to analyze polymorphisms and is used in molecular biology to detect genomic sequences (Watanabe *et al*., 2003). Restriction endonucleases recognise specific 4-base (tetramer), 5-base (pentamer), or 6-base (hexamer) sites located on the DNA, and make double-stranded cuts. The sites are short enough that they can be found randomly in the DNA of any organism, including the organism that produces the restriction endonucleases. During restriction, the endonucleases must cut each of the strands to generate a double-strand cut. Cleavage is the result of hydrolysis, a reaction in which water is added across a bond, cleaving the two adjacent nucleotides (Maniatis *et al*., 1982; Davis *et al*., 1980). Previous workers have reported the efficiency of RFLP for the genotyping of *E. coli*, other bacteria (Pourahmad and Richards, 2013; Strydom *et al*., 2013; Marty *et al*., 2012; Morandi *et al*., 2010; Figueras *et al*., 2008; Mobius *et al*., 2006; Tamanai-Shacoori *et al*., 2006; Farnia *et al*., 2004; Atanassova *et al*., 2001; Studer *et al*., 1998), plant (Sarin *et al*., 2013; Wang *et al*., 2007) and animals (Thaenkham *et al*., 2011).

7.3 Objectives

1) To investigate the genetic relatedness among *E. coli* isolates of estuarine, seafood and clinical sources using ERIC PCR.

2) To investigate the genetic relatedness among *E. coli* isolates of estuarine, seafood and clinical sources using RAPD with random primer 1 and 2.

3) To investigate the genetic relatedness among *E. coli* isolates of estuarine, seafood and clinical sources by RFLP using *EcoR I*.

4) To investigate the genetic relatedness among *E. coli* isolates of estuarine, seafood and clinical sources by RFLP using *Hind III*.

7.4 Materials and Methods

7.4.1 Isolation of DNA from *E. coli*

DNA from the bacterial genome was extracted as described in 2.4.6.1.

7.4.2 PCR Amplification of 16S rRNA gene

The universal primers (forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CTTGT GCGGGCCCCCGTCAATTC-3') were used for the amplification of 16S rRNA. The optimized protocol was carried out with a PCR mix of 25 µl contained 2.5 mM $MgCl_2$, 2.5 µl of Taq buffer, 2.5 mM each of dNTP mixture, 1 pmol/µl of each of the primers, 1 U of Taq polymerase and 1 µl of the DNA template. PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation for 1 min at 94 °C, primer annealing for 30 sec at 55 °C and extention for 1 min at 72 °C, and a final extention at 72 °C for 10 min. PCR products were then electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized by Gel Documentation (BioRad Gel Doc^{TM} EZ Imager, USA).

7.4.3 Genotyping of *E. coli* isolates using ERIC-PCR

Genotyping was performed using the ERIC2 fingerprinting assay, which uses one 22 bp primer designed to the conserved ERIC region (Manges *et al*., 2001; Johnson and O'Bryan, 2000). The PCR amplifications were performed in 25 µl volumes containing 5 mM $MgCl_2$, 2 U of *Taq* polymerase, 0.4 mM deoxynucleoside triphosphates, 10 ng of crude template DNA, and 25 pmol of the ERIC2 primer (5′-AAGTAAGTGACTGGGGTGAGCG-3′). A negative control

consisting of the same reaction mixture without a DNA template was included in each PCR. PCR amplification included an initial denaturation at 94 °C, denaturation at 94 °C for 30 sec, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min and, after 35 cycles, a final extension for 4.5 min at 72 °C. PCR products were then electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by Gel Documentation (BioRad Gel Doc™ EZ Imager, USA).

7.4.4 Genotyping of *E. coli* isolates using RAPD

Randomly selected *E. coli* isolates were analyzed by RAPD (random amplified polymorphic DNA) typing using primer 1 and 2. Two random primers, primer 1 (5-CCGCAGCCAA) and primer 2 (5-AAGAGCCCGT), described by Regua-Mangia *et al*., (2008) were used separately in each PCR assay performed in a 50 µl final reaction volume containing 1x buffer (10 mM Tris-HCl [pH 8.8], 3 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 200 mM of each deoxynucleoside triphosphate, 25 pmol of primer, 2 U of DNA polymerase, and 3 µl of template. The amplification program was as follows: 4 cycles of 5 min at 94 °C, 5 min at 37 °C, and 5 min at 72 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 37 °C, and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. PCR products were then electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by Gel Documentation (BioRad Gel Doc™ EZ Imager, USA).

7.4.5 Genotyping of *E. coli* isolates using RFLP

After verification of PCR amplification of 16S rRNA by gel electrophoresis, 1 U of restriction endonuclease (*EcoR I* and *Hind III* separately) was mixed with 3 µl of amplicon and the final volume was made to 20 µl the diluting buffer

provided with the enzyme. The enzyme used to digest the PCR products of the 16S rRNA were *EcoR I* and *Hind III* (Sigma). The incubation temperature for both enzymes was 37 °C for 1h. Seven microlitre of the digested PCR product mixed with 1 µl of gel loading dye (Genei, India) was loaded into a well of the gel. In order to determine the size of bands visually, 5 µl of standard DNA size marker (100 bp and Supermix DNA ladder, Sigma) was added to two of the wells. The 2.5% gel was run at 70 V for 2.5 hours; the resulting digest was visualized by Gel Documentation system (BioRad Gel Doc™ EZ Imager, USA).

7.4.6 Cladogram construction

Gel images of the *E. coli* isolates, were analyzed using the GelQuest software package (Germany). Cladograms for cluster analysis of all the isolates were constructed by using SquentiX Cluster Vis software (version 1.8.1, Germany) by unweighted pairgroup method arithmetic mean (UPGMA) (Romesbrug, 1984).

7.5 Results

The result of the study revealed marked genetic variability among the tested *E. coli*, highlighted by three well-known typing techniques such as ERIC PCR, RAPD and RFLP.

7.5.1 Enterobacterial repetitive intergenic consensus (ERIC) PCR of *E. coli* isolates

The genome variability in the *E. coli* strains was reflected by ERIC PCR analysis. There were 3-12 bands with molecular weight ranging from 200 bp - 1.25 kb generated by ERIC primers (Figure 7.1 A - D).

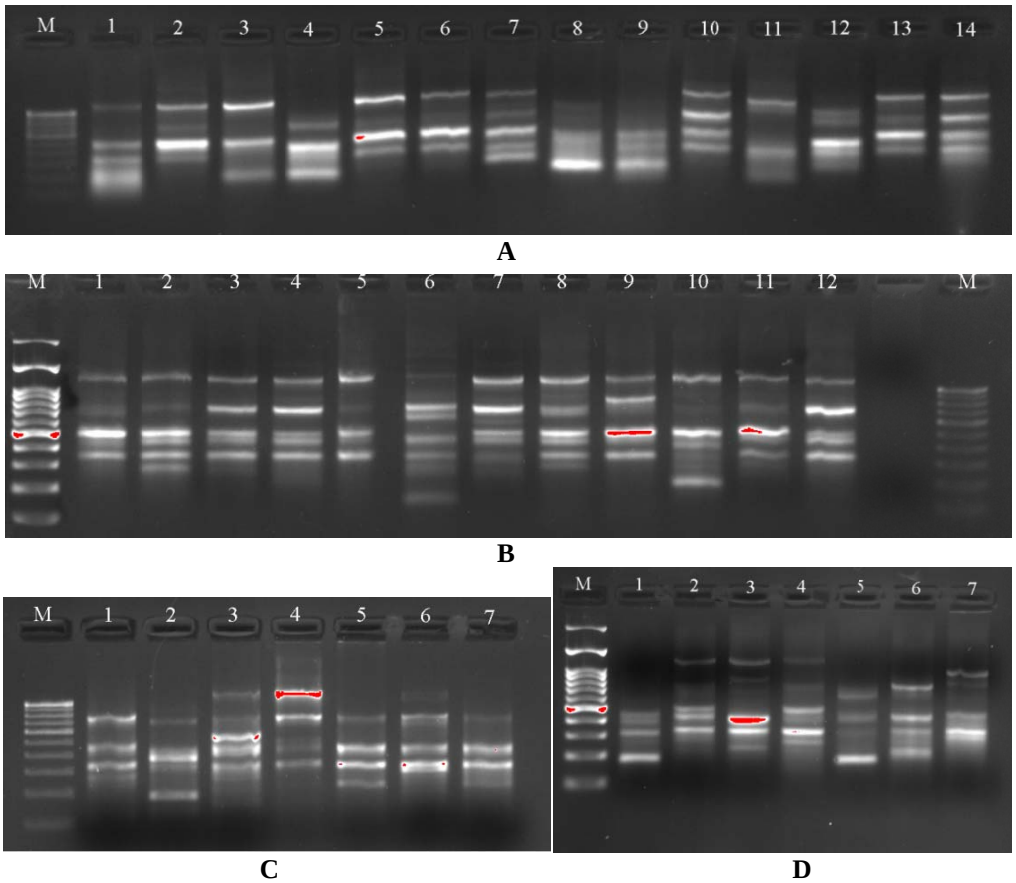


Figure 7.1 Gel image showing ERIC-PCR fingerprint of *E. coli* isolates. **A**: Lanes: M) 100 bp marker, 1) ES127[a]; 2) EC50[a]; 3) A49[a]; 4) A24[a]; 5)A36[a]; 6) A80[a]; 7) EFB2[b]; 8) N7[a]; 9) ES67[a]; 10) N16[a];11) EC81[a]; 12) N13[a]; 13) ES133[a]; 14) EFK5[b]. **B**: Lanes: M) 100 bp – 3kb marker, 1) EFB31[b]; 2) A53[a]; 3) A58[a]; 4) EF28 [b]; 5) EC6[a]; 6) ES11[a]; 7) EC37[a]; 8) EFB15[b]; 9) ES72[a]; 10) A51[a]; 11) EC68[a]; 12) B405[a]; M)100 bp. **C**: Lanes: M) 100 bp marker, 1) C13[c]; 2) EC24[a]; 3) EF23[b]; 4) EC21[a]; 5) C22[c]; 6) C83[c]; 7) C21[c]. **D**. Lanes: M) 100 bp – 3kb marker, 1) EF17[b]; 2) A15[a]; 3) C8[c]; 4) EF14[b]; 5) EF10[b]; 6) EF16[b]; 7) C6[c].

[a] Estuarine *E. coli* isolates; [b] Seafood *E. coli* isolates; [c] Clinical *E. coli* isolates

7.5.1.1 ERIC PCR of *E. coli* isolates from Cochin estuary

Randomly selected 68 strains of *E. coli* isolated over the period of three years were subjected to ERIC PCR. ERIC PCR also showed three major clusters (Figure 7.2). Cluster 1 could be further divided into three sub clusters: Ia, Ib and Ic having mixed strains of phylogenetic groups A, B1, B2 and D. Cluster 2 had 20 strains felled into two sub clusters. Phylogenetic group D strains were absent in cluster 2. Cluster 3 further divided into three sub clusters: 3a, 3b and 3c.

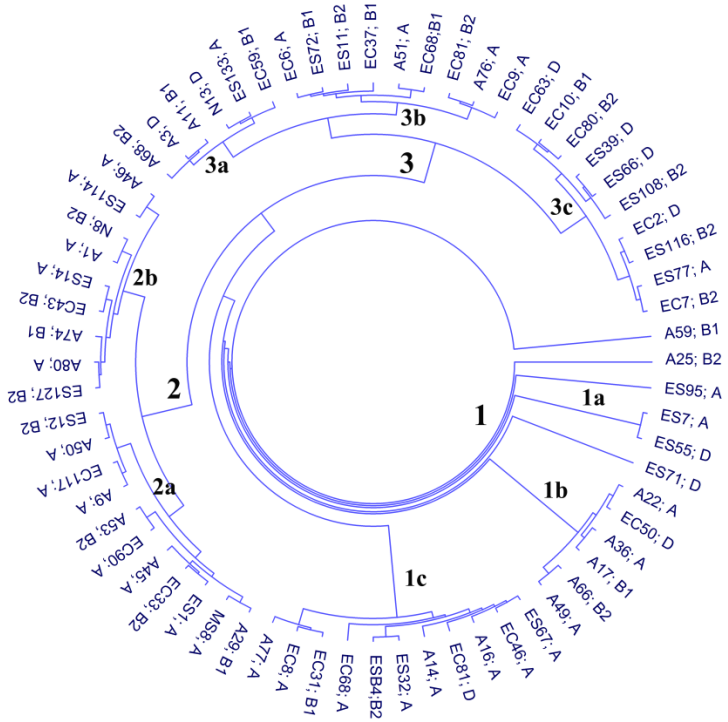


Figure 7.2 Circular cladogram of randomly selected 68 *E. coli* isolates from Cochin estuary, based on ERIC PCR.

7.5.1.2 ERIC PCR of *E. coli* isolates from seafood sources

Randomly selected 15 strains of *E. coli* isolates from seafood sources were subjected to ERIC PCR. ERIC PCR showed three major clusters (Figure 7.3). Cluster 1 represented by B2 and D strains. Cluster 2 represented by phylogenetic

group A strains only. Cluster 3 could be further divided into three sub clusters: 3a, 3b and 3c having mixed strains of phylogenetic groups A, B1, B2 and D. Phylogenetic group D strains were absent in cluster 2. Cluster 3 further divided into three sub clusters: 3a, 3b and 3c.

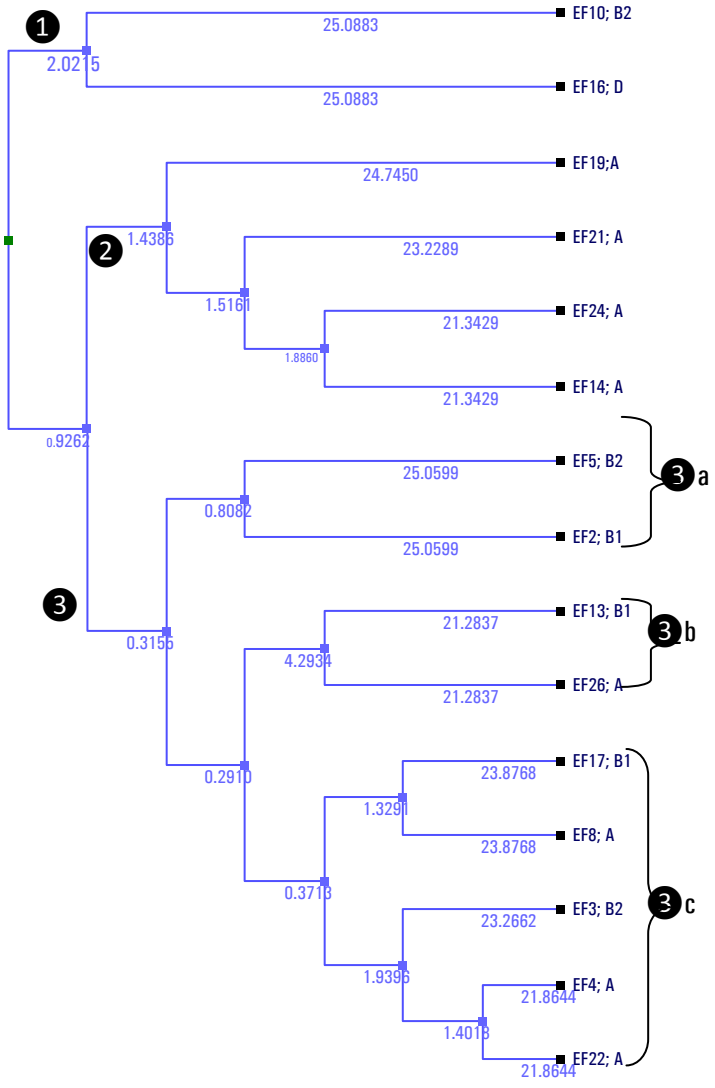


Figure 7.3 Rectangular cladogram of randomly selected 15 *E. coli* isolates from seafood sources based on ERIC PCR.

7.5.1.3 ERIC PCR of *E. coli* isolates from clinical sources

Randomly selected 23 strains of *E. coli* isolates from clinical sources were subjected to ERIC PCR. ERIC PCR showed three major clusters (Figure 7.4).

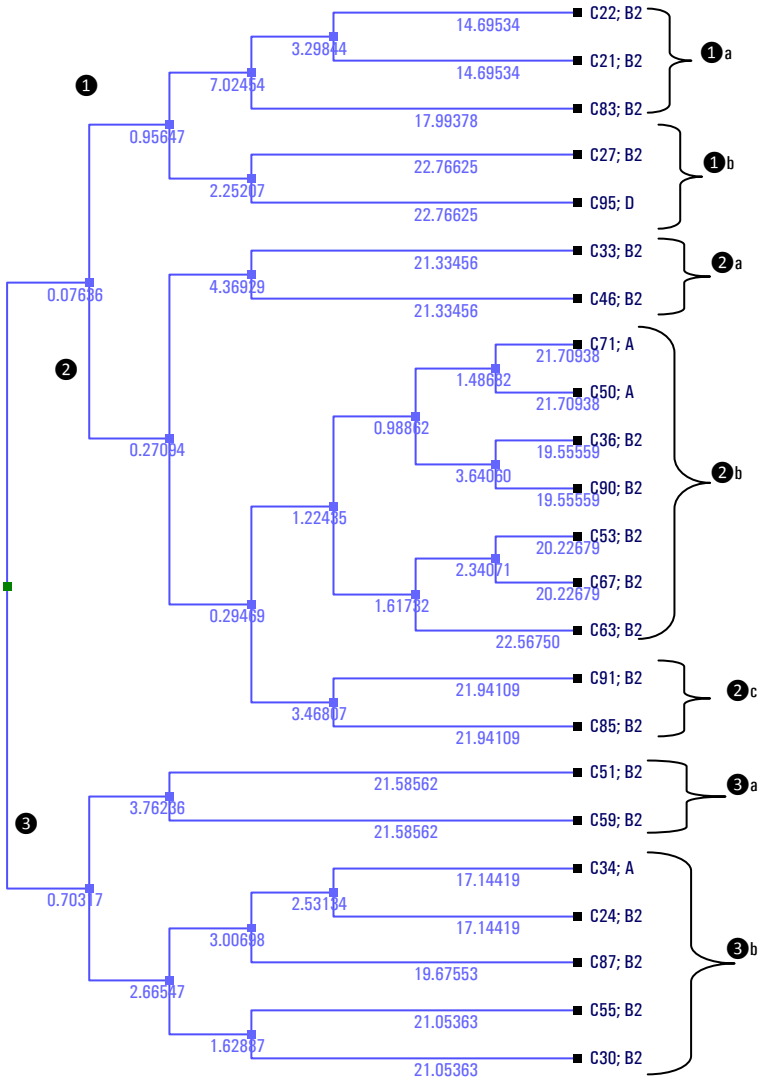


Figure 7.4 Rectangular cladogram of randomly selected 23 *E. coli* isolates from clinical sources based on ERIC PCR.

Cluster 1 could be further divided into two sub clusters: Ia having B2 strains only, and Ib having B2 and D strains. Cluster 2 divided into three sub clusters: 2a having B2 strains only, 2b having A and B2 strains, and 2c having B2 strains only. Cluster 3 further divided into two sub clusters: 3a having B2 strains and 3b having B2 and A strains.

7.5.1.4 A comparison: ERIC PCR of *E. coli* isolates from different sources (estuary, seafood and clinical)

Randomly selected 104 strains of *E. coli* isolates of different sources (estuary, n = 67; seafood n = 15, clinical n = 22) were subjected to ERIC PCR. There were 3-12 bands with molecular weight ranging from 200 bp - 1.25 kb generated by ERIC primers. ERIC PCR showed two major clusters (Figure 7.5).

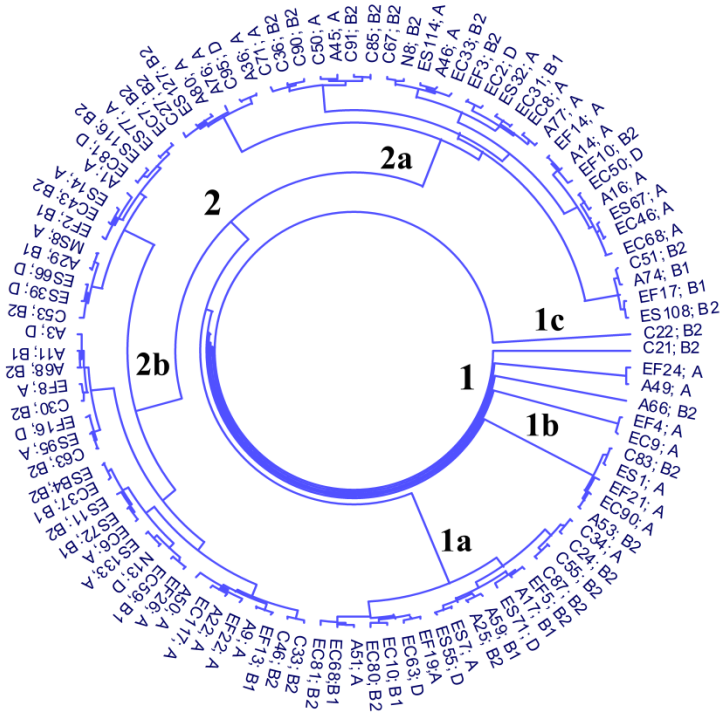


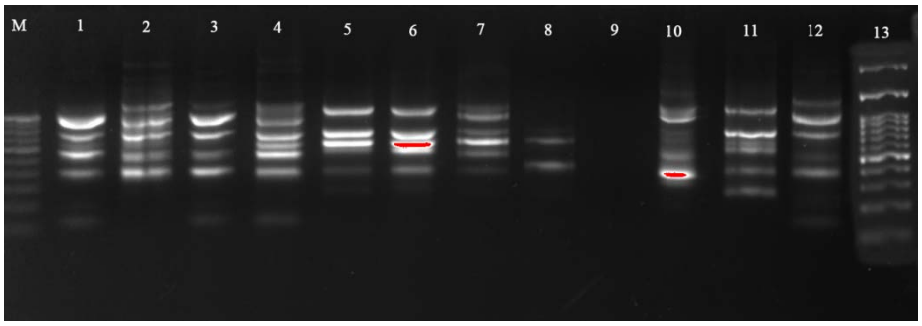
Figure 7.5 Circular cladogram of randomly selected *E. coli* isolates from different sources, based on ERIC PCR.

Cluster 1 could be further divided into three sub clusters: all three sub clusters were represented by mix population. Cluster 2 divided into two sub clusters; 2a and 2b also having mix population. Cluster 1 grouped by 7 clinical, 18 estuarine and 5 seafood strains. Cluster 2 comprised of 49 estuarine, 10 seafood and 15 clinical isolates.

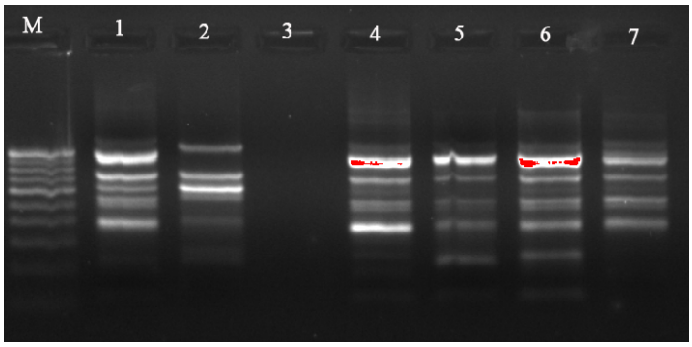
7.5.2 Randomly amplified polymorphic DNA (RAPD) analysis of *E. coli* isolates

7.5.2.1 RAPD analysis using primer 1

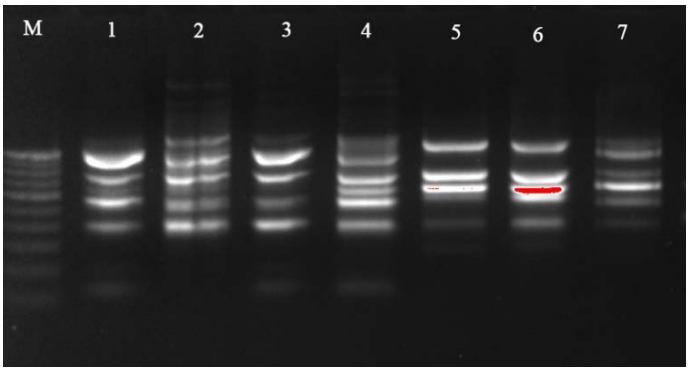
The genome variability in the *E. coli* strains was reflected by RAPD analysis. There were 2-12 bands with molecular weight ranging from 150 bp - 1.5 kb generated by random primer 1. (Figure 7.6 A - C).



A



B



C

Figure 7.6 Gel image showing RAPD fingerprint of *E. coli* isolates using primer 1. **A**: Lanes: M) 100 bp marker, 1) MS8[a]; 2) EC38[a]; 3) ES64[a]; 4) C24[c]; 5) C51[c]; 6) C33[c]; 7) C26[c]; 8) EF14[b]; 9) ES63[a]; 10) C52[c]; 11) EF19[b]; 12) EF8[b]; M) 100 bp - 3kb. **B**: Lanes: M) 100 bp marker, 1) ES10[a]; 2) EC37[a]; 3) A75[a]; 4) EF1[b]; 5) C6[c]; 6) A77[a]; 7) F4. **C**: Lanes: M) 100 bp marker, 1) ES57[a]; 2) EF20[b]; 3) C53[c]; 4) EF11[b]; 5) N8[a]; 6) ES9[a]; 7) C2[c].

[a] Estuarine *E. coli* isolates; [b] Seafood *E. coli* isolates; [c] Clinical *E. coli* isolates

7.5.2.1.1 RAPD of *E. coli* isolates from different sources using primer 1

Randomly selected 50 strains of *E. coli* isolated over the period of three years were subjected to RAPD. RAPD showed three major clusters (Figure 7.7). Cluster 1 could be further divided into five sub clusters: Ia represented by estuarine strain only. Id contained estuarine and seafood strains, whereas 1b, c and e carried mixed population. Cluster 2 felled into two sub clusters and group D strains were absent in cluster 2. Cluster 3 further divided into two sub clusters. Cluster 1 grouped by 3 clinical, 12 estuarine and 6 seafood strains. Cluster 2 comprised of 4 estuarine, 6 seafood and 8 clinical isolates. Cluster 3 represented by 4 estuarine, 5 seafood and 2 clinical strains.

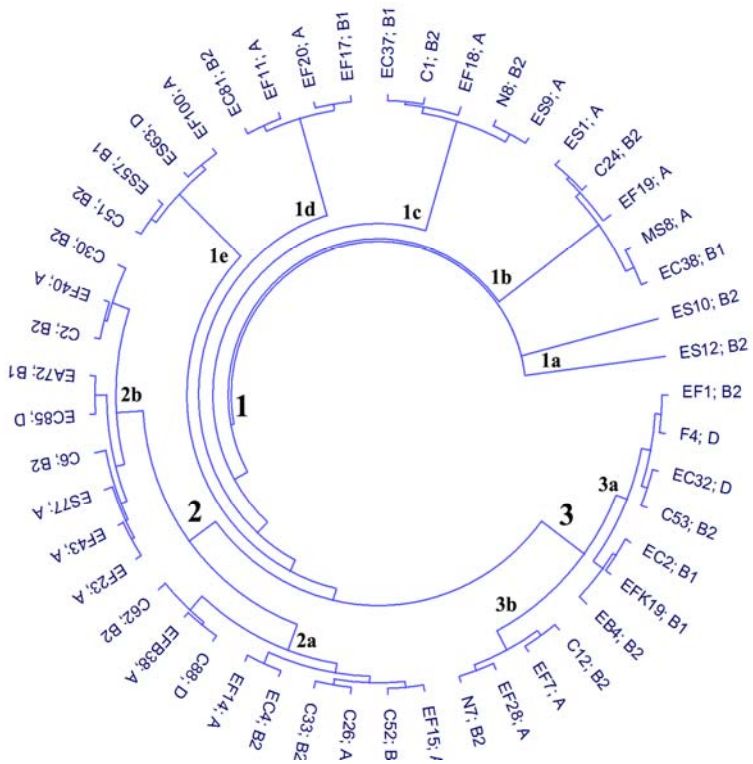
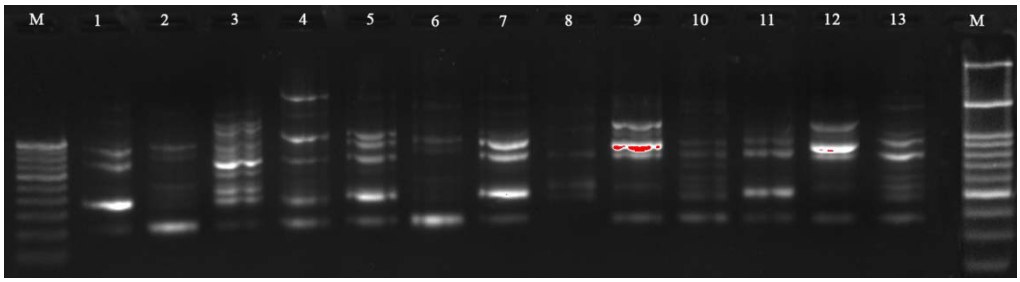


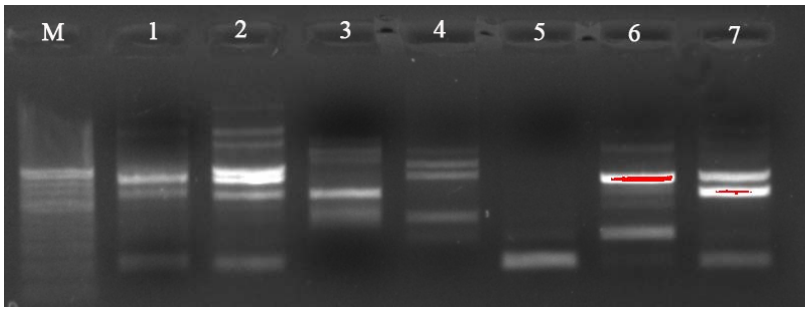
Figure 7.7 Circular cladogram of randomly selected *E. coli* isolates from different sources, based on RAPD using primer 1

7.5.2.2 RAPD analysis using primer 2

The genome variability in the *E. coli* strains was also reflected by RAPD analysis with primer 2. There were 2 12 bands with molecular weight ranging from 200 bp - 1.7 kb generated by random primer 2. (Figure 7.8 A - B).



A



B

Figure 7.8 Gel image showing RAPD fingerprint of *E. coli* isolates using primer 2. **A**: Lanes: M) 100 bp marker, 1) B405[a]; 2) ES63[a]; 3) C53[c]; 4) EF18[b]; 5) ES57[a]; 6) N13[a]; 7) ES77[a]; 8) ES1[a]; 9) EF8[b]; 10) ES69[a]; 11) N8[a]; 12) EF19[b]; 13) MS8[a]; M) 100 bp - 3kb marker. **B** : Lanes: M) 100 bp marker, 1) EF32[b]; 2) EC2[b]; 3) C2[c]; 4) EC85[a]; 5) EF2[b]; 6) A75[a]; 7) ES10[a].

[a] Estuarine *E. coli* isolates; [b] Seafood *E. coli* isolates; [c] Clinical *E. coli* isolates

7.5.2.2.1 RAPD of *E. coli* isolates from different sources using primer 2

Randomly selected 50 strains of *E. coli* isolated over the period of three years were subjected to RAPD with primer. RAPD showed three major clusters (Figure 7.9). Cluster 1 could be further divided into three sub clusters: Ia represented by estuarine and seafood strains. Sub cluster Ib contained estuarine isolates only, whereas 1c carried mixed population. Cluster 1 grouped by 1 clinical, 11 estuarine and 4 seafood strains. Cluster 2 felled into three sub clusters. Cluster 2 comprised of 5 estuarine, 3 seafood and 3 clinical isolates. Cluster 3 divided into three sub clusters. Cluster 3 represented by 12 estuarine, 3 seafood and 8 clinical strains.

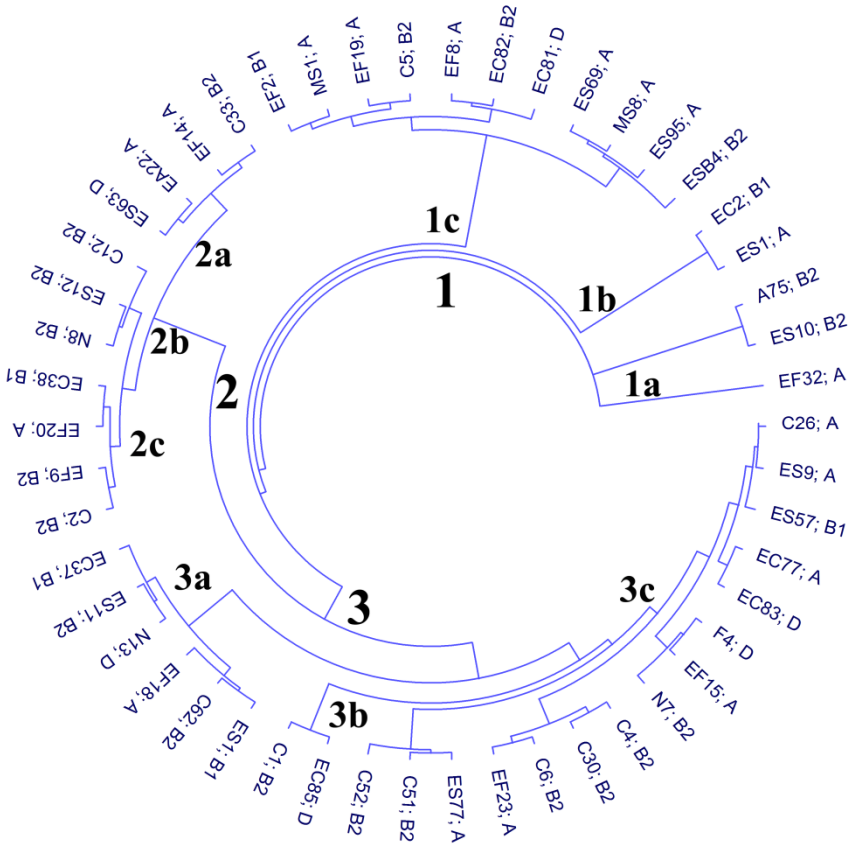
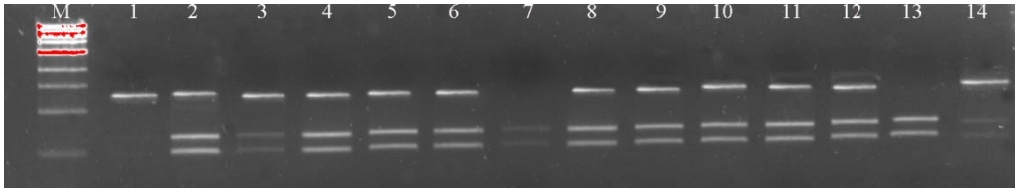


Figure 7.9 Circular cladogram of randomly selected *E. coli* isolates from different sources, based on RAPD using primer 2.

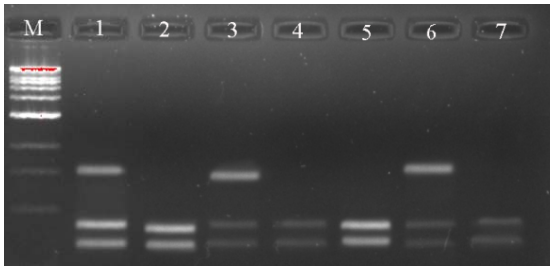
7.5.3 Restriction Fragment Length Polymorphism of *E. coli* isolates

7.5.3.1 RFLP using *EcoR I*

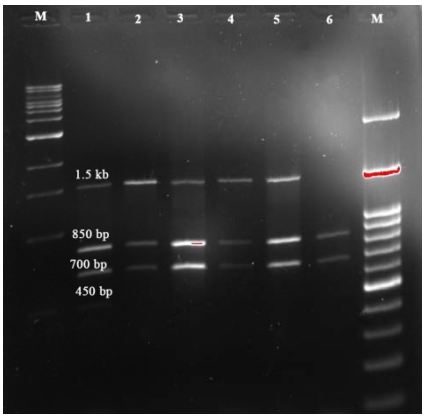
The digestive patterns obtained for restriction enzyme *EcoR I* with a random selection of *E. coli* are displayed in Figure 7.10 A - C. The sizes of the amplicons obtained were 450bp, 700 bp, 850bp, 900 bp and 1.5 kb.



A



B



C

Figure 7.10 The RFLP profiles for the PCR products of 16s rRNA of *E. coli* isolates using *EcoR I* enzyme. **A**: Lanes: M) 500 bp-33.5 kb Marker (Supermix DNA ladder); 1) ES69[a]; 2) N13[a]; 3) ES72[a]; 4) N8[a]; 5) ES65[a]; 6) ES67[a]; 7) ES63[a]; 8) ES75[a]; 9) MS9[a]; 10) F2[a]; 11) MS6[a]; 12) MS5[a]; 13) ES57[a]; 14) F3[a]. **B**: Lanes: M) Supermix DNA ladder; 1) A77[a]; 2) A17[a]; 3) A44[a]; 4) A54[a]; 5) A29[a]; 6) A64[a]; 7) A13[a]. **C**: Lanes: M) Supermix DNA ladder; 1) ES11[a]; 2) ES23[a]; 3) ES9[a]; 4) E135[a]; 5) ES1[a]; 6) C1[c]; M, 100 bp -3kb ladder.

[a] Estuarine *E. coli* isolates; [b] Seafood *E. coli* isolates; [c] Clinical *E. coli* isolates

7.5.3.1.1 RFLP of *E. coli* isolates from Cochin estuary using *EcoR I*

Randomly selected 68 strains of *E. coli* isolated from estuary over the period of three years were subjected to RFLP with enzyme *EcoR I*. RFLP showed two major clusters (Figure 7.11). Cluster 1 could be further divided into three sub clusters: 1a, represented by B2 and A strains.1b contained A, B1 and B2 strains, 1c represented by all phylogenetic groups. Cluster 2 felled into five sub clusters: 2a, represented by A and B2 strains, 2b contained A, B2 and D strains, 2c represented by all phylogenetic groups, 2d represented by A, B2 and D strains, where as 2e contained group A strains only.

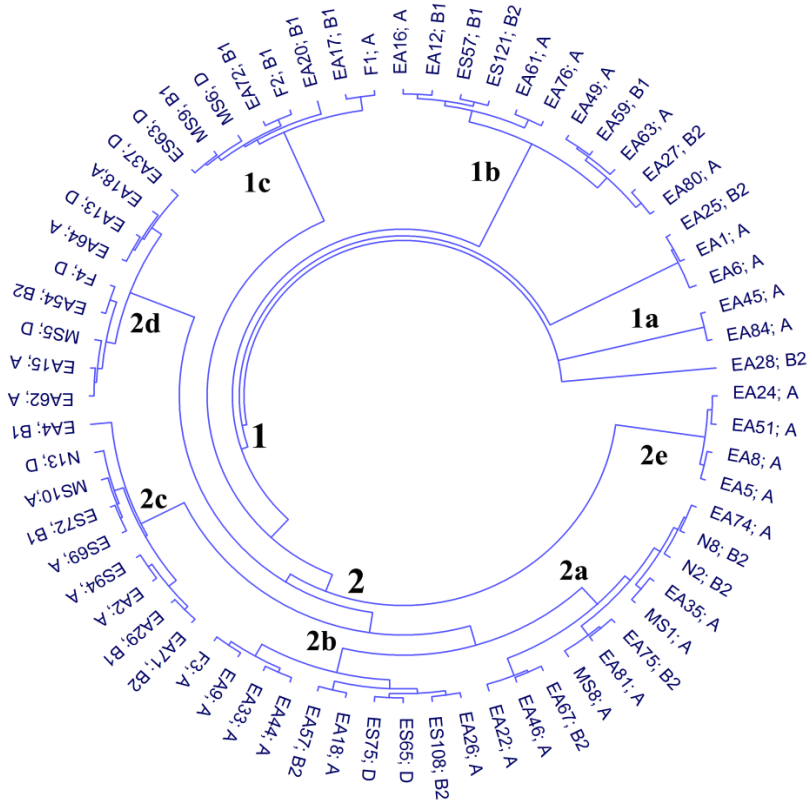


Figure 7.11 Circular cladogram of randomly selected 68 *E. coli* isolates from Cochin estuary, based on RFLP using enzyme *EcoR I*.

7.5.3.1.2 RFLP of *E. coli* isolates from seafood sources using *EcoR I*

Randomly selected 31 strains of *E. coli* isolates of seafood sources were subjected to RFLP analysis with enzyme *EcoR I*. RFLP showed two major clusters (Figure 7.12).

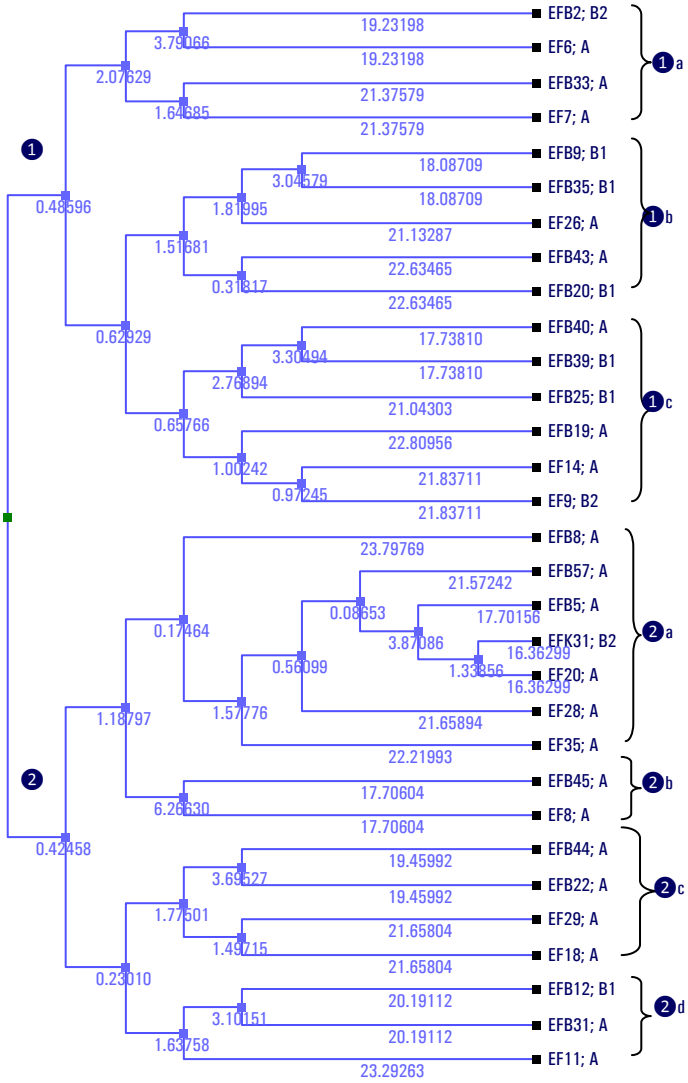


Figure 7.12 Rectangular cladogram of randomly selected 31 *E. coli* isolates from seafood sources based on RFLP using enzyme *EcoR I*.

Cluster 1 could be further divided into three sub clusters: 1a, represented by B2 and A strains, 1b contained A, and B1, 1c represented by A, B1 and B2 strains. Cluster 2 felled into four sub clusters. Sub cluster 2b and 2c represented by A strains only, where as 2d contained A and B1 strains. While sub cluster 2a represented by 6A strains and 1 B2 strain.

7.5.3.1.3 RFLP of *E. coli* isolates from clinical sources using *EcoR I*

Randomly selected 73 strains of *E. coli* isolates from clinical sources were subjected to RFLP with *EcoR I*. RFLP showed three major clusters (Figure 7.13). Cluster 1 could be further divided into two sub clusters: Ia represented by three A and one B2 strains, and Ib represented by B2 only. Cluster 2 divided into four sub clusters: 2a represented by four B2 and one A strains, 2b contained five B2 and one B1strains, and 2c and 2d contained B2 strains only. Cluster 3 further divided into three sub clusters: 3a and 3c represented by B2 strains only, whereas 3b contained one A and three B2 strains.

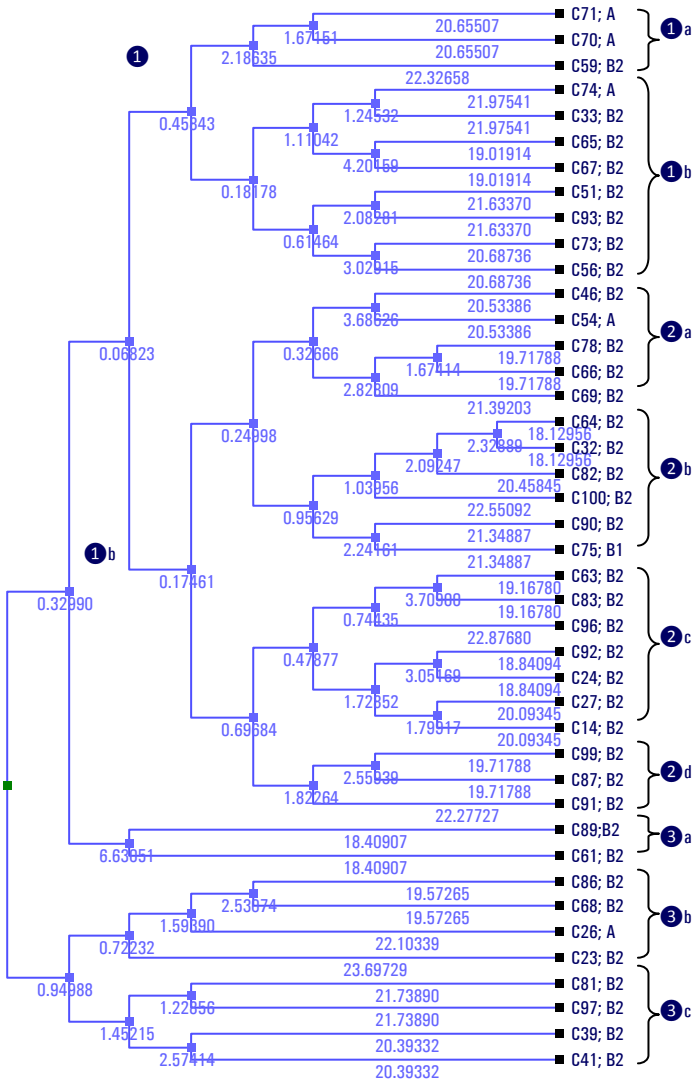


Figure 7.13 Rectangular cladogram of randomly selected 73 *E. coli* isolates from clinical sources based on RFLP using enzyme *EcoR I*.

7.5.3.1.4 A comparison: RFLP of *E. coli* isolates from different sources using *EcoR I* (estuary, seafood and clinical)

Randomly selected 147 strains of *E. coli* isolates from seafood sources were subjected to RFLP with *EcoR I*. RFLP showed five major clusters

(Figure 7.14). Cluster 1 grouped by 6 clinical, 3 estuarine and 5 seafood strains. Cluster 2 comprised of 31 estuarine, 8 seafood and 15 clinical isolates. Cluster 3 represented by 16 estuarine, 9 seafood and 10 clinical strains. Cluster 4 contained 11 estuarine, 7 clinical and 6 seafood strains. Cluster 5 grouped by 11 estuarine, 5 clinical and 4 seafood strains.

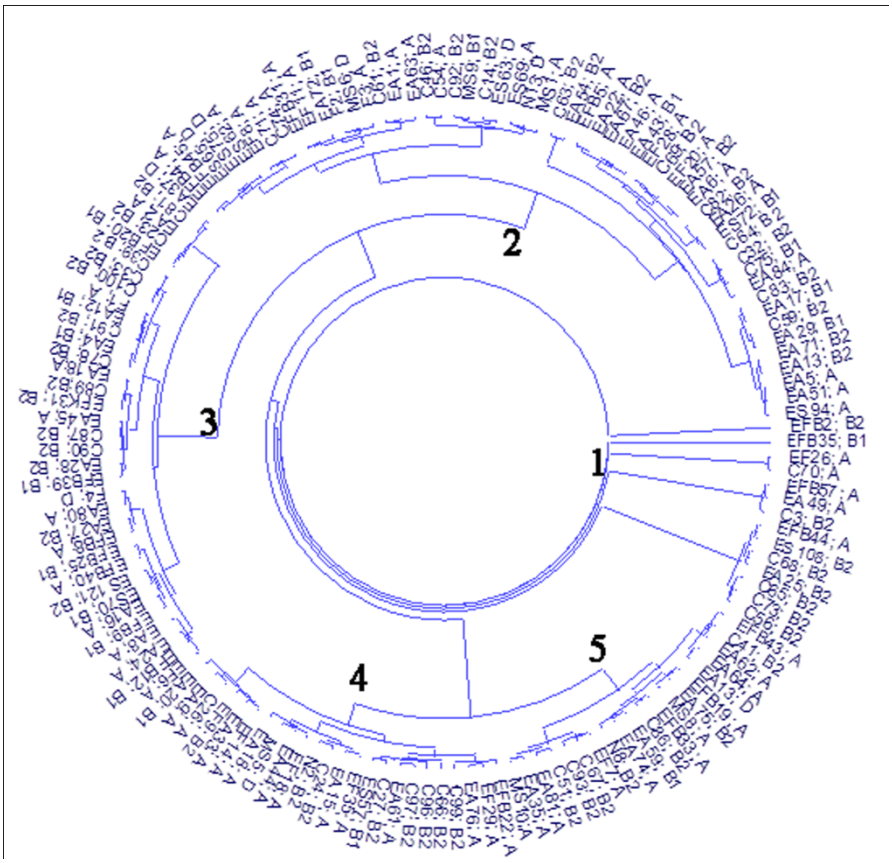
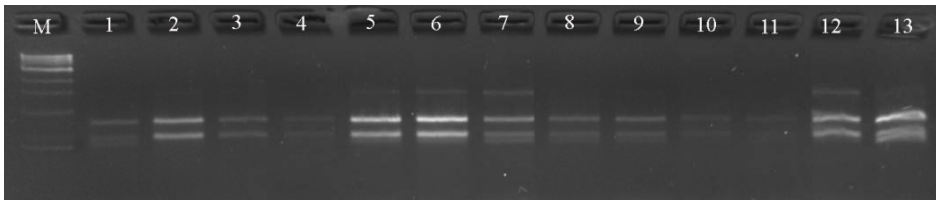


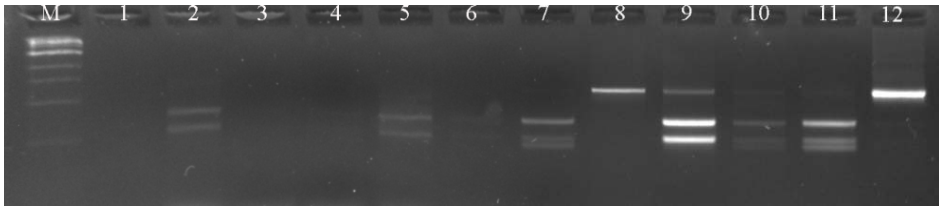
Figure 7.14 Circular cladogram of randomly selected 147 *E. coli* isolates from different sources, based on RFLP using enzyme *EcoR I*.

7.5.3.2 RFLP of *E. coli* isolates using *Hind III*

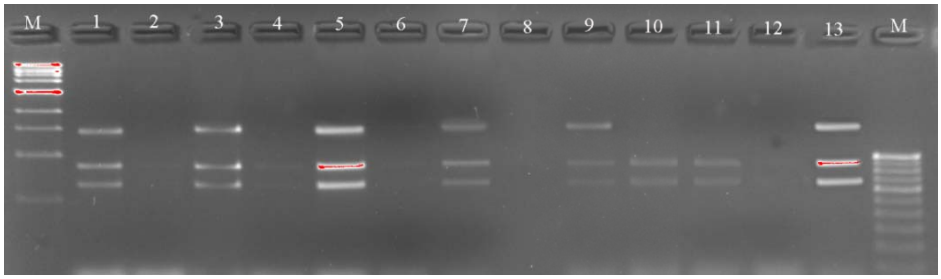
The digestive patterns obtained for restriction enzyme *Hind III* with a random selection of *E. coli* are displayed in Figure 7.15A - D. The sizes of the amplicons obtained were 500 bp, 600 bp, 650 bp, 700 bp, 900 bp and 1.5 kb.



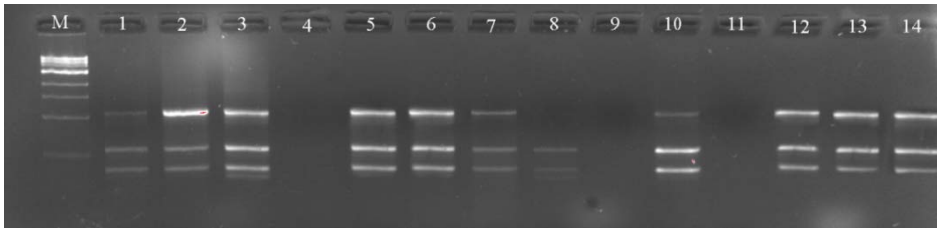
A



B



C



D

Figure 7.15 The RFLP profiles for the PCR products of 16s rRNA of *E. coli* isolates using *Hind III* enzyme. **A**: Lanes: M) Supermix DNA ladder; 1) EA4[a]; 2) EA29[a]; 3) EA35[a]; 4) EA37[a]; 5) EA51[a]; 6) EA52[a]; 7) EA65[a]; 8) EA67[a]; 9) EA68[a]; 10) EA74[a]; 11) EA84[a]; 12) C70[c]; 13) C71[c]. **B**: Lanes: M) Supermix DNA ladder; 2) EF19[b]; 5) EF8[b]; 7) EF35[b]; 8) EF37[b]; 9) EF8[b]; 10) EFK31[b]; 11) EFB2[b]; 12) EFB13[b]. **C**: Lanes: M) Supermix DNA ladder; 1) C33[c]; 3) C39[c]; 5) C41[c]; 7) C87[c]; 9) C45[c]; 10) C46[c]; 11) C27[c]; 13) C55[c]; M) 100 bp Marker. **D**: Lanes: M) Supermix DNA ladder; 1) EC9[a]; 2) EC90[a]; 3) EC7[a]; 5) EFK19[b]; 6) EFK5[b]; 7) C66[c]; 8) C60[c]; 10) C10[c]; 11) C68[c]; 12) F4[a]; 13) F3[a]; 14) M10[a]

[a] Estuarine *E. coli* isolates; [b] Seafood *E. coli* isolates; [c] Clinical *E. coli* isolates

7.5.3.2.1 RFLP of *E. coli* isolates from Cochin estuary using *Hind III*

Randomly selected 65 strains of *E. coli* isolated over the period of three years were subjected to RFLP with enzyme *Hind III*. RFLP analysis showed three major clusters (Figure 7.16). Cluster 1 could be further divided into three sub clusters: Ia represented by A, B1 and B2 strains, Ib represented by A and B1, and 1c represented by B1 and B2. Cluster 2 felled into three sub clusters: 2a and 2c represented by all phylogenetic groups, and 2b contained A, B1 and D. Cluster 3 divided into two clusters: 3a and 3b represented by various phylogenetic groups.

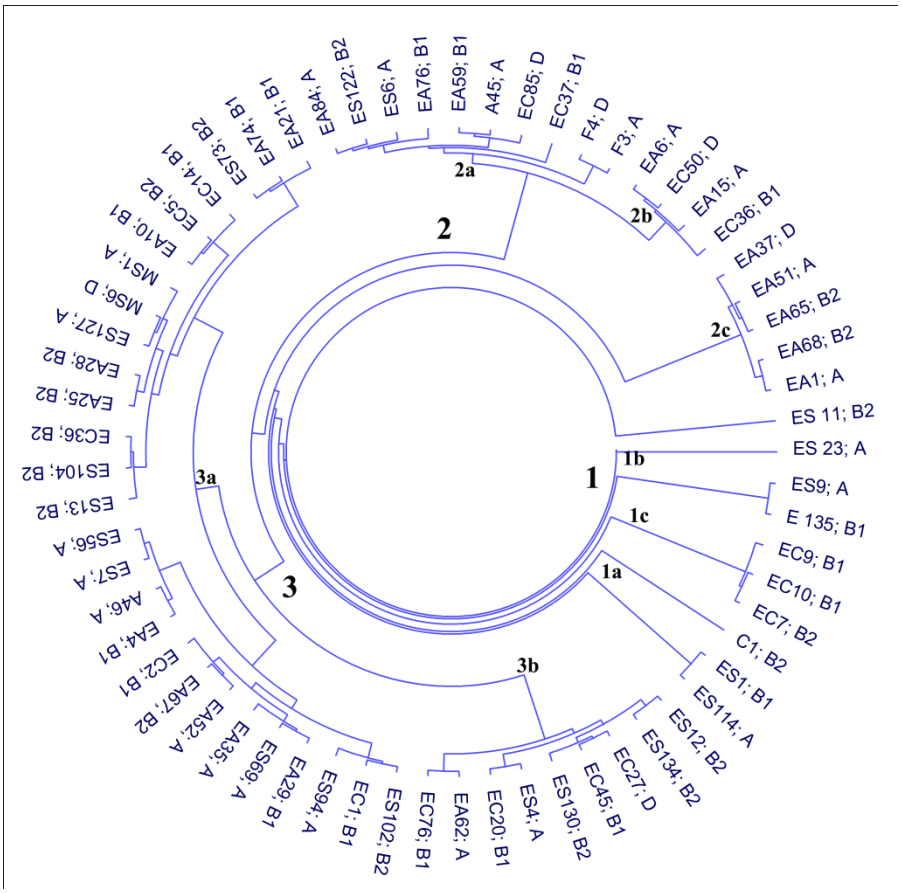


Figure 7.16 Circular cladogram of randomly selected 65 *E. coli* isolates from Cochin estuary, based on RFLP using enzyme *Hind III*.

7.5.3.2.2 RFLP of *E. coli* isolates from seafood sources using *Hind III*

Randomly selected 32 strains of *E. coli* isolates of seafood origin were subjected to RFLP with enzyme *Hind III*. RFLP analysis showed three major clusters (Figure 7.17).

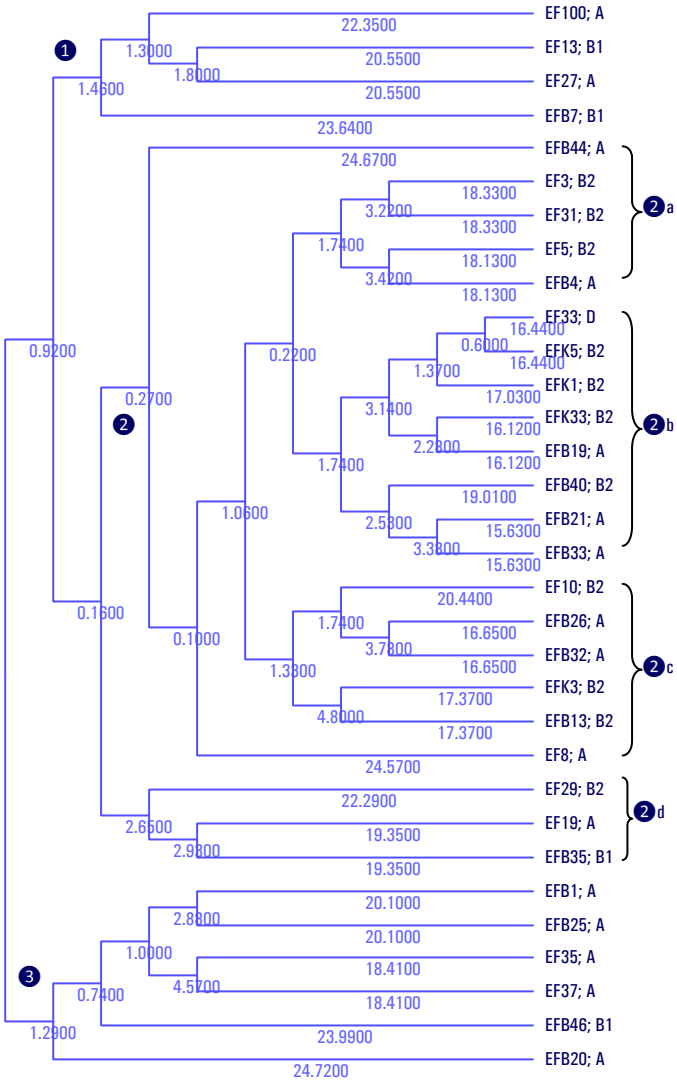


Figure 7.17 Rectangular cladogram of randomly selected 32 *E. coli* isolates from seafood sources based on RFLP using enzyme *Hind III*

Cluster 1 represented by A, and B1 strains. Cluster 2 felled into four sub clusters and represented by A, B1, B2 and D group strains. Cluster 3 contained one B1 and five A strains.

7.5.3.2.3 RFLP of *E. coli* isolates from clinical sources using *Hind III*

Randomly selected 47 strains of *E. coli* isolates of food origin were subjected to RFLP with *EcoR I*. RFLP showed three major clusters (Figure 7.18). Cluster 1 could be further divided into two sub clusters: 1a represented by B2 strains only, whereas Ib represented by one A, one B, and five B2 strains. Cluster 2 divided into four sub clusters: 2a and 2d represented by B2 strains only, 2b contained one B2, one B1 and three A strains, and 2c contained one A, two D and B2 strains. Cluster 3 further divided into two sub clusters: 3a and 3b represented by B2 strains only.

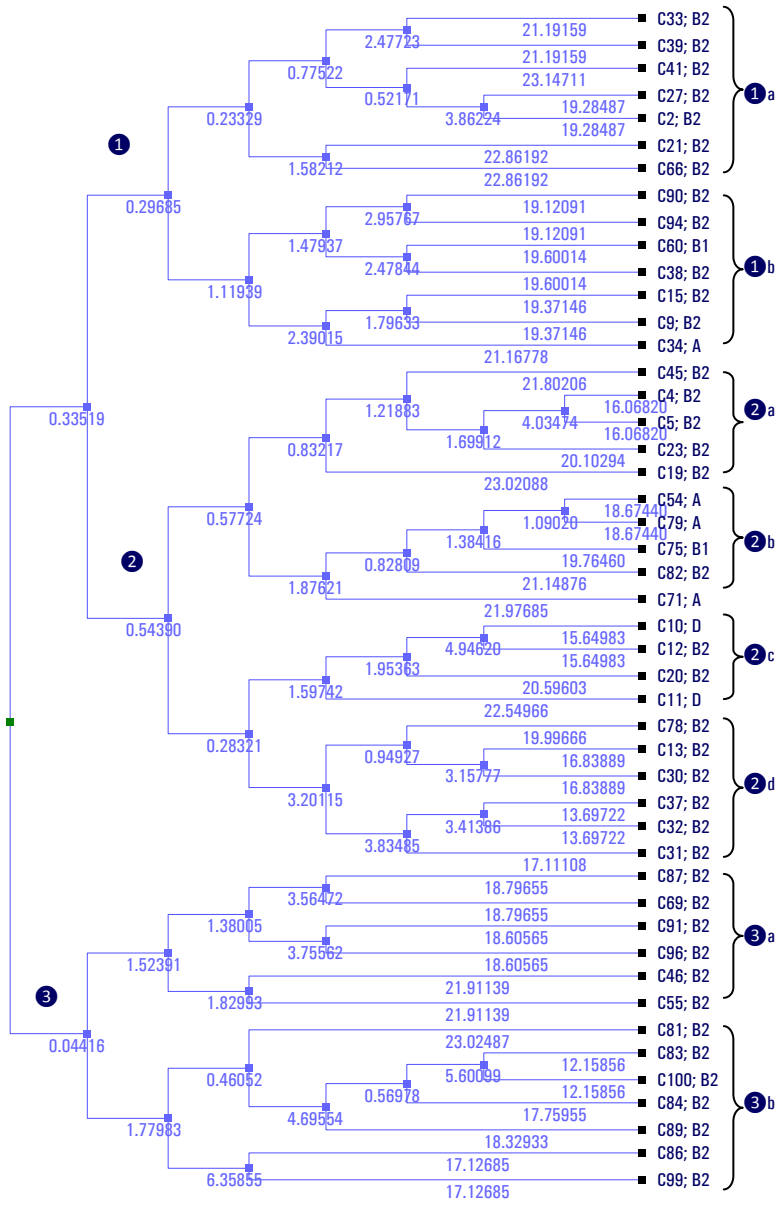


Figure 7.18 Rectangular cladogram of randomly selected 47 *E. coli* isolates from clinical sources based on RFLP using enzyme *Hind III*

7.5.3.2.4 A comparison: RFLP of *E. coli* isolates from different sources using *Hind III* (estuary, seafood and clinical)

Randomly selected 144 strains of *E. coli* isolates of different origin were subjected to RFLP with *Hind III*. RFLP showed four major clusters (Figure 7.19). Cluster 1 grouped by one clinical and five estuarine strains. Cluster 2 comprised of 16 estuarine, 4 seafood and 9 clinical isolates. Cluster 3 represented by 33 estuarine, 20 seafood and 19 clinical strains. Cluster 4 contained 10 estuarine, 19 clinical and 8 seafood strains.

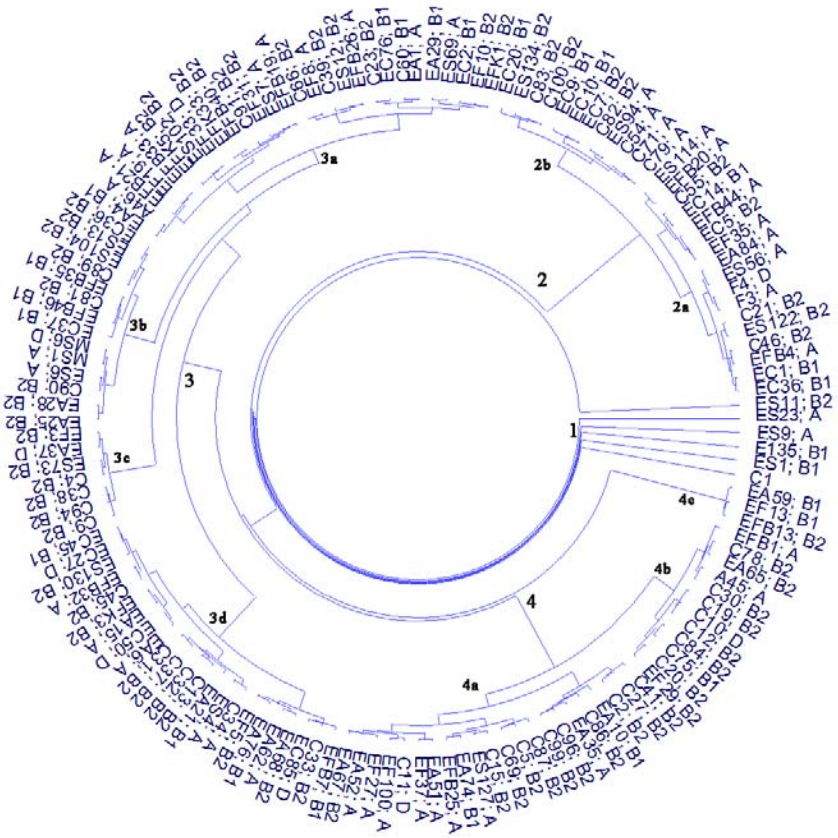


Figure 7.19 Circular cladogram of randomly selected 144 *E. coli* isolates from different sources, based on RFLP using enzyme *Hind III*.

7.6 Discussion

Compared to phenotypical methods, genotypical methods such as ERIC-PCR, RAPD and RFLP, have provided more rapid, reliable and cost-effective alternatives for detection and identification of clonal variation in *E. coli*.

7.6.1 ERIC PCR

7.6.1.1 ERIC PCR of *E. coli* isolates from Cochin estuary

ERIC-PCR analysis revealed high heterogeneity among the *E. coli* isolates. Cluster analysis indicated the presence of similar and different clones among *E. coli* isolates from Cochin estuary. *E. coli* of estuarine origin grouped into 3 clusters and clusters were represented by A, B1, B2 and D strains. Strains belonged to various phylogenetic groups clustered together in clusters 1 and 2, while cluster 3 did not contain phylogenetic group D isolates. From our genotyping analysis by ERIC-PCR, it was clear that within various phylogenetic groups of *E. coli* whether it is pathogenic group or not, there is a difference and similarity. It might be due to various genetic changes occurring within the isolates. In our analysis, we found out that some of the potent pathogenic strains which belonged either B2 or D, showed similarity with non-pathogenic strains of group A/B1. These non-pathogenic strains might have the ability to acquire virulence genes and might be converted to pathogenic groups in near future. By genotyping analysis using ERIC-PCR, we could identify the changes occurring in the isolates, which might pose risk to people.

We could not find a difference between pathogenic and non-pathogenic strains from estuarine isolates. Generally, genotypic methods have greater discriminatory power than phenotypic methods. However, combining a genotypic method with phylogenetic analysis usually results in greater

discriminatory power and more complete strain identification than genotyping alone. Similarly, combining two independent genetic methods can also improve discrimination. Gordon *et al*., (2002) found that the types of *E. coli* from human faeces and septic systems for two households differed. In another study it has been reported that, *E. coli* isolates from surface water tend to be more similar to each other than isolates from wastewater or animal faeces, suggesting that there may be selective survival of some strains in the environment (McLellan, 2004).

7.6.1.2 ERIC PCR of *E. coli* isolates from seafood sources

E. coli of seafood origin were clustered into 3 clusters and we could observe a clear difference between non pathogenic and pathogenic isolates. Genotyping analysis by ERIC-PCR of seafood *E. coli* isolates, revealed that within seafood isolates diversity is less when compared to those from estuarine source. Cluster 1 represented by pathogenic groups (B2 and D) only, whereas cluster 2 represented by non-pathogenic group (A) only. Only cluster 3 represents mixed population comprised of pathogenic and non pathogenic phylogenetic groups. da Silveira *et al*., (2002) suggested that ERIC–PCR has a better discriminating capacity and could substitute either RAPD–PCR or RFLP, which are claimed to show a lower level of genetic variability (Maurer *et al*., 1998). da Silveira *et al*., (2002) also suggested that molecular typing methods such as ERIC–PCR are particularly useful in discriminating between avian pathogenic and commensal nontypable *E. coli* isolates.

7.6.1.3 ERIC PCR of *E. coli* isolates from clinical sources

E. coli of clinical origin showed a marked difference in ERIC patterns and grouped into 3 clusters. Most of the B2 strains were clustered together,

while some of the B2 strains was clustered with non pathogenic A strains. This indicates that the non pathogenic isolates are potent enough to acquire the virulent factors or it is in the middle of such transition stage. Rodriguez *et al*., (2012) reported that the analysis on genetic diversity by ERIC-PCR demonstrated a clonal relationship among pathogenic *E. coli* carrying the same virulence factors and similar antibiotic resistance. Jerek *et al*., (1999) analyzed *L. monocytogenes* from different sources with ERIC-PCR and reported that there was a high similarity between one strains of *L. monocytogenes* isolated from chicken with strains isolated from human. The difference between pathogenic and non-pathogenic strains was obvious in *E. coli* isolates from clinical sources.

7.6.1.4 A comparison: ERIC PCR of *E. coli* isolates from different sources

ERIC PCR analysis of *E. coli* from different sources revealed two major clusters. Cluster 1 grouped by 7 clinical, 18 estuarine and 5 seafood strains. Cluster 2 comprised of 49 estuarine, 10 seafood and 15 clinical isolates. This result revealed the similarity between certain strains of *E. coli* from different sources. Our results indicate that there is a similarity between *E. coli* isolates from various sources. From our results it was clear that pathogen cycling is occurring through food, water and clinical sources. Estuarine environment is a melting pot for waste input, both organic and inorganic. Waste water from various sources such as houses, industries, hospitals and sewage are released into this water body and there is a possibility of *E. coli* from various sources thus offers a conducive environment for gene transfer. Opportunistic pathogens might acquire resistant and virulence genes. People who use this water for fishing and recreation might get infected and these environmental isolates could colonise the human body, possibly harbouring various virulence

genes. Food chain also plays a role in the transit of virulence genes from the environments to the human.

7.6.2 RAPD

As in the case of ERIC-PCR, RAPD analysis also revealed that there is a similarity between certain strains of *E. coli* isolates from various sources. This also underlines the occurrence of pathogen cycling within the system. RAPD of *E. coli* isolated from different origin using primer 1 showed that cluster 1 grouped by 3 clinical, 12 estuarine and 6 seafood strains. Cluster 2 comprised of 4 estuarine, 6 seafood and 8 clinical isolates. Cluster 3 represented by 4 estuarine, 5 seafood and 2 clinical strains. While RAPD of *E. coli* isolated from different sources using primer 2 revealed that cluster 1 grouped by 1 clinical, 11 estuarine and 4 seafood strains. Cluster 2 comprised of 5 estuarine, 3 seafood and 3 clinical isolates. Cluster 3 represented by 12 estuarine, 3 seafood and 8 clinical strains. Detection of pathogenic as well as non pathogenic (mixed population) isolates in a same cluster indicates that the genetic changes (mutation/rearrangements) are occurring amongst the strains belonging to the clone making them pathogenic or non-pathogenic.

RAPD has been shown to be one of the most discriminating methods for typing *Campylobacter* isolates (Nielsen *et al*., 2000; Madden *et al*., 1998). Nielsen *et al*., (2000) compared six typing methods for their ability to discriminate among *Campylobacter* spp. and found PFGE and RAPD to be the best. Chansiripornchai *et al*., (2001), who used RAPD–PCR and did not find differences between avian pathogenic and nonpathogenic *E. coli* isolates. Khoodoo *et al*., (2002) also tested 26 *Salmonella* isolates and found a lack of significant correlation between the RAPD profiles obtained and the serogroups of the isolates. However, in study by Albufera

et al., (2009) the RAPD-PCR could discriminate *Salmonella* isolates of the same serogroups by generating different profiles.

7.6.3 RFLP

7.6.3.1 RFLP of *E. coli* isolates from Cochin estuary

RFLP of *E. coli* isolates from Cochin estuary using *EcoR I* showed two major clusters. Clusters were represented by all phylogenetic groups. It is very difficult to explain why all clusters have all the phylogenetic group strains. Whereas RFLP of *E. coli* isolated from Cochin estuary using *Hind III* showed three major clusters. Cluster analysis indicated the presence of similar and different clones among *E. coli* isolates from Cochin estuary. As in the case of ERIC-PCR analysis, RFLP also revealed that within various phylogenetic groups of *E. coli* whether it is pathogenic group or not, there is a difference and similarity. This strongly indicates that estuarine isolates are more diverse and gene transfer is constantly occurring in estuarine environment.

7.6.3.2 RFLP of *E. coli* isolates from seafood sources

RFLP of *E. coli* isolates of seafood origin using *EcoR I* showed two major clusters while RFLP using *Hind III* showed three major clusters. Most of the strains belonged to non pathogenic groups such A and B1 clustered together. But Some B2 strains also clustered with non-pathogenetic groups. RFLP analysis also showed a similar result as that of ERIC-PCR of *E. coli* from seafood sources.

7.6.3.3 RFLP of *E. coli* isolates from clinical sources

RFLP of *E. coli* isolates from clinical sources using *EcoR I* as well as *Hind IIII* showed three major clusters. In both cases, most of the B2 strains were grouped together to form many clusters. While some of the strains

belonged to A and B1 groups also clustered with pathogenic groups. RFLP analysis also showed a similar result as that of ERIC-PCR of *E. coli* from clinical sources.

7.6.3.4 A comparison: RFLP of *E. coli* isolates from different sources

As in the case of ERIC-PCR and RAPD, RFLP analysis also revealed that there is a similarity between *E. coli* isolates from various sources. This also underlines the occurrence of pathogen cycling among various sources. RFLP using *EcoR I* showed five major clusters whereas RFLP using *Hind III* showed four major clusters. All clusters were comprised of mixed population.

The results of the genotyping of *E. coli* isolates from estuary, seafood and clinical samples by ERIC PCR, RAPD and RFLP revealed the similarity of some of the strains from these three sources. Though the antibiotic resistance analysis (antibiotic resistance and genes responsible for resistance) as well as analysis of virulence genes among the *E. coli* from these sources also pointed to some sharing of characteristics among these strains, genotyping conclusively revealed the pathogen cycling through these three sources. Considering the rich fish and shellfish resources the estuary has and the large number of people using the estuary for recreation, it is of utmost importance to prevent the release of potentially pathogenic *E. coli* into the natural water body from diverse sources.

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Chapter 8

Characterization of a multidrug-resistant shiga toxin-producing ExPEC strain (CUSMBES11)

8.1 Introduction

E. coli are natural habitants of the digestive tract of humans and animals. However, certain strains can be pathogenic for humans (Kaper *et al*., 2004). Pathogenic *E. coli* can be categorized as intestinal pathogenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). Among the intestinal pathogenic *E. coli*, shiga toxin-producing *E. coli* (STEC) are responsible for severe clinical symptoms, such as hemorrhagic colitis and the potentially lethal hemolytic uremic syndrome (Karmali *et al*., 2010, 1989; Paton and Paton, 2002, 1998; Nataro and Kaper, 1998). ExPEC strains have the ability to cause extraintestinal infections such as urinary tract infections, neonatal meningitis, sepsis, and wound infections which can lead to serious

complications and death (Wiles *et al*., 2008; Ron, 2006; Bonacorsi and Bingen, 2005).

The escalating problem of emergence of antibiotic resistant bacteria and their resistant genes is becoming a major global health issue (Levy, 2002; Chee-Sanford *et al*., 2001). Links have been found to exist between antibiotic use and the emergence of antibiotic resistant bacterial pathogens (Peak *et al*., 2007; Levy, 2002; Seveno *et al*., 2002; Aminov *et al*., 2001). Studies have proven increase in antibiotic resistance of strains that belong to pathogenic bacteria (Blasco *et al*., 2008) and over the years, nearly every bacterial pathogen has developed resistance to one or more clinical antibiotics (Todar, 2008).

Here we characterized an *E. coli* strain ES11 isolated from surface water of Cochin estuary and screened for genes conferring resistance to clinically important antibiotics (third generation cephalosporins, tetracyclines, aminoglycosides, phenicol, trimethoprim and sulphonamides) and integrons.

8.2 Review of literature

Antibiotic resistance genes can be studied by real time polymerase reaction (qPCR), which is rapid, effective, and handy tool, compared to conventional methods that are used in microbiological research (Martinez *et al*., 2007). Real time PCR technology has allowed the development of a quick and reliable alternative to traditional methods for detection of various genes. Multiplex assays can amplify a group of 3-5 genes simultaneously with conventional PCR followed by time-consuming agarose gel electrophoresis

analysis (Strommenger *et al*., 2003; Ng *et al*., 2001; Warsa *et al*., 1996; Roberts *et al*., 1993). The development of a multiplex PCR-based assay significantly saves time and labour compared to individual PCR assays (Son *et al*., 2014; Obeng *et al*., 2012; Vidal *et al*., 2005). However, this method is still labour intensive in that it requires conventional PCR and gel electrophoresis to identify positive samples. Using the intercalating fluorescence dye SYBR Green I and the Lightcycler system, the accumulation of amplicons in the reaction can be monitored over time. SYBR Green I obviates the need to examine PCR products on time-consuming agarose gels. After PCR amplification, the Light Cycler continuously monitors the decrease of fluorescence resulting from the release of SYBR Green I during DNA melting point analysis by slowly increasing the temperature (Fan *et al*., 2007). The real-time PCR reaction is considerably faster than conventional PCR and there is less potential for false positive results due to cross-contamination between experiments since no post-PCR steps are required.

The fluorogenic real-time PCR assay has been widely used in molecular diagnosis to identify pathogens and bacteria in the environment; it provided comparable sensitivity and superior reproducibility, precision and shorter performance time when compared to previous methods (Panicker *et al*., 2004; Hernandez *et al*., 2003; Jothikumar *et al*., 2003). This fluorescence-based real-time assay not only can quickly identify target genes independently but also allows for multiple PCR reactions in one tube by employing the unique melting curve analysis following DNA amplification. Real-time PCR was also reported for the diagnosis and quantification of *tet* genes (Yu *et al*., 2005; Maeda *et al*., 2003). Singleplex qPCR using SYBR

green detection has previously been used to detect certain antibiotic resistance genes in environmental samples, most frequently identified in water or waste water samples (Walsh *et al*., 2011a; Volkmann *et al*., 2007, 2004; Borjesson *et al*., 2009; Knapp *et al*., 2008; Koike *et al*., 2007; Pruden *et al*., 2006; Smith *et al*., 2004). Brolund *et al*., (2010) developed a real time SYBR Green PCR assay for rapid detection of acquired *AmpC* in *Enterobacteriaceae*.

Guarddon *et al*., (2011) used real time polymerase reaction for the quantitative detection of *tetA* and *tetB* bacterial tetracycline resistance genes in food. There are many authors who used Taqman-based qPCR in order to detect and/or quantify a particular organism from different foods (Pennacchia *et al*., 2009; Elizaquivel *et al*., 2008; Fernandez *et al*., 2006). Fan *et al*., (2007) developed a multiplex, real-time PCR assay using SYBR Green for detection of tetracycline efflux genes of Gram-negative bacteria. Grape *et al*., (2007) used real-time multiplex PCR methods for detection of trimethoprim resistance *dhfr* genes in large collections of bacteria. Birkett *et al*., (2007) used real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum β-lactamases. Real-time PCR technology has been successfully applied to the detection of *E. coli* O157:H7 in faeces, food and water samples (Fitzmaurice *et al*., 2004; Belanger *et al*., 2002; Ibekwe *et al*., 2002; Reischl *et al*., 2002; Sharma, 2002; Bellin *et al*., 2001; Fortin *et al*., 2001). Hardwick *et al*., (2008) quantified the *class 1 integrons* from natural environments using real-time quantitative PCR.

8.3 Objectives

1) To assess the presence of genes that encode for known STEC virulence factors, namely *stx1*, *stx2* by real-time PCR.
2) To investigate the distribution of various antibiotic resistance genes by Real-time PCR.
3) To evaluate the distribution of various virulence factor genes by Real-Time PCR.

8.4 Materials and Methods

8.4.1 Source and serotype of the *E. coli*.

Water samples were collected from station 2 (Bolgatty) during the study period. Samples were further analysed as described in 2.4.1.

8.4.2 Antibiotic susceptibility testing

Antibiotic susceptibility of *E. coli* isolate (ES11) was determined as described in 4.4.1. The antibiotics tested in this study are given in Table 8.1.

8.4.3 MAR indexing

Multiple antibiotic resistances (MAR) indexing of the isolate were done as described in 4.4.2.

Table 8.1 List of antibiotics used, abbreviation and their concentration tested.

Sl.No	Name of the antibiotic	Abbreviation	concentration
1	Amikacin	Ak	30 µg
2	Amoxyclave	Amc	30 µg
3	Amoxycillin	Amx	30 µg
4	Ampicillin	Amp	10 µg
5	Carbenicillin	Cb	100 µg
6	Cefepime	Cpm	30 µg
7	Cefotaxime	Ctx	30 µg
8	Cefoxitin	Cx	30 µg
9	Cefpodoxime	Cpd	10 µg
10	Ceftazidime	Caz	30 µg
11	Ceftriaxone	Ctr	30 µg
12	Cefuroxime	Cxm	30 µg
13	Cephalothin	Cep	30 µg
14	Chloramphenicol	C	30 µg
15	Ciprofloxacin	Cip	5 µg
16	Colistin	Cl	10 µg
17	Co-Trimoxazole	Co	25 µg
18	Doxycycline Hydrochloride	Do	30 µg
19	Erythromycin	E	15 µg
20	Gatifloxacin	Gat	5 µg
21	Gentamicin	Gen	10 µg
22	Kanamycin	K	30 µg
23	Levofloxacin	Le	5 µg
24	Nalidixic acid	Na	30 µg
25	Neomycin	N	30 µg
26	Netillin	Net	30 µg
27	Nitrofurantoin	Nf	300 µg
28	Novobiocin	Nv	30 µg
29	Rifampicin	Rf	5 µg
30	Streptomycin	S	10 µg
31	Sulphamethizole	Sm	300 µg
32	Sulphafurazole	Sf	300 µg
33	Tetracycline	Te	30 µg
34	Trimethoprim	Tr	5 µg
35	Vancomycin	Va	30 µg

8.4.4 Isolation of DNA from *E. coli*

DNA from the bacterial genome was extracted as described in 2.4.6.1.

8.4.5 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was extracted as described in 5.4.2.

8.4.6 Phylogenetic analysis

The phylogenetic group was determined as described in 3.4.2. The primers used in this study are given in Table 8.2.

Table 8.2 Details of primers used for phylogenetic analysis

Genes investigated	Primer sequence (5′ - 3′)		Reference
	Forward	reverse	
ChuA	gacgaaccaacggtcaggat	tgccgccagtaccaaagaca	Clermont *et al*., (2000)
YjaA	tgaagtgtcaggagacgctg	atggagaatgcgttcctcaac	Clermont *et al*., (2000)
TspE4C2	gagtaatgtcggggcattca	cgcgccaacaaagtattacg	Clermont *et al*., (2000)

8.4.7 PCR amplification of 16S rRNA gene

Amplification of 16S rRNA was carried out as described in 7.4.2.

8.4.8 DNA sequencing

The 16S rRNA amplified product was used for the sequencing with the 16S rRNA Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CTTGT GCGGGCCCCCGTCAATTC-3' by ABI DNA sequencer. A comparison of the 16S rRNA gene sequence of the isolate with the non-reductant collection (Genbank, EMBL) of sequences was performed using BLAST.The accession number of the strain submitted to NCBI GenBank is JX183942.1.

8.4.9 RNA isolation

Bacterial cultures were inoculated into Luria Bertani broth (HiMedia, India) and incubated in an orbital shaker incubator (Orbitek, India) at 37 °C, 110 rpm for 12 hours. After incubation the cells were harvested by centrifugation at 15000 × g for 10 minutes. Bacteria were lysed by passing the cell pellets several times through a pipette. Two hundred microlitre of chloroform per 1 ml of trizol reagent (Ambion, Life Technologies) were then added and the tube was vortexed for 15 sec. After centrifugation for 15 min at 12,000 × g, the aqueous phase was collected and processed for RNA extraction. RNA from the aqueous phase was precipitated with 500 ml of isopropanol per ml trizol. Samples were incubated 10 min at room temperature and RNA was pelleted by centrifugation for 10 min at 12,000 × g. RNA pellets were then washed with 75% ethanol per 1 ml of trizol, and centrifuged at 7500 × g for 10 min. RNA pellets were air-dried for 10 min and resuspended in 100 ml of DEPC (Diethylpyrocarbonate) treated water (Ambion, Life Technologies).

8.4.10 RNA concentration

Purified RNA was diluted (1/10) in DEPC-treated water (Ambion, Life technologies) and OD 260/280 readings were obtained using a spectrophotometer (Nano Drop Spectrophotometer). Samples with an OD 260/280 ratio of 1.8 or greater and a minimum concentration of 0.5 mg/ml were used for subsequent analyses.

8.4.11 cDNA synthesis

RNA was converted to cDNA using the Superscript RT III (Invitrogen, Life Technologies) reverse transcriptase. Reverse transcription was set-up using 5 µg of RNA, 1 µl random hexamer, 10mM dNTP and DEPC water to

10 µl. Mixture was heated at 65 °C min for 5 min and incubated on ice for 1min. The contents of the tube was collected by brief centrifugation and 4 µl 5X first strand buffer, 1 µl 0.1 M DTT, 1 µl RNase OUT, and 1 µl superscript III RT (200 units/ µl) was added to the tube. The mixture was incubated at 50 °C for 30-60 minutes and inactivated the reaction by heating at 70 °C for 15 minutes. cDNA was then diluted by addition of 20 µl of DEPC-treated water (Ambion, Life Technologies).

8.4.12 Real time PCR

qPCR assays were performed on a Biorad cycler Real Time PCR Detection System (Bio-Rad, USA) with SYBR Green I detection. Sample was measured in quandary duplicates in a 96-well plate (Bio-Rad, USA). Real-time PCR mastermix for a 10 µl reaction was prepared by mixing; 5 µl 2x Power SYBR Green mastermix (Applied Biosystems, Life Technologies), 1 µl of each primer (100 µM), 0.5 µl DEPC water and 2.5 µl template. The thermal cycling conditions were: 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C, 30 sec), annealing (53 °C, 30 sec), extension (72 °C, 45 sec) and final extension (72 °C, 10 min). Primers used in the real-time PCR reactions are listed in Table 8.3, 8.4 and 8. 5. External positive and negative controls were included in all real-time PCR reactions. After real-time data acquisition, the cycle threshold (C_t) value was calculated by determining the point at which fluorescence exceeds an arbitrary threshold signal. The C_t value is predictive of the quantity of target gene copies in the PCR sample.

Table 8.3 Primers for house-keeping genes used in Real Time PCR

Genes investigated	Primer sequence (5′ - 3′)		Reference
	Forward	reverse	
uidA	aaaacggcaagaaaaagcag	acgcgtggttacagcttgcg	Bej *et al*., (1991)
frr	gcgtagaagcgttcaaaacc	caagatcggacgccataatc	Liu *et al*., (2005)

Table 8.4 Primers for antibiotic resistance genes used in Real Time PCR

Genes investigated	Primer sequence (5′ - 3′)		Reference
	Forward	reverse	
Betalactams			
bla_{TEM}	gagtattcaacattttcgt	accaatgcttaatcagtga	Marynard *et al*., (2003)
$bla_{CTX\text{-}M}$	cgatgtgcagtaccagtaa	ttagtgaccagaatcagcgg	Batchelor *et al*., (2005)
Tetracycline			
tet(A)	gtgaaacccaacatacccc	gaaggcaagcaggatgtag	Marynard *et al*., (2003)
tet(B)	ccttatcatgccagtcttgc	actgccgtttttttcgcc	Marynard *et al*., (2003)
Gentamicin			
aphA2	gaacaagatggattgcacgc	gctcttcagcaatatcacgg	Marynard *et al*., (2003)
Streptomycin			
str A	cctggtgataacggcaattc	ccaatcgcagatagaaggc	Rosengren *et al*., (2009)
Trimethoprim			
dhfrIa	gtgaaactatcactaatgg	ttaacccttttgccagattt	Navia *et al*., (2003)
dhfrVII	ttgaaaatttcattgattg	ttagccttttttccaaatct	Navia *et al*., (2003)
Sulphonamide			
sul1	ttcggcattctgaatctcac	atgatctaaccctcggtctc	Marynard *et al*., (2003)
sul2	cggcatcgtcaacataacc	gtgtgcggatgaagtcag	Marynard *et al*. (2003)
Phenicol			
catI	agttgctcaatgtacctataacc	ttgtaattcattaagcattctgcc	Marynard *et al*., (2003)
Integrons 1, 2 & 3			
int1	cctcccgcacgatgatc	tccacgcatcgtcaggc	Kraft *et al*., (1986)
int2	ttattgctgggattaggc	acggctaccctctgttatc	Goldstein *et al*., (2001)
int3	agtgggtggcgaatgagtg	tgttcttgtatcggcaggtg	Goldstein *et al*., (2001)
Integron variable regions			
int1 CS	ggcatccaagcagcaag	aagcagacttgacctga	Levesque *et al*., (1995).
In 2CS	cgggatcccggacggcatgca cgatttgta	gatgccatcgcaagtacgag	White *et al*., (2001)

Table 8.5 Primers for virulence genes used in Real Time PCR

Genes investigated	Primer sequence (5′ - 3′)		Reference
	Forward	reverse	
Virulence factors genes			
papAH	atggcagtggtgtcttttggtg	cgtcccaccatacgtgctcttc	Johnson and Stell, (2000)
papC	gtggcagtatgagtaatgaccgtta	atatcctttctgcagggatgcaata	Johnson and Stell, (2000)
sfa/focDE	ctccggagaactgggtgcatcttac	cggaggagtaattacaaacctggca	Johnson and Stell, (2000)
iutA	ggctggacatcatgggaactgg	cgtcgggaacgggtagaatcg	Johnson and Stell, (2000)
kpsMT II	gcgcatttgctgatactgttg	catccagacgataagcatgagca	Johnson and Stell, (2000)
Shiga toxin-producing genes			
stx1	cgctgaatgtcattcgctctgc	cgtggtatagctactgtcacc	Blanco *et al*., (2003)
*stx*2	cttcggtatcctattcccgg	ctgctgtgacagtgacaaaacgc	Blanco *et al*., (2003)

8.4.13 Statistical analysis

Statistical analysis was performed using SPSS software 13 (Statistical Package for the Social Science) and significance level was set at $\alpha = 0.05$. One-Sample Kolmogorov-Smirnov test was applied to test the normal distribution in this study.

8.5 Results

8.5.1 Serotype

Strain was identified as *E. coli* by 16S rRNA sequencing. However, serotyping could not be done as it was found to be rough.

8.5.2 Antibiotic resistance

The strain ES11 was found to be resistant to the all class of antibiotics tested. MAR index was 1.

8.5.3 Phylogenetic group

Strain ES11 belonged to B2 phylogenetic group and it carried *TspE4C2*, *chu*A and *yjaA* genes.

8.5.4 Real time PCR

Two house-keeping genes, seven virulence genes and fourteen antibiotic resistance genes were individually and successfully amplified from *E. coli* strain ES11. The C_t values (threshold cycles) for these genes are given in Table 8.6.

Table 8.6 C_t (threshold cycles) of genes detected for strain CUSMBES11

Genes screened	1	2	3	4
uidA	28.65	29.66	23.19	25.66
frr	22.28	19.59	20.96	22.46
papAH	24.2	23.54	24.8	24.25
papC	27.21	27	29.07	27.51
sfa/focDE	23.59	25.22	24.65	26.4
iutA	26.23	25.74	29.67	29.41
kpsMT II	25.63	24.13	24.34	23.74
stx1	29.99	28.94	26.89	26.71
bla_{TEM}	25.5	24.04	25.71	25.66
bla_{CTXM}	28.35	28.01	27.41	27.21
sul1	25.89	24.05	26.63	26.67
sul2	29.09	29.01	27.52	27.41
tetA	29.23	27.37	27.75	28.74
tetB	25.27	25.67	26.25	24.57
strA	29.17	27.76	26.85	26.67
aphA2	29.97	29.96	29.5	29.42
cat1	28.39	28.49	27.64	28.29
dhfrIa	27.88	28.1	28.01	26.96
dhfrVII	26.96	28.69	25.26	23.17
class 1 integrons	25.77	26.03	27.54	29
int1	28.09	28.73	26.82	27.05
int3	26.67	27.15	26.79	26.69

8.5.5 Antibiotic resistance genes

The strain ES11 carried all the resistance genes analysed. β-lactam resistance was mediated by genes bla_{TEM} and $bla_{CTX\text{-}M}$. Tetracycline resistance was associated with *tetA* and *tetB*. Sulphonamide resistance was associated with *sul1* and *sul2*. Trimethoprim resistance genes *dhfrIa* and *dhfrVII* were also found. Streptomycin resistance and gentamicin resistance was mediated by *strA* and *aphA2*, respectively. Chloramphenicol resistance gene *cat I* was also detected in strain ES11. These results showed that the strain carried *class 1 integrons* and *class 3 integrons* which possessed one or more inserted genes, suggesting the presence of multiresistance integrons in these strain. Amplicons with the size of 1.6 Kb, 500 bp, 250 bp, 150 bp was obtained for *int 1 CS* (Integrons variable regions). While this strain did not contain class 2 integrons. There was no significant difference ($p > 0.05$) in the Ct value for antibiotic resistance genes. It showed a normal distribution.

8.5.6 House-keeping genes

There was no significant difference ($p > 0.05$) in the C_t value for house-keeping genes. It showed a normal distribution.

8.5.7 Virulence factor genes

The strain ES11 harboured virulence factor genes such as, *papAH* and *papC* (P fimbriae major structural subunit and assembly), *sfa/focDE* (S and F1C fimbriae), *iutA* (aerobactin receptor) and *kpsMT II* (group II capsule). There was no significant difference ($p > 0.05$) in the Ct value for virulence genes. It showed a normal distribution.

8.5.8 Shiga toxin-producing genes

The strain carried shiga toxin- producing gene *stx1*. While *stx2* gene was absent in the strain.

8.6 Discussion

The choice of *frr* as house-keeping gene standard in real time PCR was based on results from Liu *et al*., (2005). Additionally, Chouikha *et al*., (2008) also investigated other microarray datasets dealing with the transcriptome of *E. coli* under different growth conditions such as aerobic/anaerobic growth, biofilm formation, heat-shock and acid-shock: under all these conditions they found that *frr* was the least variable gene. *E. coli* β-glucourodinase gene was also used as house-keeping gene.

The serotyping result showed that strain ES11 was rough hence not typable. A specific combination of O and H antigens defines the serotype of an isolate. The O antigen, as part of the LPS in the outer membrane of Gram-negative bacteria, is a major target of both the immune system and bacteriophages, and plays an important role in the bacterium-host interplay. It is one of the most variable cell constituents and also plays an important role in virulence (Feng *et al*., 2004). Although, serotyping plays an important role in the characterization of pathogenic *E. coli*, there are many limitations in conventional serogrouping for it to be used as a regular diagnostic or epidemiological tool (Gordon, 2010). The transition of *E. coli* from smooth (S) to rough (R) form is another challenging phenomenon in serotyping of *E. coli*. The R forms do not synthesise O-antigen due to specific mutations in genes within the *rfb* gene cluster which encodes the O-antigen. As a result, the R strains cannot be typed using conventional serological methods (Orskov and

Orskov, 1978). The strain ES 11 was identified as *E. coli* by sequencing the 16S RNA gene.

The isolate belonged to pathogenetic phylogenetic group B2. Strains belong to phylogenetic group B2 carry the greatest number of virulence factors and followed by those in group D, whereas most commensal strains belong to groups A and B1 (Moreno *et al*., 2008; Johnson *et al*., 2005b; Moreno *et al*., 2005; Sannes *et al*., 2004; Johnson *et al*., 2002a; Johnson and Stell, 2000).

The strain ES11 was resistant to all class of antibiotics tested. Tang *et al*., (2011) reported that the most common resistance pattern observed among the *E. coli* isolates was multiresistance to Amp + Kan +S + Neo + Gen + Cip + Ofx + Te + Tr + Sdm. Several reports have been published on antibiotic resistance among *E. coli* isolated from rivers (Korzeniewska *et al*., 2013; Tacao *et al*., 2013; Koczura *et al*., 2012; Su *et al*., 2012; Tacao *et al*., 2012; Garcia-Aljaro *et al*., 2009; Olaniran *et al*., 2009; Hu *et al*., 2008; Nazir *et al*., 2005; Goni-Urriza *et al*., 2000). Previous studies have reported that *E. coli* isolates from drinking water (Coleman *et al*., 2013; Talukdar *et al*., 2013; Sahoo *et al*., 2012; Mataseje *et al*., 2009; Ozgumus *et al*., 2007, Gaur *et al*., 1992) can harbour resistance determinants to many classes of antibiotic agents. Several studies been reported on antibiotic resistance characterization of *E. coli* isolated from spring water (Wicki *et al*., 2011; Ozgumus *et al*., 2007), waste water (Moura *et al*., 2012; Sabate *et al*., 2008), and irrigation water (Roe *et al*., 2003).

The strain ES11 carried all the antibiotic resistance genes and virulence factor genes tested. In consistent with our study, Diallo *et al*., (2013) reported co-occurrence of extra intestinal virulence genes and antibiotic resistance

genes in *E. coli* isolated from waste water. But the co-occurrence of antibiotic resistance genes was high in our isolate. Obeng *et al*., (2012) also reported co-occurrence of extraintestinal virulence genes and antibiotic resistance genes in *E. coli* isolated from the faeces of intensively farmed and free range poultry. The distribution found in their study was *iutA* + *kpsMT II* + *papC* + *tet(B)* + *tet(C)* + *sulII* + *aphA1* + *aadA2*. In our study the exhibited distribution was *papAH* + *papC* + *sfa/focDE* + *iutA* + *kpsMT II* + bla_{TEM} + bla_{CTX-M} + *sul1*+ *sul2* + *tetA* + *tetB* + *strA* + *aphA2* + *cat1* + *dhfrIa* + *dhfrVII* + *class 1 integrons* + *int1* + *int3*. Gerjets *et al*., (2011) reported the co-occurrence of virulence factor genes in *E. coli* isolates from cows with coliform mastitis and healthy cows. Mora *et al*., (2013) reported the co-occurrence of different virulent factor gene in multidrug *E. coli* isolates from poultry. Martinez-Medina *et al*., (2009b) observed the co-occurrence of *papC*, *papGIII*, *sfafocDE*, and *papC* in *E. coli* isolated from human extraintestinal infections and urinary tract infections. Bashir *et al*., (2012) reported the presence of different virulence gene such as *sfaDE*, *afaBC3*, *papC*, *eaeA*, *cnf1*, *hlyA* in one of the uropathogenic *E. coli* isolates.

The isolate harboured both *class 1* and *3 integrons*. While the strain ES11 was found to be negative for *class 2 integrons*. Most common integrons present in resistant *Enterobacteriaceae* are *class 1 Integrons* (Leverstein-van Hall *et al*., 2003; Hansson *et al*., 2002; Bass *et al*., 1999; Martinez-Freijo *et al*., 1998). *Class 2 integrons*, which possess a defective integrase gene, are less prevalent and are associated with transposons of Tn7 family. *Class 3 integrons* are the least common (Cambray *et al*., 2010). Isolates with *class 1 integrons* expressed significantly broader antibiotic resistance ranges compared to those harboring *class 2 integrons*. This may be explained by

greater diversity of the gene cassette content of *class 1 integrons* (Ozgumus *et al*., 2009; Gu *et al*., 2008; Moura *et al*., 2007).

The isolate carried the shiga toxin-producing gene *stx1*. Shiga toxin-producing *E. coli* (STEC) emerged in 1982 as human pathogens causing hemorrhagic colitis and the hemolytic uremic syndrome (Riley *et al*., 1983; Wells *et al*., 1983). Since then, these strains have gained relevance because of the high number of STEC outbreaks involving hundreds of different *E. coli* serotypes carrying shiga toxin genes (*stx*) (Caprioli *et al*., 1998). STEC strains produce one or both of two major types of shiga toxin, designated *stx1* and *stx2*, and the production of the latter is associated with an increased risk of developing hemolytic uremic-syndrome (Boerlin *et al*., 1999; Ostroff *et al*., 1989; Kleanthous *et al*., 1985).

This is the first report in which an isolate carried both intestinal (*stx1*) and extraintestinal pathogenic genes (*papAH , papC , sfa/focDE , iutA , kpsMT II*). Interestingly, the isolate also carried resistance genes such as bla_{TEM} , $bla_{CTX\text{-}M}$, *sul1, sul2 , tetA , tetB , strA , aphA2 ,cat1 , dhfrIa* ,and *dhfrVII*. The isolate also found to carry both *class 1* and *3 integrons*. These results are in favour of the possibility that multidrug *E. coli* strains of estuarine origin are virulent for humans and consequently constitute a zoonotic risk. Given the severity of the clinical manifestations of the disease in humans and the inability and/or the potential risks of antibiotic administration for treatment, it appears that the most direct and effective measure towards ensuring public health is the prevention of STEC and ExPEC infections in humans.

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Chapter 9

Summary and Conclusion

9.1 Summary

In the present study diversity of *E. coli* in the water samples of Cochin estuary were studied for a period of 3 years ranging from January 2010-December 2012. The stations were selected based on the closeness to satellite townships and waste input. Two of the stations (Chitoor and Thevara) were fixed upstream, two in the central part of the estuary namely Bolgatty and Off Marine Science Jetty, and one at the Barmouth. Diversity was assessed in terms of serotypes, phylogenetic groups and genotypes. Two groups of seafood samples such as fish and shellfish collected from the Cochin estuary were used for isolation of *E. coli*. One hundred clinical *E. coli* isolates were collected from one public health centre, one hospital and five medical labs in and around Cochin City, Kerala. Samples were analysed for faecal coliforms by MPN method. Biochemically confirmed *E. coli* cultures were serotyped at National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli, Himachal Pradesh. Molecular identification of *E. coli* was done by the amplification of *uidA* gene. Phylogenetic grouping of *E. coli* isolates was

determined by a triplex PCR method. Prevalence of antibiotic resistance among *E. coli* from the above sources was determined by disc diffusion method and multiple antibiotic resistance (MAR) indexing. *E. coli* isolates from different sources such as estuarine, seafood and clinical were also analyzed for the presence of drug resistance genes such as bla_{TEM}, $bla_{CTX\text{-}M}$, *tetA, tetB, sul1, sul2, strA, aphA2, catI, dhfrIa, dhfrVII, class 1 integrase gene* and *class 1 integron variable regions* and for the presence of virulence factor genes such as *papAH* and *papC* (P fimbriae major structural subunit and assembly), *sfa/focDE* (S and F1C fimbriae), *iutA* (aerobactin receptor) and *kpsMT II* (group II capsule). Genetic relatedness among *E. coli* isolates of estuarine, seafood and clinical sources were analyzed using ERIC PCR, RAPD and RFLP. One strain (CUSMBES11) which was resistant to all the antibiotics and had all the virulence factor genes was further characterized in a detailed manner using Real-time PCR. Statistical analysis of the results of the study was carried out with SPSS (Statistical Package for the Social Science).

- Serotyping of *E. coli* isolates from Cochin estuary revealed that they belonged to 58 different serotypes. Pathogenic serotypes such as O25, O60, O75 were isolated with high frequency, where as O157, O153, O148 were encountered very rarely.
- Spatial variation in the distribution of *E. coli* serotypes revealed, presence of more pathogenic *E. coli* serotypes at stations 2 (Bolgatty) and 3 (Off Marine Science Jetty), which are close to Cochin City.
- It was found that all the stations showed high prevalence either in pre-monsoon or post-monsoon period, but showed comparatively low diversity in monsoon season. In station 1 (Chitoor), 2 (Bolgatty)

and 5 (Barmouth), more diverse serotypes were obtained during pre-monsoon period, while post-monsoon period yielded diverse serotypes in station 3 and 4.

- Games-Howell analysis showed that the distribution of phylogenetic group A was significantly higher than phylogenetic group D among different stations ($p < 0.05$).
- Phylogenetic grouping of *E. coli* revealed that A and B2 group strains were the most common (35.66% and 25.66% respectively) phylogenetic groups in estuarine water, followed by B1 and D strains (23% and 15.66%) respectively.
- *E. coli* belonging to phylogenetic group A was the most common (52%) in seafood, followed by B1 (23%), B2 (16%), and D (9%). While *E. coli* isolates from clinical sources revealed that 80% of the isolates belonged to phylogenetic group B2, followed by group A (12%), D (6%), and B1 (2%).
- Games-Howell analysis showed that the prevalence of resistance to ampicillin, cefoxitin and cefpodoxime was significantly higher among *E. coli* isolates from estuary, than to cefotaxime, ceftazidime, ceftriaxone, cefuroxime, and chloramphenicol ($p < 0.05$). A highly significant association was found between ceftazidime with ceftriaxone ($p = 0.002$). Furthermore, a significant association was found between co-trimoxazole with trimethoprim and nalidixic acid ($p < 0.05$). There was also a significant association seen between cefotaxime with cefuroxime and gentamicin ($p < 0.05$).

- Antibiotic resistance of *E. coli* belonged to phylogenetic group A and D was significantly varied among all stations in Cochin estuary. But there was no significant difference in antibiotic resistance of *E. coli* belonged to phylogenetic group B1 and B2.
- *E. coli* isolates from station 2 (Bolgatty) and station 3 (Off Marine Science Jetty), compared to those recovered from the other stations, were more frequently resistant to cefotaxime, co-trimoxazole, trimethoprim and fluoroquinolones.
- There was significant difference in antibiotic resistance among the *E. coli* isolates from different sources. Antibiotic resistance of *E. coli* isolated from clinical sources was significantly higher than those from seafood and estuary ($p < 0.001$).
- *E. coli* isolates from station 2 (Bolgatty) showed higher prevalence for antibiotic resistance genes such as, bla_{TEM}, *tetB* and *aphA2*. While isolates from station 3 (Off Marine Science jetty), showed higher prevalence for *strA*. Whereas *E. coli* isolates from station 4 (Thevara), exhibited higher prevalence for sulphonamide resistance genes, such as *sul1* and *sul2*. Statistical analysis of the result revealed a highly significant association was found between bla_{TEM} and *sul1* gene ($p = 0.25$).
- Clinical *E. coli* isolates carried significantly high prevalence of resistance genes than seafood ($p = 0.000$) and estuarine isolates ($p = 0.002$).
- Distribution of virulence factor genes significantly varied among different phylogenetic groups. *E. coli* isolates belonged to group B2,

carried more virulence genes than group A, B1 and D ($p < 0.001$). Clinical isolates carried significantly more virulence factor genes than seafood and estuarine isolates ($p < 0.05$).

- Prevalence of virulence factor genes was significantly high in station 2 (Bolgatty), which is located close to Cochin city.
- *E. coli* belonged to phylogenetic group B2 and D harboured more virulence factor genes than non pathognic groups A and B1.
- The aerobactin receptor gene (*iutA*) was positively associated with resistance to cefotaxime ($p = 0.010$) and cepfodoxime ($p = 0.036$). Group 2 capsule synthesis gene *(kpsMT II)* on the other hand showed an association with resistance to ciprofloxacin, cefoxitin, cepfodoxime, and cefotaxime ($p < 0.05$). Whereas adhesion related genes, (*papAH*, and *papC*) and S/F1C fimbriae gene (*sfa/focDE*) did not showed any positive association with any antibiotic resistance traits.
- ERIC-PCR analysis revealed high heterogeneity among the *E. coli* isolates.
- Genotyping analysis revealed that despite marked genetic variability among *E. coli* from three different sources, there were strains sharing similar features.
- Detection of pathogenic as well as non pathogenic (mixed population) isolates in a same cluster indicates that the genetic changes (mutation/rearrangements) are occurring amongst the strain belonging to the clone making them pathogenic or non-pathogenic.

By genotyping analysis using ERIC-PCR, we could identify the changes occurring in the isolates, which might pose risk to people.

- We could not find a difference between pathogenic and non-pathogenic strains from estuarine isolates. Whereas the difference was obvious in *E. coli* isolates from seafood and clinical sources.
- Detailed characterisation of a multidrug-resistant *E. coli* (ES11 – Genbank Accession No. JX183942.1) revealed the presence of two house-keeping genes (*uid A, frr*), five virulence factor genes (*papAH, papC, sfa/focDE, iutA* and *kps MT II*) and fourteen antibiotic resistance genes (bla_{TEM}, bla_{CTXM}, *tetA, tetB, sul1, sul2, strA, aphA2, catI, dhfrIa, dhfrVII, class 1 integrase gene, class 1 integron variable regions*, and *class 3 integrase gene*). This strain also carried shiga toxin- producing gene *stx1*. This is the first report in which an isolate carried both intestinal (*stx1*) and extraintestinal pathogenic genes (*papAH, papC, sfa/focDE, iutA, kpsMT II*).

9.2 Conclusion

From our results it was clear that pathogen cycling is occurring through food, water and clinical sources. Pathogen cycling through food is very common and fish and shellfish that harbour these strains might pose potential health risk to consumer. Estuarine environment is a melting pot for various kinds of wastes, both organic and inorganic. Mixing up of waste water from various sources such as domestic, industries, hospitals and sewage released into these water bodies resulting in the co-existence of *E. coli* from various sources thus offering a conducive environment for horizontal gene transfer.

Opportunistic pathogens might acquire genes for drug resistance and virulence turning them to potential pathogens. Prevalence of ExPEC in the Cochin estuary, pose threat to people who use this water for fishing and recreation. Food chain also plays an important role in the transit of virulence genes from the environments to the human. Antibiotic resistant *E. coli* are widespread in estuarine water, seafood and clinical samples, for reasons well known such as indiscriminate use of antibiotics in animal production systems, aquaculture and human medicine. Since the waste water from these sources entering the estuary provides selection pressure to drug resistant mutants in the environment. It is high time that the authorities concerned should put systems in place for monitoring and enforcement to curb such activities. Microbial contamination can limit people's enjoyment of coastal waters for contact recreation or shellfish-gathering. *E. coli* can make people sick if they are present in high levels in water used for contact recreation or shellfish-gathering. When feeding, shellfish can filter large volumes of seawater, so any microorganisms present in the water become accumulated and concentrated in the shellfish flesh. If *E. coli* contaminated shellfish are consumed the impact to human health includes gastroenteritis, urinary tract infections (UTIs), and bacteraemia.

In conclusion, the high prevalence of various pathogenic serotypes and phylogenetic groups, multidrug-resistance, and virulence factor genes detected among *E. coli* isolates from stations close to Cochin city is a matter of concern, since there is a large reservoir of antibiotic resistance genes and virulence traits within the community, and that the resistance genes and plasmid-encoded genes for virulence were easily transferable to other strains. Given the severity of the clinical manifestations of the disease in humans and

the inability and/or the potential risks of antibiotic administration for treatment, it appears that the most direct and effective measure towards prevention of STEC and ExPEC infections in humans and ensuring public health may be considered as a priority.

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Appendix -1

Table 1.1a Anova for distribution of various phylogenetic groups of *E. coli* isolates among different stations set at Cochin Estuary

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1077.165	3	359.055	3.262	.049
Within Groups	1761.169	16	110.073		
Total	2838.335	19			

Table 1.1b Games-Howell analysis of distribution of various phylogenetic groups of *E. coli* isolates among different stations set at Cochin Estuary

Games-Howell

(I) phylogroups	(J) phylogroups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	11.32800	7.06998	.435	-12.0581	34.7141
	3.00	12.15000	7.12901	.388	-11.4744	35.7744
	4.00	20.64400*	4.30662	.015	5.2333	36.0547
2.00	1.00	-11.32800	7.06998	.435	-34.7141	12.0581
	3.00	.82200	8.33736	1.000	-25.8780	27.5220
	4.00	9.31600	6.10211	.492	-13.8260	32.4580
3.00	1.00	-12.15000	7.12901	.388	-35.7744	11.4744
	2.00	-.82200	8.33736	1.000	-27.5220	25.8780
	4.00	8.49400	6.17040	.564	-14.9402	31.9282
4.00	1.00	-20.64400*	4.30662	.015	-36.0547	-5.2333
	2.00	-9.31600	6.10211	.492	-32.4580	13.8260
	3.00	-8.49400	6.17040	.564	-31.9282	14.9402

*. The mean difference is significant at the .05 level.

1-Phylogenetic group A, 2- Phylogenetic group B1, 3- Phylogenetic group B2, 4- Phylogenetic group D.

Table 1.2 Anova for distribution of various phylogenetic groups of *E. coli* isolates among different sources

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1826.769	3	608.923	1.379	.317
Within Groups	3532.538	8	441.567		
Total	5359.307	11			

Appendix -2

Table 2.1a Anova for antibiotic resistance of *E. coli* isolates from Cochin Estuary

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9952.587	14	710.899	17.663	.000
Within Groups	2414.882	60	40.248		
Total	12367.469	74			

Table 2.1b Games-Howell analysis of antibiotic resistance of *E. coli* isolates from Cochin Estuary

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
1.00	2.00	38.47400(*)	3.98714	.009	14.4367	62.5113
	3.00	20.39400	4.14872	.080	-2.7358	43.5238
	4.00	26.83200(*)	4.00628	.033	2.9313	50.7327
	5.00	40.42400(*)	4.00606	.007	16.5218	64.3262
	6.00	39.37800(*)	4.04582	.007	15.7326	63.0234
	7.00	38.06600(*)	4.11423	.007	14.7853	61.3467
	8.00	37.83000(*)	4.12402	.007	14.5943	61.0657
	9.00	32.98400(*)	5.16204	.008	9.1041	56.8639
	10.00	23.46600	5.97435	.109	-4.0405	50.9725
	11.00	37.65000(*)	4.25452	.005	14.8652	60.4348
	12.00	15.73200	6.12425	.486	-12.5647	44.0287
	13.00	26.51600(*)	4.90311	.024	3.4254	49.6066
	14.00	12.34600	6.17689	.761	-16.2344	40.9264
	15.00	23.25600	5.37140	.068	-1.4215	47.9335
2.00	1.00	-38.47400(*)	3.98714	.009	-62.5113	-14.4367
	3.00	-18.08000(*)	1.32718	.001	-25.2318	-10.9282
	4.00	-11.64200(*)	.77450	.000	-15.2605	-8.0235
	5.00	1.95000	.77339	.509	-1.6621	5.5621
	6.00	.90400	.95825	.998	-3.8389	5.6469
	7.00	-.40800	1.21511	1.000	-6.8232	6.0072
	8.00	-.64400	1.24783	1.000	-7.2743	5.9863
	9.00	-5.49000	3.34608	.883	-25.5297	14.5497
	10.00	-15.00800	4.49915	.304	-42.2247	12.2087
	11.00	-.82400	1.62805	1.000	-9.9447	8.2967
	12.00	-22.74200	4.69638	.105	-51.1811	5.6971
	13.00	-11.95800	2.93088	.173	-29.3939	5.4779
	14.00	-26.12800	4.76482	.070	-54.9910	2.7350
	15.00	-15.21800	3.66080	.166	-37.2231	6.7871

3.00	1.00	-20.39400	4.14872	.080	-43.5238	2.7358
	2.00	18.08000(*)	1.32718	.001	10.9282	25.2318
	4.00	6.43800	1.38362	.074	-.6212	13.4972
	5.00	20.03000(*)	1.38300	.000	12.9704	27.0896
	6.00	18.98400(*)	1.49425	.000	11.8434	26.1246
	7.00	17.67200(*)	1.67064	.000	9.9901	25.3539
	8.00	17.43600(*)	1.69459	.000	9.6561	25.2159
	9.00	12.59000	3.53706	.225	-6.5351	31.7151
	10.00	3.07200	4.64294	1.000	-23.2702	29.4142
	11.00	17.25600(*)	1.99125	.001	7.9928	26.5192
	12.00	-4.66200	4.83431	.996	-32.2421	22.9181
	13.00	6.12200	3.14716	.778	-10.4410	22.6850
	14.00	-8.04800	4.90082	.885	-36.0576	19.9616
	15.00	2.86200	3.83615	1.000	-18.2248	23.9488
4.00	1.00	-26.83200(*)	4.00628	.033	-50.7327	-2.9313
	2.00	11.64200(*)	.77450	.000	8.0235	15.2605
	3.00	-6.43800	1.38362	.074	-13.4972	.6212
	5.00	13.59200(*)	.86667	.000	9.6190	17.5650
	6.00	12.54600(*)	1.03500	.000	7.6775	17.4145
	7.00	11.23400(*)	1.27651	.003	4.8682	17.5998
	8.00	10.99800(*)	1.30769	.004	4.4313	17.5647
	9.00	6.15200	3.36887	.819	-13.7337	26.0377
	10.00	-3.36600	4.51612	1.000	-30.4583	23.7263
	11.00	10.81800(*)	1.67437	.022	1.8524	19.7836
	12.00	-11.10000	4.71264	.608	-39.4189	17.2189
	13.00	-.31600	2.95686	1.000	-17.5860	16.9540
	14.00	-14.48600	4.78085	.379	-43.2302	14.2582
	15.00	-3.57600	3.68164	.996	-25.4359	18.2839
5.00	1.00	-40.42400(*)	4.00606	.007	-64.3262	-16.5218
	2.00	-1.95000	.77339	.509	-5.5621	1.6621
	3.00	-20.03000(*)	1.38300	.000	-27.0896	-12.9704
	4.00	-13.59200(*)	.86667	.000	-17.5650	-9.6190
	6.00	-1.04600	1.03417	.997	-5.9123	3.8203
	7.00	-2.35800	1.27583	.821	-8.7236	4.0076
	8.00	-2.59400	1.30703	.763	-9.1607	3.9727
	9.00	-7.44000	3.36861	.666	-27.3274	12.4474
	10.00	-16.95800	4.51593	.221	-44.0516	10.1356
	11.00	-2.77400	1.67386	.885	-11.7409	6.1929
	12.00	-24.69200	4.71246	.080	-53.0122	3.6282
	13.00	-13.90800	2.95657	.106	-31.1798	3.3638
	14.00	-28.07800	4.78067	.054	-56.8235	.6675
	15.00	-17.16800	3.68141	.114	-39.0295	4.6935
6.00	1.00	-39.37800(*)	4.04582	.007	-63.0234	-15.7326
	2.00	-.90400	.95825	.998	-5.6469	3.8389
	3.00	-18.98400(*)	1.49425	.000	-26.1246	-11.8434
	4.00	-12.54600(*)	1.03500	.000	-17.4145	-7.6775
	5.00	-1.04600	1.03417	.997	-3.8203	5.9123

	7.00	-1.31200	1.39566	.998	-7.8651	5.2411
	8.00	-1.54800	1.42424	.994	-8.2686	5.1726
	9.00	-6.39400	3.41580	.803	-26.0053	13.2173
	10.00	-15.91200	4.55124	.263	-42.7660	10.9420
	11.00	-1.72800	1.76690	.997	-10.5866	7.1306
	12.00	-23.64600	4.74630	.091	-51.7331	4.4411
	13.00	-12.86200	3.01022	.135	-29.8512	4.1272
	14.00	-27.03200	4.81403	.060	-55.5466	1.4826
	15.00	-16.12200	3.72463	.137	-37.7162	5.4722
7.00	1.00	-38.06600(*)	4.11423	.007	-61.3467	-14.7853
	2.00	.40800	1.21511	1.000	-6.0072	6.8232
	3.00	-17.67200(*)	1.67064	.000	-25.3539	-9.9901
	4.00	-11.23400(*)	1.27651	.003	-17.5998	-4.8682
	5.00	2.35800	1.27583	.821	-4.0076	8.7236
	6.00	1.31200	1.39566	.998	-5.2411	7.8651
	8.00	-.23600	1.60832	1.000	-7.6110	7.1390
	9.00	-5.08200	3.49655	.940	-24.3378	14.1738
	10.00	-14.60000	4.61216	.332	-41.0975	11.8975
	11.00	-.41600	1.91837	1.000	-9.4751	8.6431
	12.00	-22.33400	4.80475	.108	-50.0694	5.4014
	13.00	-11.55000	3.10156	.193	-28.2144	5.1144
	14.00	-25.72000	4.87167	.070	-53.8848	2.4448
	15.00	-14.81000	3.79883	.179	-36.0402	6.4202
8.00	1.00	-37.83000(*)	4.12402	.007	-61.0657	-14.5943
	2.00	.64400	1.24783	1.000	-5.9863	7.2743
	3.00	-17.43600(*)	1.69459	.000	-25.2159	-9.6561
	4.00	-10.99800(*)	1.30769	.004	-17.5647	-4.4313
	5.00	2.59400	1.30703	.763	-3.9727	9.1607
	6.00	1.54800	1.42424	.994	-5.1726	8.2686
	7.00	.23600	1.60832	1.000	-7.1390	7.6110
	9.00	-4.84600	3.50806	.955	-24.0617	14.3697
	10.00	-14.36400	4.62089	.346	-40.8158	12.0878
	11.00	-.18000	1.93927	1.000	-9.2919	8.9319
	12.00	-22.09800	4.81313	.111	-49.7879	5.5919
	13.00	-11.31400	3.11453	.207	-27.9459	5.3179
	14.00	-25.48400	4.87993	.072	-53.6034	2.6354
	15.00	-14.57400	3.80942	.188	-35.7610	6.6130
9.00	1.00	-32.98400(*)	5.16204	.008	-56.8639	-9.1041
	2.00	5.49000	3.34608	.883	-14.5497	25.5297
	3.00	-12.59000	3.53706	.225	-31.7151	6.5351
	4.00	-6.15200	3.36887	.819	-26.0377	13.7337
	5.00	7.44000	3.36861	.666	-12.4474	27.3274
	6.00	6.39400	3.41580	.803	-13.2173	26.0053
	7.00	5.08200	3.49655	.940	-14.1738	24.3378
	8.00	4.84600	3.50806	.955	-14.3697	24.0617
	10.00	-9.51800	5.56703	.880	-35.6804	16.6444
	11.00	4.66600	3.66059	.976	-14.2201	23.5521

	12.00	-17.25200	5.72760	.319	-44.3608	9.8568
	13.00	-6.46800	4.39767	.950	-26.7350	13.7990
	14.00	-20.63800	5.78385	.178	-48.0827	6.8067
	15.00	-9.72800	4.91436	.770	-32.3114	12.8554
10.00	1.00	-23.46600	5.97435	.109	-50.9725	4.0405
	2.00	15.00800	4.49915	.304	-12.2087	42.2247
	3.00	-3.07200	4.64294	1.000	-29.4142	23.2702
	4.00	3.36600	4.51612	1.000	-23.7263	30.4583
	5.00	16.95800	4.51593	.221	-10.1356	44.0516
	6.00	15.91200	4.55124	.263	-10.9420	42.7660
	7.00	14.60000	4.61216	.332	-11.8975	41.0975
	8.00	14.36400	4.62089	.346	-12.0878	40.8158
	9.00	9.51800	5.56703	.880	-16.6444	35.6804
	11.00	14.18400	4.73773	.367	-11.7729	40.1409
	12.00	-7.73400	6.46927	.989	-37.4069	21.9389
	13.00	3.05000	5.32781	1.000	-22.6025	28.7025
	14.00	-11.12000	6.51913	.884	-41.0344	18.7944
	15.00	-.21000	5.76168	1.000	-26.9521	26.5321
11.00	1.00	-37.65000(*)	4.25452	.005	-60.4348	-14.8652
	2.00	.82400	1.62805	1.000	-8.2967	9.9447
	3.00	-17.25600(*)	1.99125	.001	-26.5192	-7.9928
	4.00	-10.81800(*)	1.67437	.022	-19.7836	-1.8524
	5.00	2.77400	1.67386	.885	-6.1929	11.7409
	6.00	1.72800	1.76690	.997	-7.1306	10.5866
	7.00	.41600	1.91837	1.000	-8.6431	9.4751
	8.00	.18000	1.93927	1.000	-8.9319	9.2919
	9.00	-4.66600	3.66059	.976	-23.5521	14.2201
	10.00	-14.18400	4.73773	.367	-40.1409	11.7729
	12.00	-21.91800	4.92541	.112	-49.1039	5.2679
	13.00	-11.13400	3.28538	.235	-27.5814	5.3134
	14.00	-25.30400	4.99071	.070	-52.9169	2.3089
	15.00	-14.39400	3.95034	.200	-35.1798	6.3918
12.00	1.00	-15.73200	6.12425	.486	-44.0287	12.5647
	2.00	22.74200	4.69638	.105	-5.6971	51.1811
	3.00	4.66200	4.83431	.996	-22.9181	32.2421
	4.00	11.10000	4.71264	.608	-17.2189	39.4189
	5.00	24.69200	4.71246	.080	-3.6282	53.0122
	6.00	23.64600	4.74630	.091	-4.4411	51.7331
	7.00	22.33400	4.80475	.108	-5.4014	50.0694
	8.00	22.09800	4.81313	.111	-5.5919	49.7879
	9.00	17.25200	5.72760	.319	-9.8568	44.3608
	10.00	7.73400	6.46927	.989	-21.9389	37.4069
	11.00	21.91800	4.92541	.112	-5.2679	49.1039
	13.00	10.78400	5.49538	.775	-15.9094	37.4774
	14.00	-3.38600	6.65677	1.000	-33.9040	27.1320
	15.00	7.52400	5.91698	.981	-20.0885	35.1365
13.00	1.00	-26.51600(*)	4.90311	.024	-49.6066	-3.4254

	2.00	11.95800	2.93088	.173	-5.4779	29.3939
	3.00	-6.12200	3.14716	.778	-22.6850	10.4410
	4.00	.31600	2.95686	1.000	-16.9540	17.5860
	5.00	13.90800	2.95657	.106	-3.3638	31.1798
	6.00	12.86200	3.01022	.135	-4.1272	29.8512
	7.00	11.55000	3.10156	.193	-5.1144	28.2144
	8.00	11.31400	3.11453	.207	-5.3179	27.9459
	9.00	6.46800	4.39767	.950	-13.7990	26.7350
	10.00	-3.05000	5.32781	1.000	-28.7025	22.6025
	11.00	11.13400	3.28538	.235	-5.3134	27.5814
	12.00	-10.78400	5.49538	.775	-37.4774	15.9094
	14.00	-14.17000	5.55398	.503	-41.2306	12.8906
	15.00	-3.26000	4.64162	1.000	-24.8501	18.3301
14.00	1.00	-12.34600	6.17689	.761	-40.9264	16.2344
	2.00	26.12800	4.76482	.070	-2.7350	54.9910
	3.00	8.04800	4.90082	.885	-19.9616	36.0576
	4.00	14.48600	4.78085	.379	-14.2582	43.2302
	5.00	28.07800	4.78067	.054	-.6675	56.8235
	6.00	27.03200	4.81403	.060	-1.4826	55.5466
	7.00	25.72000	4.87167	.070	-2.4448	53.8848
	8.00	25.48400	4.87993	.072	-2.6354	53.6034
	9.00	20.63800	5.78385	.178	-6.8067	48.0827
	10.00	11.12000	6.51913	.884	-18.7944	41.0344
	11.00	25.30400	4.99071	.070	-2.3089	52.9169
	12.00	3.38600	6.65677	1.000	-27.1320	33.9040
	13.00	14.17000	5.55398	.503	-12.8906	41.2306
	15.00	10.91000	5.97144	.836	-17.0131	38.8331
15.00	1.00	-23.25600	5.37140	.068	-47.9335	1.4215
	2.00	15.21800	3.66080	.166	-6.7871	37.2231
	3.00	-2.86200	3.83615	1.000	-23.9488	18.2248
	4.00	3.57600	3.68164	.996	-18.2839	25.4359
	5.00	17.16800	3.68141	.114	-4.6935	39.0295
	6.00	16.12200	3.72463	.137	-5.4722	37.7162
	7.00	14.81000	3.79883	.179	-6.4202	36.0402
	8.00	14.57400	3.80942	.188	-6.6130	35.7610
	9.00	9.72800	4.91436	.770	-12.8554	32.3114
	10.00	.21000	5.76168	1.000	-26.5321	26.9521
	11.00	14.39400	3.95034	.200	-6.3918	35.1798
	12.00	-7.52400	5.91698	.981	-35.1365	20.0885
	13.00	3.26000	4.64162	1.000	-18.3301	24.8501
	14.00	-10.91000	5.97144	.836	-38.8331	17.0131

* The mean difference is significant at the .05 level.

1-Ampicillin; 2-Cefotaxime; 3-Cefoxitin; 4-Cefpodoxime; 5-Ceftazidime; 6-Ceftriaxone; 7- Cefuroxime; 8-Chloramphenicol; 9-Ciprofloxacin; 10-Co-Trimoxazole; 11-Gentamicin; 12- Nalidixic acid; 13-Streptomycin; 14-Tetracycline; 15-Trimethoprim

Table 2.2 Anova for overall antibiotic resistance of *E. coli* from different stations

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	794.565	4	198.641	1.202	.318
Within Groups	11572.802	70	165.326		
Total	12367.368	74			

Table 2.3a Anova for antibiotic resistance of *E. coli* isolates in five different stations set at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S1	Between Groups	6614.517	14	472.466	3.371	.001
	Within Groups	6307.814	45	140.174		
	Total	12922.331	59			
S2	Between Groups	16481.452	14	1177.247	9.207	.000
	Within Groups	5753.919	45	127.865		
	Total	22235.371	59			
S3	Between Groups	8407.320	14	600.523	4.206	.000
	Within Groups	6424.310	45	142.762		
	Total	14831.630	59			
S4	Between Groups	8707.544	14	621.967	3.437	.001
	Within Groups	8142.420	45	180.943		
	Total	16849.964	59			
S5	Between Groups	11184.904	14	798.922	5.352	.000
	Within Groups	6717.523	45	149.278		
	Total	17902.427	59			

S1-Chittor, S2-Bolgatty, S3-Off Marine Science Jetty, S4-Thevara, S5-Barmouth

Table 2.3b Games-Howell analysis of antibiotic resistance of *E. coli* isolates from station 2 (Bolgatty)

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
1.00	2.00	56.61250	11.18507	.130	-23.8110	137.0360
	3.00	41.10250	13.88828	.364	-31.0199	113.2249
	4.00	46.53750	12.39675	.211	-25.1360	118.2110
	5.00	58.71000	11.08663	.121	-23.1451	140.5651
	6.00	58.71000	11.08663	.121	-23.1451	140.5651
	7.00	57.39500	11.13368	.127	-23.7563	138.5463
	8.00	53.22250	11.70762	.143	-21.7842	128.2292
	9.00	37.03500	12.04055	.368	-35.9449	110.0149
	10.00	27.08000	12.82822	.706	-43.9093	98.0693
	11.00	51.18500	11.43068	.163	-26.2855	128.6555
	12.00	21.15500	11.64184	.818	-54.3653	96.6753
	13.00	48.22500	12.92233	.199	-22.7211	119.1711
	14.00	24.29750	12.57509	.780	-46.9887	95.5837
	15.00	31.97500	13.78712	.613	-39.9092	103.8592
2.00	1.00	-56.61250	11.18507	.130	-137.0360	23.8110
	3.00	-15.51000	8.56622	.817	-75.6960	44.6760
	4.00	-10.07500	5.84595	.850	-48.7601	28.6101
	5.00	2.09750	1.84641	.986	-8.8427	13.0377
	6.00	2.09750	1.84641	.986	-8.8427	13.0377
	7.00	.78250	2.11061	1.000	-10.1542	11.7192
	8.00	-3.39000	4.19097	.999	-28.7092	21.9292
	9.00	-19.57750	5.04685	.220	-51.8226	12.6676
	10.00	-29.53250	6.71261	.172	-75.1373	16.0723
	11.00	-5.42750	3.34009	.893	-23.9917	13.1367
	12.00	-35.45750(*)	4.00352	.011	-59.2669	-11.6481
	13.00	-8.38750	6.89074	.972	-55.4059	38.6309
	14.00	-32.31500	6.21521	.106	-73.9571	9.3271
	15.00	-24.63750	8.40121	.436	-83.5359	34.2609
3.00	1.00	-41.10250	13.88828	.364	-113.2249	31.0199
	2.00	15.51000	8.56622	.817	-44.6760	75.6960
	4.00	5.43500	10.09721	1.000	-48.5604	59.4304
	5.00	17.60750	8.43728	.717	-44.3425	79.5575
	6.00	17.60750	8.43728	.717	-44.3425	79.5575
	7.00	16.29250	8.49901	.779	-44.7713	77.3563
	8.00	12.12000	9.23811	.963	-42.9173	67.1573
	9.00	-4.06750	9.65656	1.000	-58.0727	49.9377
	10.00	-14.02250	10.62250	.969	-68.9959	40.9509
	11.00	10.08250	8.88452	.984	-46.9665	67.1315
	12.00	-19.94750	9.15460	.679	-75.3492	35.4542
	13.00	7.12250	10.73596	1.000	-48.1702	62.4152
	14.00	-16.80500	10.31539	.896	-71.0967	37.4867
	15.00	-9.12750	11.76260	1.000	-68.5469	50.2919
4.00	1.00	-46.53750	12.39675	.211	-118.2110	25.1360

	2.00	10.07500	5.84595	.850	-28.6101	48.7601
	3.00	-5.43500	10.09721	1.000	-59.4304	48.5604
	5.00	12.17250	5.65533	.693	-28.6959	53.0409
	6.00	12.17250	5.65533	.693	-28.6959	53.0409
	7.00	10.85750	5.74701	.790	-28.8442	50.5592
	8.00	6.68500	6.79247	.996	-29.3832	42.7532
	9.00	-9.50250	7.35147	.974	-47.0070	28.0020
	10.00	-19.45750	8.58094	.635	-63.1652	24.2502
	11.00	4.64750	6.30315	1.000	-31.5929	40.8879
	12.00	-25.38250	6.67845	.182	-61.3403	10.5753
	13.00	1.68750	8.72100	1.000	-42.8842	46.2592
	14.00	-22.24000	8.19770	.444	-63.7149	19.2349
	15.00	-14.56250	9.95760	.940	-67.5435	38.4185
5.00	1.00	-58.71000	11.08663	.121	-140.5651	23.1451
	2.00	-2.09750	1.84641	.986	-13.0377	8.8427
	3.00	-17.60750	8.43728	.717	-79.5575	44.3425
	4.00	-12.17250	5.65533	.693	-53.0409	28.6959
	6.00	.00000	1.10309	1.000	-5.5714	5.5714
	7.00	-1.31500	1.50407	.999	-9.5646	6.9346
	8.00	-5.48750	3.92069	.938	-32.9784	22.0034
	9.00	-21.67500	4.82475	.175	-56.1737	12.8237
	10.00	-31.63000	6.54727	.150	-79.2902	16.0302
	11.00	-7.52500	2.99395	.560	-27.7091	12.6591
	12.00	-37.55500(*)	3.71964	.016	-63.4732	-11.6368
	13.00	-10.48500	6.72978	.896	-59.5302	38.5602
	14.00	-34.41250	6.03625	.097	-78.1866	9.3616
	15.00	-26.73500	8.26970	.371	-87.4219	33.9519
6.00	1.00	-58.71000	11.08663	.121	-140.5651	23.1451
	2.00	-2.09750	1.84641	.986	-13.0377	8.8427
	3.00	-17.60750	8.43728	.717	-79.5575	44.3425
	4.00	-12.17250	5.65533	.693	-53.0409	28.6959
	5.00	.00000	1.10309	1.000	-5.5714	5.5714
	7.00	-1.31500	1.50407	.999	-9.5646	6.9346
	8.00	-5.48750	3.92069	.938	-32.9784	22.0034
	9.00	-21.67500	4.82475	.175	-56.1737	12.8237
	10.00	-31.63000	6.54727	.150	-79.2902	16.0302
	11.00	-7.52500	2.99395	.560	-27.7091	12.6591
	12.00	-37.55500(*)	3.71964	.016	-63.4732	-11.6368
	13.00	-10.48500	6.72978	.896	-59.5302	38.5602
	14.00	-34.41250	6.03625	.097	-78.1866	9.3616
	15.00	-26.73500	8.26970	.371	-87.4219	33.9519
7.00	1.00	-57.39500	11.13368	.127	-138.5463	23.7563
	2.00	-.78250	2.11061	1.000	-11.7192	10.1542
	3.00	-16.29250	8.49901	.779	-77.3563	44.7713
	4.00	-10.85750	5.74701	.790	-50.5592	28.8442
	5.00	1.31500	1.50407	.999	-6.9346	9.5646
	6.00	1.31500	1.50407	.999	-6.9346	9.5646
	8.00	-4.17250	4.05182	.992	-30.3541	22.0091

	9.00	-20.36000	4.93190	.200	-53.6036	12.8836
	10.00	-30.31500	6.62662	.163	-76.9074	16.2774
	11.00	-6.21000	3.16373	.766	-25.2085	12.7885
	12.00	-36.24000(*)	3.85761	.013	-60.8521	-11.6279
	13.00	-9.17000	6.80700	.949	-57.1670	38.8270
	14.00	-33.09750	6.12224	.103	-75.7477	9.5527
	15.00	-25.42000	8.33267	.409	-85.2065	34.3665
8.00	1.00	-53.22250	11.70762	.143	-128.2292	21.7842
	2.00	3.39000	4.19097	.999	-21.9292	28.7092
	3.00	-12.12000	9.23811	.963	-67.1573	42.9173
	4.00	-6.68500	6.79247	.996	-42.7532	29.3832
	5.00	5.48750	3.92069	.938	-22.0034	32.9784
	6.00	5.48750	3.92069	.938	-22.0034	32.9784
	7.00	4.17250	4.05182	.992	-22.0091	30.3541
	9.00	-16.18750	6.11827	.474	-47.6266	15.2516
	10.00	-26.14250	7.55127	.251	-67.8639	15.5789
	11.00	-2.03750	4.80819	1.000	-27.0541	22.9791
	12.00	-32.06750(*)	5.29063	.021	-58.8203	-5.3147
	13.00	-4.99750	7.71005	1.000	-47.9395	37.9445
	14.00	-28.92500	7.11276	.146	-67.3369	9.4869
	15.00	-21.24750	9.08531	.614	-75.0574	32.5624
9.00	1.00	-37.03500	12.04055	.368	-110.0149	35.9449
	2.00	19.57750	5.04685	.220	-12.6676	51.8226
	3.00	4.06750	9.65656	1.000	-49.9377	58.0727
	4.00	9.50250	7.35147	.974	-28.0020	47.0070
	5.00	21.67500	4.82475	.175	-12.8237	56.1737
	6.00	21.67500	4.82475	.175	-12.8237	56.1737
	7.00	20.36000	4.93190	.200	-12.8836	53.6036
	8.00	16.18750	6.11827	.474	-15.2516	47.6266
	10.00	-9.95500	8.05780	.980	-52.1093	32.1993
	11.00	14.15000	5.57002	.526	-16.3927	44.6927
	12.00	-15.88000	5.99142	.474	-46.9610	15.2010
	13.00	11.19000	8.20678	.961	-32.0062	54.3862
	14.00	-12.73750	7.64838	.886	-52.1302	26.6552
	15.00	-5.06000	9.51049	1.000	-57.9364	47.8164
10.00	1.00	-27.08000	12.82822	.706	-98.0693	43.9093
	2.00	29.53250	6.71261	.172	-16.0723	75.1373
	3.00	14.02250	10.62250	.969	-40.9509	68.9959
	4.00	19.45750	8.58094	.635	-24.2502	63.1652
	5.00	31.63000	6.54727	.150	-16.0302	79.2902
	6.00	31.63000	6.54727	.150	-16.0302	79.2902
	7.00	30.31500	6.62662	.163	-16.2774	76.9074
	8.00	26.14250	7.55127	.251	-15.5789	67.8639
	9.00	9.95500	8.05780	.980	-32.1993	52.1093
	11.00	24.10500	7.11433	.291	-18.6239	66.8339
	12.00	-5.92500	7.44887	.999	-47.7411	35.8911
	13.00	21.14500	9.32415	.634	-25.9631	68.2531
	14.00	-2.76250	8.63664	1.000	-47.5313	41.9663

	15.00	4.89500	10.48989	1.000	-49.1995	58.9895
11.00	1.00	-51.18500	11.43068	.163	-128.6555	26.2855
	2.00	5.42750	3.34009	.893	-13.1367	23.9917
	3.00	-10.08250	8.88452	.984	-67.1315	46.9665
	4.00	-4.64750	6.30315	1.000	-40.8679	31.5929
	5.00	7.52500	2.99395	.560	-12.6591	27.7091
	6.00	7.52500	2.99395	.560	-12.6591	27.7091
	7.00	6.21000	3.16373	.766	-12.7885	25.2085
	8.00	2.03750	4.80819	1.000	-22.9791	27.0541
	9.00	-14.15000	5.57002	.526	-44.6927	16.3927
	10.00	-24.10500	7.11433	.291	-66.8339	18.6239
	12.00	-30.03000(*)	4.64572	.018	-53.9609	-6.0991
	13.00	-2.96000	7.28264	1.000	-47.0467	41.1267
	14.00	-26.88750	6.64705	.173	-65.8641	12.0891
	15.00	-19.21000	8.72553	.671	-74.9736	36.5536
12.00	1.00	-21.15500	11.64184	.818	-96.6753	54.3653
	2.00	35.45750(*)	4.00352	.011	11.6481	59.2669
	3.00	19.94750	9.15460	.679	-35.4542	75.3492
	4.00	25.38250	6.67845	.182	-10.5753	61.3403
	5.00	37.55500(*)	3.71964	.016	11.6368	63.4732
	6.00	37.55500(*)	3.71964	.016	11.6368	63.4732
	7.00	36.24000(*)	3.85761	.013	11.6279	60.8521
	8.00	32.06750(*)	5.29063	.021	5.3147	58.8203
	9.00	15.86000	5.99142	.474	-15.2010	46.9610
	10.00	5.92500	7.44887	.999	-35.8911	47.7411
	11.00	30.03000(*)	4.64572	.018	6.0991	53.9609
	13.00	27.07000	7.60978	.239	-16.0016	70.1416
	14.00	3.14250	7.00395	1.000	-35.2539	41.5389
	15.00	10.82000	9.00038	.978	-43.3375	64.9775
13.00	1.00	-48.22500	12.92233	.199	-119.1711	22.7211
	2.00	8.38750	6.89074	.972	-38.6309	55.4059
	3.00	-7.12250	10.73596	1.000	-62.4152	48.1702
	4.00	-1.68750	8.72100	1.000	-46.2592	42.8842
	5.00	10.48500	6.72978	.896	-38.5602	59.5302
	6.00	10.48500	6.72978	.896	-38.5602	59.5302
	7.00	9.17000	6.80700	.949	-38.8270	57.1670
	8.00	4.99750	7.71005	1.000	-37.9445	47.9395
	9.00	-11.19000	8.20678	.961	-54.3862	32.0062
	10.00	-21.14500	9.32415	.634	-68.2531	25.9631
	11.00	2.96000	7.28264	1.000	-41.1267	47.0467
	12.00	-27.07000	7.60978	.239	-70.1416	16.0016
	14.00	-23.92750	8.97271	.463	-69.4587	21.6037
	15.00	-16.25000	10.60476	.926	-70.6922	38.1922
14.00	1.00	-24.29750	12.57509	.780	-95.5837	46.9887
	2.00	32.31500	6.21521	.106	-9.3271	73.9571
	3.00	16.80500	10.31539	.896	-37.4867	71.0967
	4.00	22.24000	8.19770	.444	-19.2349	63.7149
	5.00	34.41250	6.03625	.097	-9.3616	78.1866

	6.00	34.41250	6.03625	.097	-9.3616	78.1866
	7.00	33.09750	6.12224	.103	-9.5527	75.7477
	8.00	28.92500	7.11276	.146	-9.4869	67.3369
	9.00	12.73750	7.64838	.886	-26.6552	52.1302
	10.00	2.78250	8.83664	1.000	-41.9663	47.5313
	11.00	26.88750	6.64705	.173	-12.0891	65.8641
	12.00	-3.14250	7.00395	1.000	-41.5389	35.2539
	13.00	23.92750	8.97271	.463	-21.6037	69.4587
	15.00	7.67750	10.17878	1.000	-45.6567	61.0117
15.00	1.00	-31.97500	13.78712	.613	-103.8592	39.9092
	2.00	24.63750	8.40121	.436	-34.2609	83.5359
	3.00	9.12750	11.76260	1.000	-50.2919	68.5469
	4.00	14.56250	9.95760	.940	-38.4185	67.5435
	5.00	26.73500	8.26970	.371	-33.9519	87.4219
	6.00	26.73500	8.26970	.371	-33.9519	87.4219
	7.00	25.42000	8.33267	.409	-34.3665	85.2065
	8.00	21.24750	9.08531	.614	-32.5624	75.0574
	9.00	5.06000	9.51049	1.000	-47.8164	57.9364
	10.00	-4.89500	10.48989	1.000	-58.9895	49.1995
	11.00	19.21000	8.72553	.671	-36.5536	74.9736
	12.00	-10.82000	9.00038	.978	-64.9775	43.3375
	13.00	16.25000	10.60476	.926	-38.1922	70.6922
	14.00	-7.67750	10.17878	1.000	-61.0117	45.6567

* The mean difference is significant at the .05 level.

1-Ampicillin; 2-Cefotaxime; 3-Cefoxitin; 4-Cefpodoxime; 5-Ceftazidime; 6-Ceftriaxone; 7- Cefuroxime; 8-Chloramphenicol; 9-Ciprofloxacin; 10-Co-Trimoxazole; 11-Gentamicin; 12- Nalidixic acid; 13-Streptomycin; 14-Tetracycline; 15-Trimethoprim

Table 2.3c Games-Howell analysis of antibiotic resistance of *E. coli* isolates from station 3 (Off Marine Science Jetty)

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
1.00	2.00	38.51000	11.46530	.327	-39.3218	116.3418
	3.00	21.42250	14.54008	.941	-52.7975	95.6425
	4.00	32.44500	12.05587	.486	-40.9445	105.8345
	5.00	42.95000	11.44199	.256	-35.1300	121.0300
	6.00	41.63500	11.41111	.275	-36.7843	120.0543
	7.00	40.12000	11.33028	.298	-39.2457	119.4857
	8.00	44.41250	11.17738	.239	-36.9956	125.8206
	9.00	32.90000	13.34632	.553	-38.4452	104.2452
	10.00	34.57500	12.24487	.438	-37.9889	107.1389
	11.00	38.85750	12.28503	.339	-33.5605	111.2755
	12.00	18.62250	13.55878	.959	-52.9928	90.2378
	13.00	24.35750	12.40358	.766	-47.6847	96.3997
	14.00	16.94000	11.55068	.925	-60.0371	93.9171
	15.00	29.37750	13.57485	.662	-42.2632	101.0182
2.00	1.00	-38.51000	11.46530	.327	-116.3418	39.3218
	3.00	-17.08750	9.82128	.844	-81.7809	47.6059
	4.00	-6.06500	5.51250	.990	-36.2467	24.1167
	5.00	4.44000	3.99536	.992	-15.7483	24.6283
	6.00	3.12500	3.90604	1.000	-16.6538	22.9038
	7.00	1.61000	3.66319	1.000	-17.2718	20.4918
	8.00	5.90250	3.15873	.803	-12.9100	24.7150
	9.00	-5.61000	7.94784	1.000	-55.1736	43.9536
	10.00	-3.93500	5.91443	1.000	-37.2071	29.3371
	11.00	.34750	5.99714	1.000	-33.5703	34.2653
	12.00	-19.88750	8.29965	.596	-72.3001	32.5251
	13.00	-14.15250	6.23638	.640	-49.9523	21.6473
	14.00	-21.57000	4.29673	.052	-43.3670	.2270
	15.00	-9.13250	8.32588	.987	-61.7575	43.4925
3.00	1.00	-21.42250	14.54008	.941	-95.6425	52.7975
	2.00	17.08750	9.82128	.844	-47.6059	81.7809
	4.00	11.02250	10.50469	.992	-49.9088	71.9538
	5.00	21.52750	9.79407	.674	-43.4132	86.4682
	6.00	20.21250	9.75797	.722	-45.0707	85.4957
	7.00	18.69750	9.66332	.773	-47.5686	84.9636
	8.00	22.99000	9.48359	.596	-45.5224	91.5024
	9.00	11.47750	11.96365	.997	-50.2241	73.1791
	10.00	13.15250	10.72107	.978	-47.3196	73.6246
	11.00	17.43500	10.76692	.894	-42.9736	77.8436
	12.00	-2.80000	12.20020	1.000	-65.2428	59.6428
	13.00	2.93500	10.90199	1.000	-57.3478	63.2178
	14.00	-4.48250	9.92082	1.000	-68.3483	59.3833
	15.00	7.95500	12.21806	1.000	-54.5485	70.4585
4.00	1.00	-32.44500	12.05587	.486	-105.8345	40.9445
	2.00	6.06500	5.51250	.990	-24.1167	36.2467

	3.00	-11.02250	10.50469	.992	-71.9538	49.9088
	5.00	10.50500	5.46386	.785	-19.6945	40.7045
	6.00	9.19000	5.39890	.867	-21.0678	39.4478
	7.00	7.67500	5.22589	.932	-22.9617	38.3117
	8.00	11.96750	4.88554	.582	-20.7683	44.7033
	9.00	.45500	8.77833	1.000	-47.0909	48.0009
	10.00	2.13000	6.99081	1.000	-33.2996	37.5596
	11.00	6.41250	7.06093	.998	-29.4234	42.2484
	12.00	-13.82250	9.09808	.924	-63.7768	36.1318
	13.00	-8.08750	7.26522	.991	-45.1523	28.9773
	14.00	-15.50500	5.68795	.450	-45.7812	14.7712
	15.00	-3.06750	9.12202	1.000	-53.2038	47.0688
5.00	1.00	-42.95000	11.44199	.256	-121.0300	35.1300
	2.00	-4.44000	3.99536	.992	-24.6283	15.7483
	3.00	-21.52750	9.79407	.674	-86.4682	43.4132
	4.00	-10.50500	5.46386	.785	-40.7045	19.6945
	6.00	-1.31500	3.83709	1.000	-20.7124	18.0824
	7.00	-2.83000	3.58958	1.000	-21.2352	15.5752
	8.00	1.46250	3.07307	1.000	-16.6623	19.5873
	9.00	-10.05000	7.91418	.968	-59.8281	39.7281
	10.00	-8.37500	5.86913	.944	-41.7200	24.9700
	11.00	-4.09250	5.95247	1.000	-38.0930	29.9080
	12.00	-24.32750	8.26742	.416	-76.9650	28.3100
	13.00	-18.59250	6.19343	.379	-54.5008	17.3158
	14.00	-26.01000(*)	4.23415	.021	-47.5568	-4.4632
	15.00	-13.57250	8.29376	.880	-66.4231	39.2781
6.00	1.00	-41.63500	11.41111	.275	-120.0543	36.7843
	2.00	-3.12500	3.90604	1.000	-22.9038	16.6538
	3.00	-20.21250	9.75797	.722	-85.4957	45.0707
	4.00	-9.19000	5.39890	.867	-39.4478	21.0678
	5.00	1.31500	3.83709	1.000	-18.0824	20.7124
	7.00	-1.51500	3.48990	1.000	-19.2974	16.2674
	8.00	2.77750	2.95601	.997	-14.4121	19.9671
	9.00	-8.73500	7.86947	.986	-58.8203	41.3503
	10.00	-7.06000	5.80869	.978	-40.5351	26.4151
	11.00	-2.77750	5.89289	1.000	-36.9208	31.3658
	12.00	-23.01250	8.22463	.461	-75.9694	29.9444
	13.00	-17.27750	6.13620	.439	-53.3617	18.8067
	14.00	-24.69500(*)	4.14998	.025	-45.9378	-3.4522
	15.00	-12.25750	8.25110	.922	-65.4282	40.9132
7.00	1.00	-40.12000	11.33028	.298	-119.4857	39.2457
	2.00	-1.61000	3.66319	1.000	-20.4918	17.2718
	3.00	-18.69750	9.66332	.773	-84.9636	47.5686
	4.00	-7.67500	5.22589	.932	-38.3117	22.9617
	5.00	2.83000	3.58958	1.000	-15.5752	21.2352
	6.00	1.51500	3.48990	1.000	-16.2674	19.2974
	8.00	4.29250	2.62675	.890	-10.3119	18.8969
	9.00	-7.22000	7.75180	.996	-58.2466	43.8066

	10.00	-5.54500	5.64825	.995	-39.5749	28.4849
	11.00	-1.26250	5.73480	1.000	-35.9909	33.4659
	12.00	-21.49750	8.11211	.512	-75.4159	32.4209
	13.00	-15.76250	5.98454	.507	-52.5097	20.9847
	14.00	-23.18000(*)	3.92226	.030	-43.8298	-2.5302
	15.00	-10.74250	8.13895	.957	-64.8759	43.3909
8.00	1.00	-44.41250	11.17738	.239	-125.8206	36.9956
	2.00	-5.90250	3.15873	.803	-24.7150	12.9100
	3.00	-22.99000	9.48359	.596	-91.5024	45.5224
	4.00	-11.96750	4.88554	.582	-44.7033	20.7683
	5.00	-1.46250	3.07307	1.000	-19.5873	16.6623
	6.00	-2.77750	2.95601	.997	-19.9671	14.4121
	7.00	-4.29250	2.62675	.890	-18.8969	10.3119
	9.00	-11.51250	7.52655	.905	-64.9837	41.9587
	10.00	-9.83750	5.33492	.806	-46.1518	26.4768
	11.00	-5.55500	5.42647	.991	-42.5954	31.4854
	12.00	-25.79000	7.89715	.358	-82.1250	30.5450
	13.00	-20.05500	5.68976	.297	-59.1784	19.0684
	14.00	-27.47250(*)	3.45583	.020	-48.6826	-6.2624
	15.00	-15.03500	7.92472	.786	-71.5827	41.5127
9.00	1.00	-32.90000	13.34632	.553	-104.2452	38.4452
	2.00	5.61000	7.94784	1.000	-43.9536	55.1736
	3.00	-11.47750	11.96365	.997	-73.1791	50.2241
	4.00	-.45500	8.77833	1.000	-48.0009	47.0909
	5.00	10.05000	7.91418	.968	-39.7281	59.8281
	6.00	8.73500	7.86947	.986	-41.3503	58.8203
	7.00	7.22000	7.75180	.996	-43.8066	58.2466
	8.00	11.51250	7.52655	.905	-41.9587	64.9837
	10.00	1.67500	9.03614	1.000	-46.1141	49.4641
	11.00	5.95750	9.09050	1.000	-41.9187	53.8337
	12.00	-14.27750	10.74968	.969	-68.6228	40.0678
	13.00	-8.54250	9.25007	.998	-56.7379	39.6529
	14.00	-15.96000	8.07052	.760	-64.8540	32.9340
	15.00	-3.52250	10.76994	1.000	-57.9780	50.9330
10.00	1.00	-34.57500	12.24487	.438	-107.1389	37.9889
	2.00	3.93500	5.91443	1.000	-29.3371	37.2071
	3.00	-13.15250	10.72107	.978	-73.6246	47.3196
	4.00	-2.13000	6.99081	1.000	-37.5596	33.2996
	5.00	8.37500	5.86913	.944	-24.9700	41.7200
	6.00	7.06000	5.80869	.978	-26.4151	40.5351
	7.00	5.54500	5.64825	.995	-28.4849	39.5749
	8.00	9.83750	5.33492	.806	-26.4768	46.1518
	9.00	-1.67500	9.03614	1.000	-49.4641	46.1141
	11.00	4.28250	7.37899	1.000	-32.9917	41.5567
	12.00	-15.95250	9.34708	.869	-65.9890	34.0840
	13.00	-10.21750	7.57471	.966	-48.5443	28.1093
	14.00	-17.63500	6.07829	.395	-50.7983	15.5283
	15.00	-5.19750	9.37038	1.000	-55.4046	45.0096

11.00	1.00	-38.85750	12.28503	.339	-111.2755	33.5605
	2.00	-.34750	5.99714	1.000	-34.2653	33.5703
	3.00	-17.43500	10.76692	.894	-77.8436	42.9736
	4.00	-6.41250	7.06093	.998	-42.2484	29.4234
	5.00	4.09250	5.95247	1.000	-29.9080	38.0930
	6.00	2.77750	5.89289	1.000	-31.3658	36.9208
	7.00	1.26250	5.73480	1.000	-33.4659	35.9909
	8.00	5.55500	5.42647	.991	-31.4854	42.5954
	9.00	-5.95750	9.09050	1.000	-53.8337	41.9187
	10.00	-4.28250	7.37899	1.000	-41.5567	32.9917
	12.00	-20.23500	9.39964	.688	-70.3247	29.8547
	13.00	-14.50000	7.63947	.800	-53.1226	24.1226
	14.00	-21.91750	6.15881	.229	-55.6897	11.8547
	15.00	-9.48000	9.42281	.996	-59.7379	40.7779
12.00	1.00	-18.62250	13.55878	.959	-90.2378	52.9928
	2.00	19.88750	8.29965	.596	-32.5251	72.3001
	3.00	2.80000	12.20020	1.000	-59.6428	65.2428
	4.00	13.82250	9.09808	.924	-36.1318	63.7768
	5.00	24.32750	8.26742	.416	-28.3100	76.9650
	6.00	23.01250	8.22463	.461	-29.9444	75.9694
	7.00	21.49750	8.11211	.512	-32.4209	75.4159
	8.00	25.79000	7.89715	.358	-30.5450	82.1250
	9.00	14.27750	10.74968	.969	-40.0678	68.6228
	10.00	15.95250	9.34708	.869	-34.0840	65.9890
	11.00	20.23500	9.39964	.688	-29.8547	70.3247
	13.00	5.73500	9.55405	1.000	-44.5748	56.0448
	14.00	-1.66250	8.41720	1.000	-53.3791	50.0141
	15.00	10.75500	11.03212	.997	-44.9658	66.4758
13.00	1.00	-24.35750	12.40358	.766	-96.3997	47.6847
	2.00	14.15250	6.23638	.640	-21.6473	49.9523
	3.00	-2.93500	10.90199	1.000	-63.2178	57.3478
	4.00	8.08750	7.26522	.991	-28.9773	45.1523
	5.00	18.59250	6.19343	.379	-17.3158	54.5008
	6.00	17.27750	6.13620	.439	-18.8067	53.3617
	7.00	15.76250	5.98454	.507	-20.9847	52.5097
	8.00	20.05500	5.68976	.297	-19.0684	59.1784
	9.00	8.54250	9.25007	.998	-39.6529	56.7379
	10.00	10.21750	7.57471	.966	-28.1093	48.5443
	11.00	14.50000	7.63947	.800	-24.1226	53.1226
	12.00	-5.73500	9.55405	1.000	-56.0448	44.5748
	14.00	-7.41750	6.39200	.985	-42.9737	28.1387
	15.00	5.02000	9.57685	1.000	-45.4509	55.4909
14.00	1.00	-16.94000	11.55068	.925	-93.9171	60.0371
	2.00	21.57000	4.29673	.052	-.2270	43.3670
	3.00	4.48250	9.92082	1.000	-59.3833	68.3483
	4.00	15.50500	5.68795	.450	-14.7712	45.7812
	5.00	26.01000(*)	4.23415	.021	4.4632	47.5568
	6.00	24.69500(*)	4.14998	.025	3.4522	45.9378

	7.00	23.18000(*)	3.92226	.030	2.5302	43.8298
	8.00	27.47250(*)	3.45583	.020	6.2624	48.6826
	9.00	15.96000	8.07052	.760	-32.9340	64.8540
	10.00	17.63500	6.07829	.395	-15.5283	50.7983
	11.00	21.91750	6.15881	.229	-11.8547	55.6897
	12.00	1.68250	8.41720	1.000	-50.0141	53.3791
	13.00	7.41750	6.39200	.985	-28.1387	42.9737
	15.00	12.43750	8.44307	.928	-39.4684	64.3434
15.00	1.00	-29.37750	13.57485	.682	-101.0182	42.2632
	2.00	9.13250	8.32588	.987	-43.4925	61.7575
	3.00	-7.95500	12.21806	1.000	-70.4585	54.5485
	4.00	3.06750	9.12202	1.000	-47.0688	53.2038
	5.00	13.57250	8.29376	.880	-39.2781	66.4231
	6.00	12.25750	8.25110	.922	-40.9132	65.4282
	7.00	10.74250	8.13895	.957	-43.3909	64.8759
	8.00	15.03500	7.92472	.786	-41.5127	71.5827
	9.00	3.52250	10.76994	1.000	-50.9330	57.9780
	10.00	5.19750	9.37038	1.000	-45.0096	55.4046
	11.00	9.48000	9.42281	.996	-40.7779	59.7379
	12.00	-10.75500	11.03212	.997	-66.4758	44.9658
	13.00	-5.02000	9.57685	1.000	-55.4909	45.4509
	14.00	-12.43750	8.44307	.928	-64.3434	39.4684

* The mean difference is significant at the .05 level.

1-Ampicillin; 2-Cefotaxime; 3-Cefoxitin; 4-Cefpodoxime; 5-Ceftazidime; 6-Ceftriaxone; 7- Cefuroxime; 8-Chloramphenicol; 9-Ciprofloxacin; 10-Co-Trimoxazole; 11-Gentamicin; 12- Nalidixic acid; 13-Streptomycin; 14-Tetracycline; 15-Trimethoprim

Table 2.4 Anova for antibiotic resistance of *E. coli* isolates of different phylogenetic groups in Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	2465.333	4	616.333	2.508	.050
	Within Groups	17199.937	70	245.713		
	Total	19665.271	74			
B1	Between Groups	2650.381	4	662.595	1.846	.130
	Within Groups	25129.054	70	358.986		
	Total	27779.435	74			
B2	Between Groups	597.861	4	149.465	.718	.583
	Within Groups	14579.970	70	208.285		
	Total	15177.831	74			
D	Between Groups	3132.283	4	783.071	2.640	.041
	Within Groups	20760.272	70	296.575		
	Total	23892.555	74			

Table 2.5 Anova for antibiotic resistance of *E. coli* isolates of different phylogenetic groups in five different stations set at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S1	Between Groups	1353.823	3	451.274	2.184	.100
	Within Groups	11568.508	56	206.581		
	Total	12922.331	59			
S2	Between Groups	1478.792	3	492.931	1.330	.274
	Within Groups	20756.579	56	370.653		
	Total	22235.371	59			
S3	Between Groups	189.282	3	63.094	.241	.867
	Within Groups	14642.348	56	261.471		
	Total	14831.630	59			
S4	Between Groups	2326.226	3	775.409	2.990	.039
	Within Groups	14523.737	56	259.352		
	Total	16849.964	59			
S5	Between Groups	1724.367	3	574.789	1.990	.126
	Within Groups	16178.060	56	288.894		
	Total	17902.427	59			

S1-Chittor, S2-Bolgatty, S3-Off Marine Science Jetty, S4-Thevara, S5-Barmouth

Table 2.6a Anova for antibiotic resistance of *E. coli* isolates from clinical sources

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32947.698	14	2353.407	8.525	.000
Within Groups	12422.925	45	276.065		
Total	45370.623	59			

Table 2.6b Games-Howell analysis of antibiotic resistance of *E. coli* isolates from clinical sources

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
1.00	2.00	51.87500(*)	5.71604	.003	23.0019	80.7481
	3.00	2.18750	5.76799	1.000	-26.9452	31.3202
	4.00	5.00000	6.88682	1.000	-31.0150	41.0150
	5.00	59.79250(*)	9.39845	.037	4.6184	114.9666
	6.00	68.91750(*)	8.97225	.017	17.1386	120.6964
	7.00	50.62500(*)	5.67801	.003	21.9371	79.3129
	8.00	68.33500(*)	10.03361	.032	8.0592	128.6108
	9.00	38.44000	18.05726	.701	-86.1592	163.0392
	10.00	45.73000(*)	5.20256	.004	18.9275	72.5325
	11.00	70.83500(*)	9.88593	.026	11.7487	129.9213
	12.00	17.19000	8.86907	.777	-33.7716	68.1516
	13.00	53.12500(*)	6.01925	.003	22.6406	83.6094
	14.00	36.35500	14.31123	.549	-58.4614	131.1714
	15.00	43.96000	8.08261	.054	-.8601	88.7801
2.00	1.00	-51.87500(*)	5.71604	.003	-80.7481	-23.0019
	3.00	-49.68750(*)	5.72257	.003	-78.5945	-20.7805
	4.00	-46.87500(*)	6.84883	.015	-82.8144	-10.9356
	5.00	7.91750	9.37065	.999	-47.3520	63.1870
	6.00	17.04250	8.94312	.789	-34.8136	68.8986
	7.00	-1.25000	5.63186	1.000	-29.6972	27.1972
	8.00	16.46000	10.00757	.881	-43.9328	76.8528
	9.00	-13.43500	18.04280	.999	-138.1999	111.3299
	10.00	-6.14500	5.15216	.985	-32.6197	20.3297
	11.00	18.96000	9.85950	.781	-40.2388	78.1588
	12.00	-34.68500	8.83960	.185	-85.7189	16.3489
	13.00	1.25000	5.97575	1.000	-29.0481	31.5481
	14.00	-15.52000	14.29299	.987	-110.5059	79.4659
	15.00	-7.91500	8.05026	.996	-52.7623	36.9323
3.00	1.00	-2.18750	5.76799	1.000	-31.3202	26.9452
	2.00	49.68750(*)	5.72257	.003	20.7805	78.5945
	4.00	2.81250	6.89224	1.000	-33.2138	38.8388
	5.00	57.60500(*)	9.40243	.043	2.4440	112.7660
	6.00	66.73000(*)	8.97642	.019	14.9617	118.4983
	7.00	48.43750(*)	5.68458	.004	19.7148	77.1602
	8.00	66.14750(*)	10.03733	.036	5.8880	126.4070
	9.00	36.25250	18.05933	.746	-88.3231	160.8281
	10.00	43.54250(*)	5.20973	.005	16.6930	70.3920
	11.00	68.64750(*)	9.88971	.029	9.5768	127.7182
	12.00	15.00250	8.87329	.869	-35.9493	65.9543
	13.00	50.93750(*)	6.02546	.004	20.4260	81.4490
	14.00	34.16750	14.31384	.603	-60.6248	128.9598
	15.00	41.77250	8.08723	.066	-3.0443	86.5893
4.00	1.00	-5.00000	6.88682	1.000	-41.0150	31.0150

	2.00	46.87500(*)	6.84883	.015	10.9356	82.8144
	3.00	-2.81250	6.89224	1.000	-38.8388	33.2138
	5.00	54.79250(*)	10.12740	.049	.4016	109.1834
	6.00	63.91750(*)	9.73317	.019	12.3883	115.4467
	7.00	45.62500(*)	6.81712	.017	9.7432	81.5068
	8.00	63.33500(*)	10.71945	.037	4.5141	122.1559
	9.00	33.44000	18.44717	.819	-87.2851	154.1651
	10.00	40.73000(*)	6.42650	.029	5.0779	76.3821
	11.00	65.83500(*)	10.58135	.029	8.0595	123.6105
	12.00	12.19000	9.63814	.976	-38.6623	63.0423
	13.00	48.12500(*)	7.10386	.014	11.5562	84.6938
	14.00	31.35500	14.60016	.703	-59.8876	122.5976
	15.00	38.96000	8.91975	.099	-6.9812	84.9012
5.00	1.00	-59.79250(*)	9.39845	.037	-114.9666	-4.6184
	2.00	-7.91750	9.37065	.999	-63.1870	47.3520
	3.00	-57.60500(*)	9.40243	.043	-112.7660	-2.4440
	4.00	-54.79250(*)	10.12740	.049	-109.1834	-.4016
	6.00	9.12500	11.64634	1.000	-49.7736	68.0236
	7.00	-9.16750	9.34750	.995	-64.5211	46.1861
	8.00	8.54250	12.48236	1.000	-54.6559	71.7409
	9.00	-21.35250	19.52436	.989	-135.9407	93.2357
	10.00	-14.06250	9.06656	.907	-70.8121	42.6871
	11.00	11.04250	12.36396	.999	-51.4962	73.5812
	12.00	-42.60250	11.56704	.160	-101.1428	15.9378
	13.00	-6.66750	9.55863	1.000	-61.4023	48.0673
	14.00	-23.43750	16.12288	.941	-111.4343	64.5593
	15.00	-15.83250	10.97563	.947	-72.0542	40.3892
6.00	1.00	-68.91750(*)	8.97225	.017	-120.6964	-17.1386
	2.00	-17.04250	8.94312	.769	-68.8986	34.8136
	3.00	-66.73000(*)	8.97642	.019	-118.4983	-14.9617
	4.00	-63.91750(*)	9.73317	.019	-115.4467	-12.3883
	5.00	-9.12500	11.64634	1.000	-68.0236	49.7736
	7.00	-18.29250	8.91886	.731	-70.2178	33.6328
	8.00	-.58250	12.16469	1.000	-62.4661	61.3011
	9.00	-30.47750	19.32281	.902	-145.8102	84.8552
	10.00	-23.18750	8.62397	.489	-76.3533	29.9783
	11.00	1.91750	12.04317	1.000	-59.2422	63.0772
	12.00	-51.72750	11.22349	.075	-108.4193	4.9643
	13.00	-15.79250	9.13990	.858	-67.2420	35.6570
	14.00	-32.56250	15.87822	.731	-120.6510	55.5260
	15.00	-24.95750	10.61296	.597	-78.9381	29.0231
7.00	1.00	-50.62500(*)	5.67801	.003	-79.3129	-21.9371
	2.00	1.25000	5.63186	1.000	-27.1972	29.6972
	3.00	-48.43750(*)	5.68458	.004	-77.1602	-19.7148
	4.00	-45.62500(*)	6.81712	.017	-81.5068	-9.7432
	5.00	9.16750	9.34750	.995	-46.1861	64.5211
	6.00	18.29250	8.91886	.731	-33.6328	70.2178
	8.00	17.71000	9.98590	.837	-42.7845	78.2045

	9.00	-12.18500	18.03079	1.000	-137.0890	112.7190
	10.00	-4.89500	5.10993	.997	-31.0984	21.3084
	11.00	20.21000	9.83750	.729	-39.0868	79.5068
	12.00	-33.43500	8.81506	.206	-84.5341	17.6641
	13.00	2.50000	5.93938	1.000	-27.6471	32.6471
	14.00	-14.27000	14.27782	.993	-109.3992	80.8592
	15.00	-6.66500	8.02330	.999	-51.5402	38.2102
8.00	1.00	-68.33500(*)	10.03361	.032	-128.6108	-8.0592
	2.00	-16.46000	10.00757	.881	-76.8528	43.9328
	3.00	-66.14750(*)	10.03733	.036	-126.4070	-5.8880
	4.00	-63.33500(*)	10.71945	.037	-122.1559	-4.5141
	5.00	-8.54250	12.48236	1.000	-71.7409	54.6559
	6.00	.58250	12.16469	1.000	-61.3011	62.4661
	7.00	-17.71000	9.98590	.837	-76.2045	42.7845
	9.00	-29.89500	19.83792	.924	-143.6168	83.8268
	10.00	-22.60500	9.72342	.624	-84.6720	39.4620
	11.00	2.50000	12.85340	1.000	-62.4273	67.4273
	12.00	-51.14500	12.08879	.109	-112.7402	10.4502
	13.00	-15.21000	10.18380	.926	-74.9132	44.4932
	14.00	-31.98000	16.50120	.780	-120.1667	56.2067
	15.00	-24.37500	11.52419	.704	-84.1954	35.4454
9.00	1.00	-38.44000	18.05726	.701	-163.0392	86.1592
	2.00	13.43500	18.04280	.999	-111.3299	138.1999
	3.00	-36.25250	18.05933	.746	-160.8281	88.3231
	4.00	-33.44000	18.44717	.819	-154.1651	87.2851
	5.00	21.35250	19.52436	.989	-93.2357	135.9407
	6.00	30.47750	19.32281	.902	-84.8552	145.8102
	7.00	12.18500	18.03079	1.000	-112.7190	137.0890
	8.00	29.89500	19.83792	.924	-83.8268	143.6168
	10.00	7.29000	17.88676	1.000	-119.3767	133.9567
	11.00	32.39500	19.76364	.886	-81.5021	146.2921
	12.00	-21.25000	19.27512	.988	-136.7824	94.2824
	13.00	14.68500	18.14114	.999	-108.9856	138.3556
	14.00	-2.08500	22.30877	1.000	-117.3749	113.2049
	15.00	5.52000	18.92612	1.000	-111.7753	122.8153
10.00	1.00	-45.73000(*)	5.20256	.004	-72.5325	-18.9275
	2.00	6.14500	5.15216	.985	-20.3297	32.6197
	3.00	-43.54250(*)	5.20973	.005	-70.3920	-16.6930
	4.00	-40.73000(*)	6.42650	.029	-76.3821	-5.0779
	5.00	14.06250	9.06656	.907	-42.6871	70.8121
	6.00	23.18750	8.62397	.489	-29.9783	76.3533
	7.00	4.89500	5.10993	.997	-21.3084	31.0984
	8.00	22.60500	9.72342	.624	-39.4620	84.6720
	9.00	-7.29000	17.88676	1.000	-133.9567	119.3767
	11.00	25.10500	9.57096	.515	-35.7285	85.9385
	12.00	-28.54000	8.51658	.305	-80.8366	23.7566
	13.00	7.39500	5.48662	.964	-21.3267	36.1167
	14.00	-9.37500	14.09550	1.000	-106.3946	87.6446

	15.00	-1.77000	7.69416	1.000	-47.4319	43.8919
11.00	1.00	-70.83500(*)	9.88593	.026	-129.9213	-11.7487
	2.00	-18.96000	9.85950	.781	-78.1588	40.2388
	3.00	-68.64750(*)	9.88971	.029	-127.7182	-9.5768
	4.00	-65.83500(*)	10.58135	.029	-123.6105	-8.0595
	5.00	-11.04250	12.36396	.999	-73.5812	51.4962
	6.00	-1.91750	12.04317	1.000	-63.0772	59.2422
	7.00	-20.21000	9.63750	.729	-79.5068	39.0868
	8.00	-2.50000	12.85340	1.000	-67.4273	62.4273
	9.00	-32.39500	19.76364	.886	-146.2921	81.5021
	10.00	-25.10500	9.57096	.515	-85.9385	35.7285
	12.00	-53.64500	11.96650	.086	-114.5005	7.2105
	13.00	-17.71000	10.03833	.843	-76.2518	40.8318
	14.00	-34.48000	16.41183	.710	-122.5886	53.6286
	15.00	-26.87500	11.39584	.595	-85.8337	32.0837
12.00	1.00	-17.19000	8.86907	.777	-68.1516	33.7716
	2.00	34.68500	8.83960	.185	-16.3489	85.7189
	3.00	-15.00250	8.87329	.869	-65.9543	35.9493
	4.00	-12.19000	9.63814	.976	-63.0423	38.6623
	5.00	42.60250	11.56704	.180	-15.9378	101.1428
	6.00	51.72750	11.22349	.075	-4.9643	108.4193
	7.00	33.43500	8.81506	.206	-17.6641	84.5341
	8.00	51.14500	12.08879	.109	-10.4502	112.7402
	9.00	21.25000	19.27512	.988	-94.2824	136.7824
	10.00	28.54000	8.51658	.305	-23.7566	80.8366
	11.00	53.64500	11.96650	.086	-7.2105	114.5005
	13.00	35.93500	9.03863	.169	-14.7267	86.5967
	14.00	19.16500	15.82014	.980	-68.9729	107.3029
	15.00	26.77000	10.52587	.513	-26.6919	80.2319
13.00	1.00	-53.12500(*)	6.01925	.003	-83.6094	-22.6406
	2.00	-1.25000	5.97575	1.000	-31.5481	29.0481
	3.00	-50.93750(*)	6.02546	.004	-81.4490	-20.4260
	4.00	-48.12500(*)	7.10386	.014	-84.6938	-11.5562
	5.00	6.66750	9.55863	1.000	-48.0673	61.4023
	6.00	15.79250	9.13990	.858	-35.6570	67.2420
	7.00	-2.50000	5.93938	1.000	-32.6471	27.6471
	8.00	15.21000	10.18380	.926	-44.4932	74.9132
	9.00	-14.68500	18.14114	.999	-138.3556	108.9856
	10.00	-7.39500	5.48662	.964	-36.1167	21.3267
	11.00	17.71000	10.03833	.843	-40.8318	76.2518
	12.00	-35.93500	9.03863	.169	-86.5967	14.7267
	14.00	-16.77000	14.41693	.980	-110.6601	77.1201
	15.00	-9.16500	8.26831	.990	-53.9518	35.6218
14.00	1.00	-36.35500	14.31123	.549	-131.1714	58.4614
	2.00	15.52000	14.29299	.987	-79.4659	110.5059
	3.00	-34.16750	14.31384	.603	-128.9598	60.6248
	4.00	-31.35500	14.80016	.703	-122.5976	59.8876
	5.00	23.43750	16.12288	.941	-64.5593	111.4343

	6.00	32.56250	15.87822	.731	-55.5260	120.6510
	7.00	14.27000	14.27762	.993	-60.8592	109.3992
	8.00	31.98000	16.50120	.780	-56.2067	120.1667
	9.00	2.08500	22.30877	1.000	-113.2049	117.3749
	10.00	9.37500	14.09550	1.000	-87.6446	106.3946
	11.00	34.48000	16.41183	.710	-53.6286	122.5886
	12.00	-19.16500	15.82014	.980	-107.3029	68.9729
	13.00	16.77000	14.41693	.980	-77.1201	110.6601
	15.00	7.60500	15.39302	1.000	-81.2695	96.4795
15.00	1.00	-43.96000	8.08261	.054	-88.7801	.8601
	2.00	7.91500	8.05026	.996	-36.9323	52.7623
	3.00	-41.77250	8.08723	.066	-86.5893	3.0443
	4.00	-38.96000	8.91975	.099	-84.9012	6.9812
	5.00	15.83250	10.97563	.947	-40.3892	72.0542
	6.00	24.95750	10.61296	.597	-29.0231	78.9381
	7.00	6.66500	8.02330	.999	-38.2102	51.5402
	8.00	24.37500	11.52419	.704	-35.4454	84.1954
	9.00	-5.52000	18.92612	1.000	-122.8153	111.7753
	10.00	1.77000	7.69416	1.000	-43.8919	47.4319
	11.00	26.87500	11.39584	.595	-32.0837	85.8337
	12.00	-26.77000	10.52587	.513	-80.2319	26.6919
	13.00	9.16500	8.26831	.990	-35.6218	53.9518
	14.00	-7.60500	15.39302	1.000	-96.4795	81.2695

* The mean difference is significant at the .05 level.

1-Ampicillin; 2-Cefotaxime; 3-Cefoxitin; 4-Cefpodoxime; 5-Ceftazidime; 6-Ceftriaxone; 7- Cefuroxime; 8-Chloramphenicol; 9-Ciprofloxacin; 10-Co-Trimoxazole; 11-Gentamicin; 12- Nalidixic acid; 13-Streptomycin; 14-Tetracycline; 15-Trimethoprim

Table 2.7 Anova for antibiotic resistance among different phylogenetic groups of *E. coli* isolates from clinical sources

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4716.549	3	1572.183	2.168	.102
Within Groups	40603.425	56	725.061		
Total	45319.974	59			

Table 2.8 Anova for antibiotic resistance of *E. coli* isolates from seafood sources

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32947.698	14	2353.407	8.525	.000
Within Groups	12422.925	45	276.065		
Total	45370.623	59			

Table 2.9a Anova for antibiotic resistance among different phylogenetic groups of *E. coli* isolates from seafood sources

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1227.277	3	409.092	2.341	.083
Within Groups	9786.726	56	174.763		
Total	11014.002	59			

Table 2.9b Games-Howell analysis of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from seafood sources

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	2.32333	5.91761	.979	-14.0814	18.7281
	3.00	8.00733	4.45703	.296	-4.1710	20.1857
	4.00	11.43333*	3.06218	.009	2.6560	20.2107
2.00	1.00	-2.32333	5.91761	.979	-18.7281	14.0814
	3.00	5.68400	6.10135	.788	-11.1462	22.5142
	4.00	9.11000	5.17092	.329	-5.8433	24.0633
3.00	1.00	-8.00733	4.45703	.296	-20.1857	4.1710
	2.00	-5.68400	6.10135	.788	-22.5142	11.1462
	4.00	3.42600	3.40372	.748	-6.3549	13.2069
4.00	1.00	-11.43333*	3.06218	.009	-20.2107	-2.6560
	2.00	-9.11000	5.17092	.329	-24.0633	5.8433
	3.00	-3.42600	3.40372	.748	-13.2069	6.3549

*. The mean difference is significant at the .05 level.

1-Phylogenetic group A, 2- Phylogenetic group B1, 3- Phylogenetic group B2, 4- Phylogenetic group D.

Table 2.10 Anova for antibiotic resistance of *E. coli* isolates from different sources

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10411.836	14	743.703	1.448	.171
Within Groups	23119.329	45	513.763		
Total	33531.165	59			

Table 2.11 Games-Howell analysis of antibiotic resistance of *E. coli* isolates from different sources

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	41.26667*	7.46514	.000	22.3714	60.1619
	3.00	36.02533*	7.46358	.000	17.1331	54.9176
2.00	1.00	-41.26667*	7.46514	.000	-60.1619	-22.3714
	3.00	-5.24133	4.48372	.481	-16.3356	5.8530
3.00	1.00	-36.02533*	7.46358	.000	-54.9176	-17.1331
	2.00	5.24133	4.48372	.481	-5.8530	16.3356

*. The mean difference is significant at the .05 level.

1-Clinical source, 2-Seafood source; 3-Estuarine source

Table 2.12 Games-Howell analysis of antibiotic resistance among different phylogenetic groups of *E. coli* isolates from clinical sources

Multiple Comparisons

Games-Howell

Dependent Variable	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
A	1.00	2.00	36.15533*	8.70441	.002	13.8993	58.4113
		3.00	33.31867*	8.90932	.004	10.6878	55.9495
	2.00	1.00	-36.15533*	8.70441	.002	-58.4113	-13.8993
		3.00	-2.83667	4.61150	.813	-14.2653	8.5920
	3.00	1.00	-33.31867*	8.90932	.004	-55.9495	-10.6878
		2.00	2.83667	4.61150	.813	-8.5920	14.2653
B1	1.00	2.00	60.14933*	8.30945	.000	39.5225	80.7761
		3.00	57.05800*	7.56354	.000	38.0837	76.0323
	2.00	1.00	-60.14933*	8.30945	.000	-80.7761	-39.5225
		3.00	-3.09133	6.36708	.879	-18.9202	12.7375
	3.00	1.00	-57.05800*	7.56354	.000	-76.0323	-38.0837
		2.00	3.09133	6.36708	.879	-12.7375	18.9202
B2	1.00	2.00	46.33333*	7.58628	.000	27.1616	65.5050
		3.00	37.48933*	7.22357	.000	18.9940	55.9847
	2.00	1.00	-46.33333*	7.58628	.000	-65.5050	-27.1616
		3.00	-8.84400	4.08679	.097	-19.0141	1.3261
	3.00	1.00	-37.48933*	7.22357	.000	-55.9847	-18.9940
		2.00	8.84400	4.08679	.097	-1.3261	19.0141
D	1.00	2.00	49.25600*	6.13088	.000	33.2623	65.2497
		3.00	31.42067*	7.40975	.001	12.9622	49.8791
	2.00	1.00	-49.25600*	6.13088	.000	-65.2497	-33.2623
		3.00	-17.83533*	4.29113	.002	-28.9927	-6.6780
	3.00	1.00	-31.42067*	7.40975	.001	-49.8791	-12.9622
		2.00	17.83533*	4.29113	.002	6.6780	28.9927

*. The mean difference is significant at the .05 level.

Table 2.13 Anova for MAR index of *E. coli* isolates from five different stations set at Cochin estuary

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.207	4	.052	1.809	.129
Within Groups	5.209	182	.029		
Total	5.416	186			

Table 2.14 Games-Howell analysis of MAR index of *E. coli* isolates from different sources

Multiple Comparisons

Games-Howell

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.34505*	.03335	.000	.2660	.4240
	3.00	.27771*	.02684	.000	.2142	.3412
2.00	1.00	-.34505*	.03335	.000	-.4240	-.2660
	3.00	-.06734*	.02653	.034	-.1306	-.0041
3.00	1.00	-.27771*	.02684	.000	-.3412	-.2142
	2.00	.06734*	.02653	.034	.0041	.1306

*. The mean difference is significant at the .05 level.

1-Clinical source, 2-Seafood source; 3-Estuarine source

Table 2.15 Correlation between antibiotic resistance phenotypes in *E. coli* isolates from Cochin estuary

		Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Amp	Pearson Correlation	1	-.305	.076	.200	-.490	-.548	-.305	.111	-.108	.823	-.317	.746	.156	.599	.810
	Sig. (2-tailed)		.695	.924	.800	.510	.452	.695	.889	.892	.177	.683	.254	.844	.401	.190
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Ctx	Pearson Correlation	-.305	1	.926	.816	.471	.494	1.000**	.775	-.494	.030	.977*	.030	.556	.292	-.063
	Sig. (2-tailed)	.695		.074	.184	.529	.506	.000	.225	.506	.970	.023	.970	.444	.708	.937
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cx	Pearson Correlation	.076	.926	1	.945	.327	.326	.926	.837	-.588	.340	.905	.305	.617	.521	.235
	Sig. (2-tailed)	.924	.074		.055	.673	.674	.074	.163	.412	.660	.095	.695	.383	.479	.765
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cpd	Pearson Correlation	.200	.816	.945	1	.481	.461	.816	.632	-.806	.275	.859	.195	.383	.371	.155
	Sig. (2-tailed)	.800	.184	.055		.519	.539	.184	.368	.194	.725	.141	.805	.617	.629	.845
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Caz	Pearson Correlation	-.490	.471	.327	.481	1	.998**	.471	-.183	-.798	-.693	.638	-.763	-.471	-.628	-.777
	Sig. (2-tailed)	.510	.529	.673	.519		.002	.529	.817	.202	.307	.362	.237	.529	.372	.223
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Ctr	Pearson Correlation	-.548	.494	.326	.461	.998**	1	.494	-.164	-.761	-.720	.652	-.780	-.447	-.635	-.800
	Sig. (2-tailed)	.452	.506	.674	.539	.002		.506	.836	.239	.280	.348	.220	.553	.365	.200
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cxm	Pearson Correlation	-.305	1.000**	.926	.816	.471	.494	1	.775	-.494	.030	.977*	.030	.556	.292	-.063
	Sig. (2-tailed)	.695	.000	.074	.184	.529	.506		.225	.506	.970	.023	.970	.444	.708	.937
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C	Pearson Correlation	.111	.775	.837	.632	-.183	-.164	.775	1	-.055	.593	.640	.627	.947	.814	.540
	Sig. (2-tailed)	.889	.225	.163	.368	.817	.836	.225		.945	.407	.360	.373	.053	.186	.460
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cip	Pearson Correlation	-.108	-.494	-.588	-.806	-.798	-.761	-.494	-.055	1	.149	-.652	.273	.258	.176	.263
	Sig. (2-tailed)	.892	.506	.412	.194	.202	.239	.506	.945		.851	.348	.727	.742	.824	.737
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cot	Pearson Correlation	.823	.030	.340	.275	-.693	-.720	.030	.593	.149	1	-.090	.989*	.684	.945	.992**
	Sig. (2-tailed)	.177	.970	.660	.725	.307	.280	.970	.407	.851		.910	.011	.316	.055	.008
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Gen	Pearson Correlation	-.317	.977*	.905	.859	.638	.652	.977*	.640	-.652	-.090	1	-.115	.376	.139	-.195
	Sig. (2-tailed)	.683	.023	.095	.141	.362	.348	.023	.360	.348	.910		.885	.624	.861	.805
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Na	Pearson Correlation	.746	.030	.305	.195	-.763	-.780	.030	.627	.273	.989*	-.115	1	.749	.963*	.993**
	Sig. (2-tailed)	.254	.970	.695	.805	.237	.220	.970	.373	.727	.011	.885		.251	.037	.007
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
S	Pearson Correlation	.156	.556	.617	.383	-.471	-.447	.556	.947	.258	.684	.376	.749	1	.884	.669
	Sig. (2-tailed)	.844	.444	.383	.617	.529	.553	.444	.053	.742	.316	.624	.251		.116	.331
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Te	Pearson Correlation	.599	.292	.521	.371	-.628	-.635	.292	.814	.176	.945	.139	.963*	.884	1	.928
	Sig. (2-tailed)	.401	.708	.479	.629	.372	.365	.708	.186	.824	.055	.861	.037	.116		.072
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Tr	Pearson Correlation	.810	-.063	.235	.155	-.777	-.800	-.063	.540	.263	.992**	-.195	.993**	.669	.928	1
	Sig. (2-tailed)	.190	.937	.765	.845	.223	.200	.937	.460	.737	.008	.805	.007	.331	.072	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Amp-Ampicillin; Ctx-Cefotaxime; Cx-Cefoxitin; Cpd-Cefpodoxime; Caz-Ceftazidime; Ctr-Ceftriaxone; Cxm-Cefuroxime; C-Chloramphenicol; Cip-Ciprofloxacin (, 5mcg), Co-Co-Trimoxazole; Gen-Gentamicin; Na-Nalidixic acid; S-Streptomycin; Te-Tetracycline; Tr- Trimethoprim.

Table 2.16 Correlation between antibiotic resistance phenotypes in *E. coli* isolates from clinical sources

Correlations

		Amp	Ctx	Cx	Cpd	Caz	Ctr	Cxm	C	Cip	Cot	Gen	Na	S	Te	Tr
Amp	Pearson Correlation	1	-.525	.856	.496	-.546	.445	-.449	-.177	.801	-.083	.306	.500	.733	.254	-.755
	Sig. (2-tailed)		.475	.144	.504	.454	.555	.551	.823	.199	.917	.694	.500	.267	.746	.245
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Ctx	Pearson Correlation	-.525	1	-.417	-.206	.984*	.511	.951*	.763	-.064	.437	.648	.475	-.342	.630	.783
	Sig. (2-tailed)	.475		.583	.794	.016	.489	.049	.237	.936	.563	.352	.525	.658	.370	.217
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cx	Pearson Correlation	.856	-.417	1	.873	-.345	.512	-.509	.182	.922	-.508	.324	.461	.979*	.438	-.873
	Sig. (2-tailed)	.144	.583		.127	.655	.488	.491	.818	.078	.492	.676	.539	.021	.562	.127
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cpd	Pearson Correlation	.496	-.206	.873	1	-.065	.441	-.432	.474	.794	-.776	.256	.303	.954*	.496	-.755
	Sig. (2-tailed)	.504	.794	.127		.935	.559	.568	.526	.206	.224	.744	.697	.046	.504	.245
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Caz	Pearson Correlation	-.546	.984*	-.345	-.065	1	.508	.879	.846	-.018	.267	.620	.436	-.239	.660	.700
	Sig. (2-tailed)	.454	.016	.655	.935		.492	.121	.154	.982	.733	.380	.564	.761	.340	.300
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Ctr	Pearson Correlation	.445	.511	.512	.441	.508	1	.478	.723	.804	.200	.978*	.980*	.498	.967*	-.028
	Sig. (2-tailed)	.555	.489	.488	.559	.492		.522	.277	.196	.800	.022	.020	.502	.033	.972
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cxm	Pearson Correlation	-.449	.951*	-.509	-.432	.879	.478	1	.562	-.137	.694	.646	.504	-.493	.530	.864
	Sig. (2-tailed)	.551	.049	.491	.568	.121	.522		.438	.863	.306	.354	.496	.507	.470	.136
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C	Pearson Correlation	-.177	.763	.182	.474	.846	.723	.562	1	.441	-.142	.724	.594	.310	.875	.212
	Sig. (2-tailed)	.823	.237	.818	.526	.154	.277	.438		.559	.858	.276	.406	.690	.125	.788
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cip	Pearson Correlation	.801	-.064	.922	.794	-.018	.804	-.137	.441	1	-.250	.666	.764	.898	.734	-.617
	Sig. (2-tailed)	.199	.936	.078	.206	.982	.196	.863	.559		.750	.334	.236	.102	.266	.383
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cot	Pearson Correlation	-.083	.437	-.508	-.776	.267	.200	.694	-.142	-.250	1	.370	.359	-.637	.075	.691
	Sig. (2-tailed)	.917	.563	.492	.224	.733	.800	.306	.858	.750		.630	.641	.363	.925	.309
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Gen	Pearson Correlation	.306	.648	.324	.256	.620	.978*	.646	.724	.666	.370	1	.977*	.306	.949	.176
	Sig. (2-tailed)	.694	.352	.676	.744	.380	.022	.354	.276	.334	.630		.023	.694	.051	.824
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Na	Pearson Correlation	.500	.475	.461	.303	.436	.980*	.504	.594	.764	.359	.977*	1	.410	.904	.016
	Sig. (2-tailed)	.500	.525	.539	.697	.564	.020	.496	.406	.236	.641	.023		.590	.096	.984
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
S	Pearson Correlation	.733	-.342	.979*	.954*	-.239	.498	-.493	.310	.898	-.637	.306	.410	1	.476	-.852
	Sig. (2-tailed)	.267	.658	.021	.046	.761	.502	.507	.690	.102	.363	.694	.590		.524	.148
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Te	Pearson Correlation	.254	.630	.438	.496	.660	.967*	.530	.875	.734	.075	.949	.904	.476	1	.044
	Sig. (2-tailed)	.746	.370	.562	.504	.340	.033	.470	.125	.266	.925	.051	.096	.524		.956
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Tr	Pearson Correlation	-.755	.783	-.873	-.755	.700	-.028	.864	.212	-.617	.691	.176	.016	-.852	.044	1
	Sig. (2-tailed)	.245	.217	.127	.245	.300	.972	.136	.788	.383	.309	.824	.984	.148	.956	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).

Amp-Ampicillin; Ctx-Cefotaxime; Cx-Cefoxitin; Cpd-Cefpodoxime; Caz-Ceftazidime; Ctr-Ceftriaxone; Cxm-Cefuroxime; C-Chloramphenicol; Cip-Ciprofloxacin (, 5mcg), Co-Co-Trimoxazole; Gen-Gentamicin; Na-Nalidixic acid; S-Streptomycin; Te-Tetracycline; Tr- Trimethoprim.

Appendix -3

Table 3.1 Anova for distribution of antibiotic resistance genes in *E. coli* isolates of clinical, seafood and estuarine sources

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
clinical	Between Groups	21689.701	12	1807.475	5.306	.000
	Within Groups	13285.651	39	340.658		
	Total	34975.352	51			
seafood	Between Groups	1486.280	12	123.857	4.060	.000
	Within Groups	1189.689	39	30.505		
	Total	2675.969	51			
estuary	Between Groups	2126.060	12	177.172	12.311	.000
	Within Groups	561.253	39	14.391		
	Total	2687.313	51			

Table 3.2 Games-Howell analysis of distribution of antibiotic resistance genes in *E. coli* from Cochin estuary

Multiple Comparisons

Games-Howell

(I) phylos	(J) phylos	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
tem	*ctxm*	15.62750(*)	2.66818	.029	1.8709	29.3841
	sul1	8.46750	3.55328	.540	-9.2553	26.1903
	sul2	13.92500(*)	2.53129	.048	.1760	27.6740
	tetA	1.50500	4.07271	1.000	-19.7427	22.7527
	tetB	10.52500	2.99339	.188	-4.1609	25.2109
	strA	10.91250	3.53218	.289	-6.6774	28.5024
	aphA2	16.25500(*)	2.72584	.025	2.4108	30.0992
	cat1	19.82500(*)	2.41949	.014	5.7906	33.8594
	dhfr1	19.20500(*)	2.44413	.015	5.2624	33.1476
	dhfr7	21.28000(*)	2.31375	.015	6.6043	35.9557
	class1	18.06500(*)	2.34811	.023	3.6479	32.4821
	int1	14.33000(*)	2.48601	.044	.5039	28.1561
ctxm	*tem*	-15.62750(*)	2.66818	.029	-29.3841	-1.8709
	sul1	-7.16000	3.15903	.600	-24.5152	10.1952
	sul2	-1.70250	1.93937	.998	-11.3185	7.9135
	tetA	-14.12250	3.73372	.198	-35.9030	7.6580
	tetB	-5.10250	2.51279	.699	-17.7979	7.5929
	strA	-4.71500	3.13528	.903	-21.8905	12.4605
	aphA2	.62750	2.18722	1.000	-10.0875	11.3425

	cat1	4.19750	1.79099	.562	-5.0981	13.4931
	dhfr1	3.57750	1.82415	.731	-5.7571	12.9121
	dhfr7	5.65250	1.64534	.252	-3.8148	15.1198
	class1	2.43750	1.69332	.921	-6.8976	11.7726
	int1	-1.29750	1.87988	1.000	-10.7430	8.1480
sul1	tem	-8.46750	3.55328	.540	-26.1903	9.2553
	ctxm	7.16000	3.15903	.600	-10.1952	24.5152
	sul2	5.45750	3.04429	.797	-12.2262	23.1412
	tetA	-6.96250	4.40990	.886	-28.8755	14.9505
	tetB	2.05750	3.43812	1.000	-15.3868	19.5018
	strA	2.44500	3.91619	1.000	-16.7098	21.5998
	aphA2	7.78750	3.20788	.532	-9.5055	25.0805
	cat1	11.35750	2.95198	.203	-6.8315	29.5465
	dhfr1	10.73750	2.97222	.235	-7.3185	28.7935
	dhfr7	12.81250	2.86595	.151	-6.1144	31.7394
	class1	9.59750	2.89377	.305	-9.0573	28.2523
	int1	5.86250	3.00674	.734	-11.9965	23.7215
sul2	tem	-13.92500(*)	2.53129	.048	-27.6740	-.1760
	ctxm	1.70250	1.93937	.998	-7.9135	11.3185
	sul1	-5.45750	3.04429	.797	-23.1412	12.2262
	tetA	-12.42000	3.63716	.273	-34.7296	9.8896
	tetB	-3.40000	2.36693	.924	-15.9314	9.1314
	strA	-3.01250	3.01964	.990	-20.5047	14.4797
	aphA2	2.33000	2.01797	.980	-7.7803	12.4403
	cat1	5.90000	1.57984	.157	-1.9734	13.7734
	dhfr1	5.28000	1.61733	.242	-2.7138	13.2738
	dhfr7	7.35500	1.41258	.059	-.3304	15.0404
	class1	4.14000	1.46819	.383	-3.5279	11.8079
	int1	.40500	1.67994	1.000	-7.8319	8.6419
tetA	tem	-1.50500	4.07271	1.000	-22.7527	19.7427
	ctxm	14.12250	3.73372	.198	-7.6580	35.9030
	sul1	6.96250	4.40990	.886	-14.9505	28.8755
	sul2	12.42000	3.63716	.273	-9.8896	34.7296
	tetB	9.02000	3.97264	.596	-12.2215	30.2615
	strA	9.40750	4.39292	.648	-12.4535	31.2685
	aphA2	14.75000	3.77514	.175	-6.8674	36.3674
	cat1	18.32000	3.56025	.098	-4.5929	41.2329
	dhfr1	17.70000	3.57704	.107	-5.0653	40.4653
	dhfr7	19.77500	3.48925	.083	-3.8764	43.4264
	class1	16.56000	3.51213	.133	-6.8318	39.9518
	int1	12.82500	3.60579	.252	-9.7088	35.3588
tetB	tem	-10.52500	2.99339	.188	-25.2109	4.1609
	ctxm	5.10250	2.51279	.699	-7.5929	17.7979

	sul1	-2.05750	3.43812	1.000	-19.5018	15.3868
	sul2	3.40000	2.36693	.924	-9.1314	15.9314
	tetA	-9.02000	3.97264	.596	-30.2615	12.2215
	strA	.38750	3.41631	1.000	-16.9104	17.6854
	aphA2	5.73000	2.57394	.610	-7.1180	18.5780
	cat1	9.30000	2.24696	.144	-3.3992	21.9992
	dhfr1	8.68000	2.27348	.179	-3.9513	21.3113
	dhfr7	10.75500	2.13268	.096	-2.5128	24.0228
	class1	7.54000	2.16992	.256	-5.4855	20.5655
	int1	3.80500	2.31844	.858	-8.7536	16.3636
strA	tem	-10.91250	3.53218	.289	-28.5024	6.6774
	ctxm	4.71500	3.13528	.903	-12.4605	21.8905
	sul1	-2.44500	3.91619	1.000	-21.5998	16.7098
	sul2	3.01250	3.01964	.990	-14.4797	20.5047
	tetA	-9.40750	4.39292	.648	-31.2685	12.4535
	tetB	-.38750	3.41631	1.000	-17.6854	16.9104
	aphA2	5.34250	3.18449	.845	-11.7765	22.4615
	cat1	8.91250	2.92655	.356	-9.0781	26.9031
	dhfr1	8.29250	2.94696	.417	-9.5662	26.1512
	dhfr7	10.36750	2.83975	.250	-8.3591	29.0941
	class1	7.15250	2.86782	.529	-11.3020	25.6070
	int1	3.41750	2.98178	.975	-14.2467	21.0817
aphA2	tem	-16.25500(*)	2.72584	.025	-30.0992	-2.4108
	ctxm	-.62750	2.18722	1.000	-11.3425	10.0875
	sul1	-7.78750	3.20788	.532	-25.0805	9.5055
	sul2	-2.33000	2.01797	.980	-12.4403	7.7803
	tetA	-14.75000	3.77514	.175	-36.3674	6.8674
	tetB	-5.73000	2.57394	.610	-18.5780	7.1180
	strA	-5.34250	3.18449	.845	-22.4615	11.7765
	cat1	3.57000	1.87581	.755	-6.3349	13.4749
	dhfr1	2.95000	1.90749	.893	-6.9678	12.8678
	dhfr7	5.02500	1.73728	.387	-5.1570	15.2070
	class1	1.81000	1.78280	.990	-8.2084	11.8284
	int1	-1.92500	1.96086	.994	-11.9103	8.0603
cat1	tem	-19.82500(*)	2.41949	.014	-33.8594	-5.7906
	ctxm	-4.19750	1.79099	.562	-13.4931	5.0981
	sul1	-11.35750	2.95198	.203	-29.5465	6.8315
	sul2	-5.90000	1.57984	.157	-13.7734	1.9734
	tetA	-18.32000	3.56025	.098	-41.2329	4.5929
	tetB	-9.30000	2.24696	.144	-21.9992	3.3992
	strA	-8.91250	2.92655	.356	-26.9031	9.0781
	aphA2	-3.57000	1.87581	.755	-13.4749	6.3349
	dhfr1	-.62000	1.43605	1.000	-7.6528	6.4128

	dhfr7	1.45500	1.20077	.972	-4.6943	7.6043
	class1	-1.76000	1.26573	.940	-8.0582	4.5382
	int1	-5.49500	1.50621	.167	-12.9203	1.9303
dhfr1	tem	-19.20500(*)	2.44413	.015	-33.1476	-5.2624
	ctxm	-3.57750	1.82415	.731	-12.9121	5.7571
	sul1	-10.73750	2.97222	.235	-28.7935	7.3185
	sul2	-5.26000	1.61733	.242	-13.2738	2.7138
	tetA	-17.70000	3.57704	.107	-40.4653	5.0653
	tetB	-8.68000	2.27348	.179	-21.3113	3.9513
	strA	-8.29250	2.94696	.417	-26.1512	9.5662
	aphA2	-2.95000	1.90749	.893	-12.8678	6.9678
	cat1	.62000	1.43605	1.000	-6.4128	7.6528
	dhfr7	2.07500	1.24969	.854	-4.4166	8.5666
	class1	-1.14000	1.31222	.998	-7.7354	5.4554
	int1	-4.87500	1.54549	.267	-12.4556	2.7056
dhfr7	tem	-21.28000(*)	2.31375	.015	-35.9557	-6.6043
	ctxm	-5.65250	1.64534	.252	-15.1198	3.8148
	sul1	-12.81250	2.86595	.151	-31.7394	6.1144
	sul2	-7.35500	1.41258	.059	-15.0404	.3304
	tetA	-19.77500	3.48925	.083	-43.4264	3.8764
	tetB	-10.75500	2.13268	.096	-24.0228	2.5128
	strA	-10.36750	2.83975	.250	-29.0941	8.3591
	aphA2	-5.02500	1.73728	.387	-15.2070	5.1570
	cat1	-1.45500	1.20077	.972	-7.6043	4.6943
	dhfr1	-2.07500	1.24969	.854	-8.5666	4.4166
	class1	-3.21500	1.04955	.293	-8.3900	1.9600
	int1	-6.95000	1.32972	.053	-14.0197	.1197
Class1 integrons	tem	-18.06500(*)	2.34811	.023	-32.4821	-3.6479
	ctxm	-2.43750	1.69332	.921	-11.7726	6.8976
	sul1	-9.59750	2.89377	.305	-28.2523	9.0573
	sul2	-4.14000	1.46819	.383	-11.8079	3.5279
	tetA	-16.56000	3.51213	.133	-39.9518	6.8318
	tetB	-7.54000	2.16992	.256	-20.5655	5.4855
	strA	-7.15250	2.86782	.529	-25.6070	11.3020
	aphA2	-1.81000	1.78280	.990	-11.8284	8.2084
	cat1	1.76000	1.26573	.940	-4.5382	8.0582
	dhfr1	1.14000	1.31222	.998	-5.4554	7.7354
	dhfr7	3.21500	1.04955	.293	-1.9600	8.3900
	int1	-3.73500	1.38866	.423	-10.8438	3.3738
int1	tem	-14.33000(*)	2.48601	.044	-28.1561	-.5039
	ctxm	1.29750	1.87988	1.000	-8.1480	10.7430
	sul1	-5.86250	3.00674	.734	-23.7215	11.9965
	sul2	-.40500	1.67994	1.000	-8.6419	7.8319

	tetA	-12.82500	3.60579	.252	-35.3588	9.7088
	tetB	-3.80500	2.31844	.858	-16.3636	8.7536
	strA	-3.41750	2.98178	.975	-21.0817	14.2467
	aphA2	1.92500	1.96086	.994	-8.0603	11.9103
	cat1	5.49500	1.50621	.167	-1.9303	12.9203
	dhfr1	4.87500	1.54549	.267	-2.7056	12.4556
	dhfr7	6.95000	1.32972	.053	-.1197	14.0197
	class1	3.73500	1.38866	.423	-3.3738	10.8438

* The mean difference is significant at the .05 level.

Table 3.3 Anova for distribution of antibiotic resistance genes in different phylogenetic groups of *E. coli* isolates of clinical, seafood and estuarine sources

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
clinical	Between Groups	1153.763	3	384.588	.546	.653
	Within Groups	33821.589	48	704.616		
	Total	34975.352	51			
seafood	Between Groups	169.947	3	56.649	1.085	.364
	Within Groups	2506.022	48	52.209		
	Total	2675.969	51			
estuary	Between Groups	58.867	3	19.622	.358	.783
	Within Groups	2628.446	48	54.759		
	Total	2687.313	51			

Table 3.4 Anova for distribution of antibiotic resistant genes among *E. coli* isolates in five different stations at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S1	Between Groups	2004.940	12	167.078	1.344	.234
	Within Groups	4846.696	39	124.274		
	Total	6851.636	51			
S2	Between Groups	4608.697	12	384.058	4.258	.000
	Within Groups	3518.017	39	90.206		
	Total	8126.714	51			
S3	Between Groups	2978.898	12	248.242	4.457	.000
	Within Groups	2172.101	39	55.695		
	Total	5150.999	51			
S4	Between Groups	1396.428	12	116.369	1.047	.428
	Within Groups	4334.170	39	111.133		
	Total	5730.598	51			
S5	Between Groups	4362.742	12	363.562	4.307	.000
	Within Groups	3292.408	39	84.421		
	Total	7655.149	51			

S1-Chittor, S2-Bolgatty, S3-Off Marine Science Jetty, S4-Thevara, S5-Barmouth

Table 3.5 Anova for distribution of antibiotic resistant genes among different phylogenetic groups of *E. coli* from five different stations set at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	1850.059	4	462.515	.689	.602
	Within Groups	40254.545	60	670.909		
	Total	42104.604	64			
B1	Between Groups	23474.841	4	5868.710	14.642	.000
	Within Groups	24048.652	60	400.811		
	Total	47523.493	64			
B2	Between Groups	19268.778	4	4817.195	9.530	.000
	Within Groups	30328.982	60	505.483		
	Total	49597.760	64			
D	Between Groups	9769.725	4	2442.431	5.312	.001
	Within Groups	27587.585	60	459.793		
	Total	37357.310	64			

Table 3.6 Games-Howell analysis of distribution of antibiotic resistant genes in *E. coli* isolates from station 3 (Off Marine Science Jetty)

Multiple Comparisons

Games-Howell

(I) genes	(J) genes	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
tem	*ctxm*	16.55250	8.40208	.726	-41.3290	74.4340
	sul 1	15.30000	9.76461	.886	-36.1229	66.7229
	sul 2	20.99250	8.59224	.548	-34.7861	76.7711
	tet A	7.30250	9.35347	.999	-44.4755	59.0805
	tetB	18.86750	9.41292	.711	-32.8010	70.5360
	str	11.45000	8.58456	.938	-44.4022	67.3022
	aphA2	20.43500	8.73273	.581	-34.1440	75.0140
	cat1	26.60000	8.28589	.351	-32.8966	86.0966
	dhfr 1	25.28500	8.38959	.388	-32.7569	83.3269
	dhfr 7	26.60000	8.28589	.351	-32.8966	86.0966
	class 1	23.06500	8.69147	.474	-31.8388	77.9688
	int 1	13.98500	9.33652	.904	-37.8284	65.7984
ctxm	*tem*	-16.55250	8.40208	.726	-74.4340	41.3290
	sul 1	-1.25250	5.35076	1.000	-36.0954	33.5904
	sul 2	4.44000	2.66637	.851	-9.6712	18.5512
	tet A	-9.25000	4.55745	.702	-37.9599	19.4599
	tetB	2.31500	4.67825	1.000	-27.3330	31.9630
	str	-5.10250	2.64152	.743	-19.0328	8.8278
	aphA2	3.88250	3.08925	.961	-13.4034	21.1684
	cat1	10.04750(*)	1.39245	.049	.0490	20.0460
	dhfr 1	8.73250	1.91524	.068	-.6466	18.1116
	dhfr 7	10.04750(*)	1.39245	.049	.0490	20.0460
	class 1	6.51250	2.97062	.631	-9.8686	22.8936
	int 1	-2.56750	4.52255	1.000	-31.0061	25.8711
sul 1	*tem*	-15.30000	9.76461	.886	-66.7229	36.1229
	ctxm	1.25250	5.35076	1.000	-33.5904	36.0954
	sul 2	5.69250	5.64467	.990	-27.1721	38.5571
	tet A	-7.99750	6.74708	.977	-41.3751	25.3801
	tetB	3.56750	6.82926	1.000	-30.1020	37.2370
	str	-3.85000	5.63297	1.000	-36.7656	29.0656
	aphA2	5.13500	5.85630	.997	-27.1033	37.3733
	cat1	11.30000	5.16640	.647	-25.7971	48.3971
	dhfr 1	9.98500	5.33113	.763	-25.0527	45.0227
	dhfr 7	11.30000	5.16640	.647	-25.7971	48.3971
	class 1	7.76500	5.79460	.944	-24.6029	40.1329
	int 1	-1.31500	6.72356	1.000	-34.6131	31.9831
sul 2	*tem*	-20.99250	8.59224	.548	-76.7711	34.7861
	ctxm	-4.44000	2.66637	.851	-18.5512	9.6712
	sul 1	-5.69250	5.64467	.990	-38.5571	27.1721
	tet A	-13.69000	4.89918	.404	-40.8063	13.4263
	tetB	-2.12500	5.01175	1.000	-30.1002	25.8502
	str	-9.54250	3.19520	.313	-25.1712	6.0862
	aphA2	-.55750	3.57423	1.000	-18.2829	17.1679

	cat1	5.60750	2.27389	.551	-10.7201	21.9351
	dhfr 1	4.29250	2.62675	.861	-9.8345	18.4195
	dhfr 7	5.60750	2.27389	.551	-10.7201	21.9351
	class 1	2.07250	3.47221	1.000	-15.0417	19.1867
	int 1	-7.00750	4.86674	.920	-33.8771	19.8621
tet A	tem	-7.30250	9.35347	.999	-59.0805	44.4755
	ctxm	9.25000	4.55745	.702	-19.4599	37.9599
	sul 1	7.99750	6.74708	.977	-25.3801	41.3751
	sul 2	13.69000	4.89918	.404	-13.4263	40.8063
	tetB	11.56500	6.22724	.778	-18.9020	42.0320
	str	4.14750	4.86571	.997	-22.9972	31.2922
	aphA2	13.13250	5.14159	.478	-13.8068	40.0718
	cat1	19.29750	4.33952	.173	-11.8623	50.4573
	dhfr 1	17.98250	4.53439	.196	-10.9186	46.8836
	dhfr 7	19.29750	4.33952	.173	-11.8623	50.4573
	class 1	15.76250	5.07120	.303	-11.1696	42.6946
	int 1	6.68250	6.11114	.987	-23.2079	36.5729
tetB	tem	-18.86750	9.41292	.711	-70.5360	32.8010
	ctxm	-2.31500	4.67825	1.000	-31.9630	27.3330
	sul 1	-3.56750	6.82926	1.000	-37.2370	30.1020
	sul 2	2.12500	5.01175	1.000	-25.8502	30.1002
	tet A	-11.56500	6.22724	.778	-42.0320	18.9020
	str	-7.41750	4.99858	.907	-35.4254	20.5904
	aphA2	1.56750	5.24896	1.000	-26.1486	29.2836
	cat1	7.73250	4.46621	.813	-24.3370	39.8020
	dhfr 1	6.41750	4.65578	.928	-23.4233	36.2583
	dhfr 7	7.73250	4.46621	.813	-24.3370	39.8020
	class 1	4.19750	5.18003	.998	-23.5360	31.9310
	int 1	-4.88250	6.20174	.999	-35.2313	25.4663
str	tem	-11.45000	8.58456	.938	-67.3022	44.4022
	ctxm	5.10250	2.64152	.743	-8.8278	19.0328
	sul 1	3.85000	5.63297	1.000	-29.0656	36.7656
	sul 2	9.54250	3.19520	.313	-6.0862	25.1712
	tet A	-4.14750	4.86571	.997	-31.2922	22.9972
	tctB	7.41750	4.99858	.907	-20.5904	35.4254
	aphA2	8.98500	3.55574	.479	-8.6816	26.6516
	cat1	15.15000	2.24471	.059	-.9681	31.2681
	dhfr 1	13.83500	2.60153	.051	-.1049	27.7749
	dhfr 7	15.15000	2.24471	.059	-.9681	31.2681
	class 1	11.61500	3.45317	.220	-5.4298	28.6598
	int 1	2.53500	4.85317	1.000	-24.3617	29.4317
aphA2	tem	-20.43500	8.73273	.581	-75.0140	34.1440
	ctxm	-3.88250	3.08925	.961	-21.1684	13.4034
	sul 1	-5.13500	5.85630	.997	-37.3733	27.1033
	sul 2	.55750	3.57423	1.000	-17.1679	18.2829
	tet A	-13.13250	5.14159	.478	-40.0718	13.8068
	tetB	-1.56750	5.24896	1.000	-29.2836	26.1486
	str	-8.98500	3.55574	.479	-26.6516	8.6816

	cat1	6.16500	2.75763	.629	-13.6361	25.9661
	dhfr 1	4.85000	3.05512	.874	-12.5337	22.2337
	dhfr 7	6.16500	2.75763	.629	-13.6361	25.9661
	class 1	2.63000	3.80660	1.000	-16.0056	21.2656
	int 1	-6.45000	5.11069	.963	-33.1673	20.2673
cat1	tem	-26.60000	8.28589	.351	-86.0966	32.8966
	ctxm	-10.04750(*)	1.39245	.049	-20.0460	-.0490
	sul 1	-11.30000	5.16640	.647	-48.3971	25.7971
	sul 2	-5.60750	2.27389	.551	-21.9351	10.7201
	tet A	-19.29750	4.33952	.173	-50.4573	11.8623
	tetB	-7.73250	4.46621	.813	-39.8020	24.3370
	str	-15.15000	2.24471	.059	-31.2681	.9681
	aphA2	-6.16500	2.75763	.629	-25.9661	13.6361
	dhfr 1	-1.31500	1.31500	.986	-10.7573	8.1273
	dhfr 7	.00000	.00000	.	.0000	.0000
	class 1	-3.53500	2.62406	.930	-22.3769	15.3069
	int 1	-12.61500	4.30286	.415	-43.5115	18.2815
dhfr 1	tem	-25.28500	8.38959	.388	-83.3269	32.7569
	ctxm	-8.73250	1.91524	.068	-18.1116	.6466
	sul 1	-9.98500	5.33113	.763	-45.0227	25.0527
	sul 2	-4.29250	2.62675	.861	-18.4195	9.8345
	tet A	-17.98250	4.53439	.196	-46.8836	10.9186
	tetB	-6.41750	4.65578	.928	-36.2583	23.4233
	str	-13.83500	2.60153	.051	-27.7749	.1049
	aphA2	-4.85000	3.05512	.874	-22.2337	12.5337
	cat1	1.31500	1.31500	.986	-8.1273	10.7573
	dhfr 7	1.31500	1.31500	.986	-8.1273	10.7573
	class 1	-2.22000	2.93511	.999	-18.6801	14.2401
	int 1	-11.30000	4.49931	.522	-39.9293	17.3293
dhfr 7	tem	-26.60000	8.28589	.351	-86.0966	32.8966
	ctxm	-10.04750(*)	1.39245	.049	-20.0460	-.0490
	sul 1	-11.30000	5.16640	.647	-48.3971	25.7971
	sul 2	-5.60750	2.27389	.551	-21.9351	10.7201
	tet A	-19.29750	4.33952	.173	-50.4573	11.8623
	tetB	-7.73250	4.46621	.813	-39.8020	24.3370
	str	-15.15000	2.24471	.059	-31.2681	.9681
	aphA2	-6.16500	2.75763	.629	-25.9661	13.6361
	cat1	.00000	.00000	.	.0000	.0000
	dhfr 1	-1.31500	1.31500	.986	-10.7573	8.1273
	class 1	-3.53500	2.62406	.930	-22.3769	15.3069
	int 1	-12.61500	4.30286	.415	-43.5115	18.2815
class 1 integrons	tem	-23.06500	8.69147	.474	-77.9688	31.8388
	ctxm	-6.51250	2.97062	.631	-22.8936	9.8686
	sul 1	-7.76500	5.79460	.944	-40.1329	24.6029
	sul 2	-2.07250	3.47221	1.000	-19.1867	15.0417
	tet A	-15.76250	5.07120	.303	-42.6946	11.1696
	tetB	-4.19750	5.18003	.998	-31.9310	23.5360

	str	-11.61500	3.45317	.220	-28.6598	5.4298
	aphA2	-2.63000	3.80660	1.000	-21.2656	16.0056
	cat1	3.53500	2.62406	.930	-15.3069	22.3769
	dhfr 1	2.22000	2.93511	.999	-14.2401	18.6801
	dhfr 7	3.53500	2.62406	.930	-15.3069	22.3769
	int 1	-9.08000	5.03987	.798	-35.7828	17.6228
int 1	*tem*	-13.98500	9.33652	.904	-65.7984	37.8284
	ctxm	2.56750	4.52255	1.000	-25.8711	31.0061
	sul 1	1.31500	6.72356	1.000	-31.9831	34.6131
	sul 2	7.00750	4.86674	.920	-19.8621	33.8771
	tet A	-6.68250	6.11114	.987	-36.5729	23.2079
	tetB	4.88250	6.20174	.999	-25.4663	35.2313
	str	-2.53500	4.85317	1.000	-29.4317	24.3617
	aphA2	6.45000	5.11069	.963	-20.2673	33.1673
	cat1	12.61500	4.30286	.415	-18.2815	43.5115
	dhfr 1	11.30000	4.49931	.522	-17.3293	39.9293
	dhfr 7	12.61500	4.30286	.415	-18.2815	43.5115
	class 1	9.08000	5.03987	.798	-17.6228	35.7828

* The mean difference is significant at the .05 level.

Table 3.7 Games-Howell analysis of distribution of antibiotic resistant genes in different phylogenetic groups of *E. coli* isolates in five different stations set at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S1	Between Groups	1733.439	3	577.813	5.419	.003
	Within Groups	5118.197	48	106.629		
	Total	6851.636	51			
S2	Between Groups	779.125	3	259.708	1.697	.180
	Within Groups	7347.589	48	153.075		
	Total	8126.714	51			
S3	Between Groups	57.006	3	19.002	.179	.910
	Within Groups	5093.993	48	106.125		
	Total	5150.999	51			
S4	Between Groups	2492.405	3	830.802	12.315	.000
	Within Groups	3238.193	48	67.462		
	Total	5730.598	51			
S5	Between Groups	1173.626	3	391.209	2.897	.045
	Within Groups	6481.523	48	135.032		
	Total	7655.149	51			

S1-Chittor, S2-Bolgatty, S3-Off Marine Science Jetty, S4-Thevara, S5-Barmouth

Table 3.8 Games-Howell analysis of relative prevalence of antibiotic resistance genes in *E. coli* isolates from different sources

Multiple Comparisons

Games-Howell

(I) sources	(J) sources	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
clinical	seafood	27.15385*	4.99566	.000	14.1239	40.1838
	estuary	21.52077*	5.14262	.002	8.2454	34.7961
seafood	clinical	-27.15385*	4.99566	.000	-40.1838	-14.1239
	estuary	-5.63308	2.47688	.080	-11.8423	.5761
estuary	clinical	-21.52077*	5.14262	.002	-34.7961	-8.2454
	seafood	5.63308	2.47688	.080	-.5761	11.8423

*. The mean difference is significant at the .05 level.

Table 3.9a Anova for relative prevalence of antibiotic resistance genes in different phylogenetic groups of *E. coli* isolates from different sources

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	5035.260	2	2517.630	15.631	.000
	Within Groups	5798.373	36	161.066		
	Total	10833.634	38			
B1	Between Groups	11472.714	2	5736.357	9.689	.000
	Within Groups	21312.836	36	592.023		
	Total	32785.550	38			
B2	Between Groups	5701.587	2	2850.793	21.622	.000
	Within Groups	4746.493	36	131.847		
	Total	10448.079	38			
D	Between Groups	7667.135	2	3833.567	19.442	.000
	Within Groups	7098.354	36	197.177		
	Total	14765.489	38			

Table 3.9b Gams-Howell analysis of relative prevalence of antibiotic resistance genes among different phylogenetic groups of *E. coli* isolates from different sources

Multiple Comparisons

Games-Howell

Dependent Variable	(I) sources	(J) sources	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
A	clinical	seafood	26.52846	5.47583	.001	12.0882	40.9688
		estuary	20.55615	5.98438	.008	5.2577	35.8546
	seafood	clinical	-26.52846	5.47583	.001	-40.9688	-12.0882
		estuary	-5.97231	2.92244	.133	-13.4961	1.5515
	estuary	clinical	-20.55615	5.98438	.008	-35.8546	-5.2577
		seafood	5.97231	2.92244	.133	-1.5515	13.4961
B1	clinical	seafood	38.63000	11.49798	.012	8.4687	68.7913
		estuary	33.61769	11.30006	.027	3.7401	63.4953
	seafood	clinical	-38.63000	11.49798	.012	-68.7913	-8.4687
		estuary	-5.01231	3.65330	.373	-14.2036	4.1790
	estuary	clinical	-33.61769	11.30006	.027	-63.4953	-3.7401
		seafood	5.01231	3.65330	.373	-4.1790	14.2036
B2	clinical	seafood	27.98077	5.28598	.000	14.4808	41.4808
		estuary	22.39769	4.96465	.001	9.4701	35.3253
	seafood	clinical	-27.98077	5.28598	.000	-41.4808	-14.4808
		estuary	-5.58308	2.87456	.152	-12.8362	1.6700
	estuary	clinical	-22.39769	4.96465	.001	-35.3253	-9.4701
		seafood	5.58308	2.87456	.152	-1.6700	12.8362
D	clinical	seafood	33.33000	6.53753	.001	15.8888	50.7712
		estuary	23.84154	6.74554	.009	6.1186	41.5644
	seafood	clinical	-33.33000	6.53753	.001	-50.7712	-15.8888
		estuary	-9.48846	1.66221	.000	-13.9230	-5.0539
	estuary	clinical	-23.84154	6.74554	.009	-41.5644	-6.1186
		seafood	9.48846	1.66221	.000	5.0539	13.9230

*. The mean difference is significant at the .05 level.

Table 3.10 Games-Howell analysis of distribution of different antibiotic resistant genes among *E. coli* isolates of clinical origin

Multiple Comparisons

Games-Howell

(I) phylos	(J) phylos	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower bound	Upper bound
tem	*ctxm*	53.75000(*)	10.21481	.044	1.6106	105.8894
	sul1	38.23000	8.72636	.150	-17.2734	93.7334
	sul2	47.60500	9.24162	.071	-5.0013	100.2113
	tetA	34.79250	18.08888	.745	-65.5594	135.1444
	tetB	45.62500	22.59735	.706	-89.6647	180.9147
	strA	46.56250	10.02252	.077	-5.3279	98.4529
	aphA2	75.83500(*)	9.47084	.011	23.7916	127.8784
	cat1	60.21000	12.55525	.056	-1.5176	121.9376
	dhfr1	75.94000(*)	9.22178	.013	23.2675	128.6125
	dhfr7	75.94000(*)	9.22178	.013	23.2675	128.6125
	class1	66.56250(*)	10.99663	.019	12.3051	120.8199
	int1	64.37500(*)	11.42022	.026	8.4212	120.3288
ctxm	*tem*	-53.75000(*)	10.21481	.044	-105.8894	-1.6106
	sul1	-15.52000	6.42273	.546	-53.0708	22.0308
	sul2	-6.14500	7.10701	.998	-42.8204	30.5304
	tetA	-18.95750	17.09707	.979	-123.3929	85.4779
	tetB	-8.12500	21.81152	1.000	-149.1692	132.9192
	strA	-7.18750	8.09648	.997	-46.8407	32.4657
	aphA2	22.08500	7.40262	.322	-15.1217	59.2917
	cat1	6.46000	11.07864	1.000	-51.7678	64.6878
	dhfr1	22.19000	7.08119	.289	-14.4587	58.8387
	dhfr7	22.19000	7.08119	.289	-14.4587	58.8387
	class1	12.81250	9.27513	.942	-33.2000	58.8250
	int1	10.62500	9.77362	.987	-38.5569	59.8069
sul1	*tem*	-38.23000	8.72636	.150	-93.7334	17.2734
	ctxm	15.52000	6.42273	.546	-22.0308	53.0708
	sul2	9.37500	4.72405	.720	-15.2987	34.0487
	tetA	-3.43750	16.25167	1.000	-115.8466	108.9716
	tetB	7.39500	21.15536	1.000	-141.1569	155.9469
	strA	8.33250	6.11229	.937	-26.8063	43.4713
	aphA2	37.60500(*)	5.15809	.015	9.7525	65.4575
	cat1	21.98000	9.72334	.614	-41.2233	85.1833
	dhfr1	37.71000(*)	4.68511	.007	13.3143	62.1057
	dhfr7	37.71000(*)	4.68511	.007	13.3143	62.1057
	class1	28.33250	7.60494	.221	-18.4456	75.1106
	int1	26.14500	8.20553	.327	-25.3133	77.6033
sul2	*tem*	-47.60500	9.24162	.071	-100.2113	5.0013
	ctxm	6.14500	7.10701	.998	-30.5304	42.8204
	sul1	-9.37500	4.72405	.720	-34.0487	15.2987
	tetA	-12.81250	16.53405	.998	-121.9374	96.3124
	tetB	-1.98000	21.37306	1.000	-147.7084	143.7484
	strA	-1.04250	6.82775	1.000	-35.7907	33.7057
	aphA2	28.23000	5.98865	.060	-1.2208	57.6808

	cat1	12.60500	10.18830	.962	-47.3522	72.5622
	dhfr1	28.33500(*)	5.58645	.042	1.0103	55.6597
	dhfr7	28.33500(*)	5.58645	.042	1.0103	55.6597
	class1	18.95750	8.19104	.579	-25.6282	63.5432
	int1	16.77000	8.75150	.748	-32.0681	65.6081
tetA	tem	-34.79250	18.08888	.745	-135.1444	65.5594
	ctxm	18.95750	17.09707	.979	-85.4779	123.3929
	sul1	3.43750	16.25167	1.000	-108.9716	115.8466
	sul2	12.81250	16.53405	.998	-96.3124	121.9374
	tetB	10.83250	26.43119	1.000	-121.8886	143.5536
	strA	11.77000	16.98289	.999	-93.4463	116.9863
	aphA2	41.04250	16.66326	.541	-66.8071	148.8921
	cat1	25.41750	18.59037	.940	-74.2585	125.0935
	dhfr1	41.14750	16.52297	.534	-68.0930	150.3880
	dhfr7	41.14750	16.52297	.534	-68.0930	150.3880
	class1	31.77000	17.57536	.792	-70.1570	133.6970
	int1	29.58250	17.84346	.849	-71.3964	130.5614
tetB	tem	-45.62500	22.59735	.706	-180.9147	89.6647
	ctxm	8.12500	21.81152	1.000	-132.9192	149.1692
	sul1	-7.39500	21.15536	1.000	-155.9469	141.1569
	sul2	1.98000	21.37306	1.000	-143.7484	147.7084
	tetA	-10.83250	26.43119	1.000	-143.5536	121.8886
	strA	.93750	21.72213	1.000	-140.9633	142.8383
	aphA2	30.21000	21.47316	.918	-114.3375	174.7575
	cat1	14.58500	23.00074	1.000	-118.7689	147.9389
	dhfr1	30.31500	21.36448	.914	-115.5178	176.1478
	dhfr7	30.31500	21.36448	.914	-115.5178	176.1478
	class1	20.93750	22.18842	.993	-116.9778	158.8528
	int1	18.75000	22.40137	.997	-117.7071	155.2071
strA	tem	-46.56250	10.02252	.077	-98.4529	5.3279
	ctxm	7.18750	8.09648	.997	-32.4657	46.8407
	sul1	-8.33250	6.11229	.937	-43.4713	26.8063
	sul2	1.04250	6.82775	1.000	-33.7057	35.7907
	tetA	-11.77000	16.98289	.999	-116.9863	93.4463
	tetB	-.93750	21.72213	1.000	-142.8383	140.9633
	aphA2	29.27250	7.13494	.110	-6.2239	64.7689
	cat1	13.64750	10.90160	.964	-44.5979	71.8929
	dhfr1	29.37750	6.80087	.097	-5.3248	64.0798
	dhfr7	29.37750	6.80087	.097	-5.3248	64.0798
	class1	20.00000	9.06293	.619	-25.3904	65.3904
	int1	17.81250	9.57247	.775	-30.9572	66.5822
aphA2	tem	-75.83500(*)	9.47084	.011	-127.8784	-23.7916
	ctxm	-22.08500	7.40262	.322	-59.2917	15.1217
	sul1	-37.60500(*)	5.15809	.015	-65.4575	-9.7525
	sul2	-28.23000	5.98865	.060	-57.6808	1.2208
	tetA	-41.04250	16.66326	.541	-148.8921	66.8071
	tetB	-30.21000	21.47316	.918	-174.7575	114.3375
	strA	-29.27250	7.13494	.110	-64.7689	6.2239

	cat1	-15.62500	10.39667	.901	-74.7555	43.5055
	dhfr1	.10500	5.95799	1.000	-29.2274	29.4374
	dhfr7	.10500	5.95799	1.000	-29.2274	29.4374
	class1	-9.27250	8.44881	.985	-53.7269	35.1819
	int1	-11.46000	8.99322	.959	-59.9152	36.9952
cat1	tem	-60.21000	12.55525	.056	-121.9376	1.5176
	ctxm	-6.46000	11.07864	1.000	-64.6878	51.7678
	sul1	-21.98000	9.72334	.614	-85.1833	41.2233
	sul2	-12.60500	10.18830	.962	-72.5622	47.3522
	tetA	-25.41750	18.59037	.940	-125.0935	74.2585
	tetB	-14.58500	23.00074	1.000	-147.9389	118.7689
	strA	-13.64750	10.90160	.964	-71.8929	44.5979
	aphA2	15.62500	10.39667	.901	-43.5055	74.7555
	dhfr1	15.73000	10.17031	.885	-44.3142	75.7742
	dhfr7	15.73000	10.17031	.885	-44.3142	75.7742
	class1	6.35250	11.80339	1.000	-52.9277	65.6327
	int1	4.16500	12.19900	1.000	-56.2665	64.5965
dhfr1	tem	-75.94000(*)	9.22178	.013	-128.6125	-23.2675
	ctxm	-22.19000	7.08119	.289	-58.8387	14.4587
	sul1	-37.71000(*)	4.68511	.007	-62.1057	-13.3143
	sul2	-28.33500(*)	5.58645	.042	-55.6597	-1.0103
	tetA	-41.14750	16.52297	.534	-150.3880	68.0930
	tetB	-30.31500	21.36448	.914	-176.1478	115.5178
	strA	-29.37750	6.80087	.097	-64.0798	5.3248
	aphA2	-.10500	5.95799	1.000	-29.4374	29.2274
	cat1	-15.73000	10.17031	.885	-75.7742	44.3142
	dhfr7	.00000	5.55356	1.000	-27.1625	27.1625
	class1	-9.37750	8.16865	.978	-53.9938	35.2388
	int1	-11.56500	8.73055	.947	-60.4546	37.3246
dhfr7	tem	-75.94000(*)	9.22178	.013	-128.6125	-23.2675
	ctxm	-22.19000	7.08119	.289	-58.8387	14.4587
	sul1	-37.71000(*)	4.68511	.007	-62.1057	-13.3143
	sul2	-28.33500(*)	5.58645	.042	-55.6597	-1.0103
	tetA	-41.14750	16.52297	.534	-150.3880	68.0930
	tetB	-30.31500	21.36448	.914	-176.1478	115.5178
	strA	-29.37750	6.80087	.097	-64.0798	5.3248
	aphA2	-.10500	5.95799	1.000	-29.4374	29.2274
	cat1	-15.73000	10.17031	.885	-75.7742	44.3142
	dhfr1	.00000	5.55356	1.000	-27.1625	27.1625
	class1	-9.37750	8.16865	.978	-53.9938	35.2388
	int1	-11.56500	8.73055	.947	-60.4546	37.3246
class1 integrons	tem	-66.56250(*)	10.99663	.019	-120.8199	-12.3051
	ctxm	-12.81250	9.27513	.942	-58.8250	33.2000
	sul1	-28.33250	7.60494	.221	-75.1106	18.4456
	sul2	-18.95750	8.19104	.579	-63.5432	25.6282
	tetA	-31.77000	17.57536	.792	-133.6970	70.1570
	tetB	-20.93750	22.18842	.993	-158.8528	116.9778

	strA	-20.00000	9.06293	.619	-65.3904	25.3904
	aphA2	9.27250	8.44881	.985	-35.1819	53.7269
	cat1	-6.35250	11.80339	1.000	-65.6327	52.9277
	dhfr1	9.37750	8.16865	.978	-35.2388	53.9938
	dhfr7	9.37750	8.16865	.978	-35.2388	53.9938
	int1	-2.18750	10.58807	1.000	-54.1157	49.7407
int1	*tem*	-64.37500(*)	11.42022	.026	-120.3288	-8.4212
	ctxm	-10.62500	9.77362	.987	-59.8069	38.5569
	sul1	-26.14500	8.20553	.327	-77.6033	25.3133
	sul2	-16.77000	8.75150	.748	-65.6081	32.0681
	tetA	-29.58250	17.84346	.849	-130.5614	71.3964
	tetB	-18.75000	22.40137	.997	-155.2071	117.7071
	strA	-17.81250	9.57247	.775	-66.5822	30.9572
	aphA2	11.46000	8.99322	.959	-36.9952	59.9152
	cat1	-4.16500	12.19900	1.000	-64.5965	56.2665
	dhfr1	11.56500	8.73055	.947	-37.3246	60.4546
	dhfr7	11.56500	8.73055	.947	-37.3246	60.4546
	class1	2.18750	10.58807	1.000	-49.7407	54.1157

* The mean difference is significant at the .05 level.

Table 3.11 Correlation between antibiotic resistance genes in *E. coli* isolates from Cochin estuary

Correlations

		tem	ctxm	sul1	sul2	tetA	tetB	strA	ephA2	cat1	dhfrIa	dhfrVII	class1	int1
tem	Pearson Correlation	1	-.314	.975*	.911	.786	.695	.863	.114	-.409	-.599	.095	.741	.904
	Sig. (2-tailed)		.686	.025	.089	.214	.305	.137	.886	.591	.401	.905	.259	.096
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
ctxm	Pearson Correlation	-.314	1	-.384	-.458	-.747	-.726	-.420	-.873	-.688	.408	.302	.000	-.169
	Sig. (2-tailed)	.686		.616	.542	.253	.274	.580	.127	.312	.592	.698	1.000	.831
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
sul1	Pearson Correlation	.975*	-.384	1	.980*	.876	.820	.954*	.278	-.266	-.762	-.129	.832	.956*
	Sig. (2-tailed)	.025	.616		.020	.124	.180	.046	.722	.734	.238	.871	.168	.044
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
sul2	Pearson Correlation	.911	-.458	.980*	1	.931	.908	.993**	.433	-.105	-.872	-.322	.862	.954*
	Sig. (2-tailed)	.089	.542	.020		.069	.092	.007	.567	.895	.128	.678	.138	.046
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
tetA	Pearson Correlation	.786	-.747	.876	.931	1	.984*	.916	.703	.231	-.841	-.412	.656	.780
	Sig. (2-tailed)	.214	.253	.124	.069		.016	.084	.297	.769	.159	.588	.344	.220
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
tetB	Pearson Correlation	.695	-.726	.820	.908	.984*	1	.914	.771	.322	-.909	-.569	.684	.761
	Sig. (2-tailed)	.305	.274	.180	.092	.016		.086	.229	.678	.091	.431	.316	.239
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
strA	Pearson Correlation	.863	-.420	.954*	.993**	.916	.914	1	.456	-.073	-.921	-.420	.901	.958*
	Sig. (2-tailed)	.137	.580	.046	.007	.084	.086		.544	.927	.079	.580	.099	.042
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
aphA2	Pearson Correlation	.114	-.873	.278	.433	.703	.771	.456	1	.851	-.624	-.724	.154	.185
	Sig. (2-tailed)	.886	.127	.722	.567	.297	.229	.544		.149	.376	.276	.846	.815
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
cat1	Pearson Correlation	-.409	-.688	-.266	-.105	.231	.322	-.073	.851	1	-.187	-.623	-.325	-.349
	Sig. (2-tailed)	.591	.312	.734	.895	.769	.678	.927	.149		.813	.377	.675	.651
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfrIa	Pearson Correlation	-.599	.408	-.762	-.872	-.841	-.909	-.921	-.624	-.187	1	.739	-.866	-.828
	Sig. (2-tailed)	.401	.592	.238	.128	.159	.091	.079	.376	.813		.261	.134	.172
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfrVII	Pearson Correlation	.095	.302	-.129	-.322	-.412	-.569	-.420	-.724	-.623	.739	1	-.426	-.255
	Sig. (2-tailed)	.905	.698	.871	.678	.588	.431	.580	.276	.377	.261		.574	.745
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
class1	Pearson Correlation	.741	.000	.832	.862	.656	.684	.901	.154	-.325	-.866	-.426	1	.956*
	Sig. (2-tailed)	.259	1.000	.168	.138	.344	.316	.099	.846	.675	.134	.574		.044
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
int1	Pearson Correlation	.904	-.169	.956*	.954*	.780	.761	.958*	.185	-.349	-.828	-.255	.956*	1
	Sig. (2-tailed)	.096	.831	.044	.046	.220	.239	.042	.815	.651	.172	.745	.044	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3.12 Correlation between antibiotic resistance genes in *E. coli* isolates of clinical origin

Correlations

		tem	ctxm	sul 1	sul 2	tetA	tetB	str A	aph	Cat I	dhfr 1	dhfr 7	class 1	int1
tem	Pearson Correlation	1	.730	-.201	.102	.410	.214	.457	-.574	.903	-.243	-.243	-.262	-.488
	Sig. (2-tailed)		.270	.799	.898	.590	.786	.543	.426	.097	.757	.757	.738	.512
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
ctxm	Pearson Correlation	.730	1	.212	.754	.912	.753	.451	-.745	.953 *	-.838	-.838	-.802	-.869
	Sig. (2-tailed)	.270		.788	.246	.088	.247	.549	.255	.047	.162	.162	.198	.131
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
sul 1	Pearson Correlation	-.201	.212	1	.475	.247	.053	-.724	.416	.044	-.379	-.379	-.115	.074
	Sig. (2-tailed)	.799	.788		.525	.753	.947	.276	.584	.956	.621	.621	.885	.926
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
sul 2	Pearson Correlation	.102	.754	.475	1	.938	.904	.245	-.553	.519	-.987 *	-.987 *	-.927	-.810
	Sig. (2-tailed)	.898	.246	.525		.062	.096	.755	.447	.481	.013	.013	.073	.190
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
tetA	Pearson Correlation	.410	.912	.247	.938	1	.945	.490	-.776	.755	-.981 *	-.981 *	-.970 *	-.948
	Sig. (2-tailed)	.590	.088	.753	.062		.055	.510	.224	.245	.019	.019	.030	.052
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
tetB	Pearson Correlation	.214	.753	.053	.904	.945	1	.630	-.830	.568	-.937	-.937	-.996 **	-.955 *
	Sig. (2-tailed)	.786	.247	.947	.096	.055		.370	.170	.432	.063	.063	.004	.045
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
str A	Pearson Correlation	.457	.451	-.724	.245	.490	.630	1	-.925	.486	-.360	-.360	-.591	-.741
	Sig. (2-tailed)	.543	.549	.276	.755	.510	.370		.075	.514	.640	.640	.409	.259
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
aph	Pearson Correlation	-.574	-.745	.416	-.553	-.776	-.830	-.925	1	-.723	.663	.663	.819	.935
	Sig. (2-tailed)	.426	.255	.584	.447	.224	.170	.075		.277	.337	.337	.181	.065
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
Cat	Pearson Correlation	.903	.953 *	.044	.519	.755	.568	.486	-.723	1	-.634	-.634	-.620	-.763
	Sig. (2-tailed)	.097	.047	.956	.481	.245	.432	.514	.277		.366	.366	.380	.237
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfr 1	Pearson Correlation	-.243	-.838	-.379	-.987 *	-.981 *	-.937	-.360	.663	-.634	1	1.000 **	.961 *	.885
	Sig. (2-tailed)	.757	.162	.621	.013	.019	.063	.640	.337	.366		.000	.039	.115
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfr 7	Pearson Correlation	-.243	-.838	-.379	-.987 *	-.981 *	-.937	-.360	.663	-.634	1.000 **	1	.961 *	.885
	Sig. (2-tailed)	.757	.162	.621	.013	.019	.063	.640	.337	.366	.000		.039	.115
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
class 1	Pearson Correlation	-.262	-.802	-.115	-.927	-.970 *	-.996 **	-.591	.819	-.620	.961 *	.961 *	1	.961 *
	Sig. (2-tailed)	.738	.198	.885	.073	.030	.004	.409	.181	.380	.039	.039		.039
	N	4	4	4	4	4	4	4	4	4	4	4	4	4
int1	Pearson Correlation	-.488	-.869	.074	-.810	-.948	-.955 *	-.741	.935	-.763	.885	.885	.961 *	1
	Sig. (2-tailed)	.512	.131	.926	.190	.052	.045	.259	.065	.237	.115	.115	.039	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix -4

Table 4.1a Anova for distribution of virulence factor genes in different phylogenetic groups of *E. coli* isolates from Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	358.938	4	89.734	2.346	.089
	Within Groups	764.875	20	38.244		
	Total	1123.812	24			
B1	Between Groups	1273.899	4	318.475	3.195	.035
	Within Groups	1993.413	20	99.671		
	Total	3267.312	24			
B2	Between Groups	20468.454	4	5117.114	8.135	.000
	Within Groups	12579.900	20	628.995		
	Total	33048.354	24			
D	Between Groups	887.581	4	221.895	.290	.881
	Within Groups	15289.905	20	764.495		
	Total	16177.486	24			

Table 4.1b Games-Howell analysis of distribution of virulence genes in *E. coli* isolates of different phylogenetic groups from five different stations in Cochin estuary

Games-Howell

Dependent Variable	(I) STATION	(J) STATION	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
A	CHITOOR (S1)	Bolgatty	-4.86200	2.14583	.314	-14.3921	4.6681
		MS	-9.37800	5.80030	.556	-35.1603	16.4043
		Thevara	.06600	.06600	.844	-.2274	.3594
		Barmouth	.06600	.06600	.844	-.2274	.3594
	BOLGATTY(S2)	Chittor	4.86200	2.14583	.314	-4.6681	14.3921
		MS	-4.51600	6.18380	.940	-29.1761	20.1441
		Thevara	4.92800	2.14482	.305	-4.6070	14.4630
		Barmoth	4.92800	2.14482	.305	-4.6070	14.4630
	MS (S3)	Chitoor	9.37800	5.80030	.556	-16.4043	35.1603
		Bolgatty	4.51600	6.18380	.940	-20.1441	29.1761
		Thevara	9.44400	5.79993	.551	-16.3401	35.2281
		Barmouth	9.44400	5.79993	.551	-16.3401	35.2281
	THEVARA (S4)	Chitoor	-.06600	.06600	.844	-.3594	.2274
		Bolgatty	-4.92800	2.14482	.305	-14.4630	4.6070
		MS	-9.44400	5.79993	.551	-35.2281	16.3401
		Barmouth	.00000	.00000	.	.0000	.0000
	BARMOUTH(S5)	Chittor	-.06600	.06600	.844	-.3594	.2274
		Bolgatty	-4.92800	2.14482	.305	-14.4630	4.6070
		MS	-9.44400	5.79993	.551	-35.2281	16.3401
		Thevara	.00000	.00000	.	.0000	.0000

B1	CHITOOR (S1)	Bolgatty	-2.81800	1.84286	.597	-11.0106	5.3746
		MS	-8.72200	3.86856	.318	-25.9201	8.4761
		Thevara	-18.85600	9.01714	.369	-58.9426	21.2306
		Barmouth	.00000	.00000	.	.0000	.0000
	BOLGATTY(S2)	Chittor	2.81800	1.84286	.597	-5.3746	11.0106
		MS	-5.90400	4.28508	.662	-22.2359	10.4279
		Thevara	-16.03800	9.20352	.496	-55.3553	23.2793
		Barmouth	2.81800	1.84286	.597	-5.3746	11.0106
	MS (S3)	Chitoor	8.72200	3.86856	.318	-8.4761	25.9201
		Bolgatty	5.90400	4.28508	.662	-10.4279	22.2359
		Thevara	-10.13400	9.81196	.832	-48.2691	28.0011
		Barmouth	8.72200	3.86856	.318	-8.4761	25.9201
	THEVARA (S4)	Chittor	18.85600	9.01714	.369	-21.2306	58.9426
		Bolgatty	16.03800	9.20352	.496	-23.2793	55.3553
		MS	10.13400	9.81196	.832	-28.0011	48.2691
		Barmouth	18.85600	9.01714	.369	-21.2306	58.9426
	BARMOUTH(S5)	Chitoor	.00000	.00000	.	.0000	.0000
		Bolgatty	-2.81800	1.84286	.597	-11.0106	5.3746
		MS	-8.72200	3.86856	.318	-25.9201	8.4761
		Thevara	-18.85600	9.01714	.369	-58.9426	21.2306
B2	CHITOOR (S1)	Bolgatty	-85.07800 *	6.43622	.001	-113.6861	-56.4699
		MS	-28.97800	12.14284	.282	-82.9577	25.0017
		Thevara	-17.58200	7.89762	.326	-52.6878	17.5238
		Barmouth	-39.86600	19.43627	.382	-126.2703	46.5383
	BOLGATTY(S2)	Chittor	85.07800 *	6.43622	.001	56.4699	113.6861
		MS	56.10000 *	13.74265	.034	4.7828	107.4172
		Thevara	67.49600 *	10.18745	.001	31.9485	103.0435
		Barmouth	45.21200	20.47390	.311	-37.8388	128.2628
	MS (S3)	Chittor	28.97800	12.14284	.282	-25.0017	82.9577
		Bolgatty	-56.10000 *	13.74265	.034	-107.4172	-4.7828
		Thevara	11.39600	14.48475	.927	-40.7071	63.4991
		Barmouth	-10.88800	22.91734	.987	-93.8940	72.1180
	THEVARA (S4)	Chitoor	17.58200	7.89762	.326	-17.5238	52.6878
		Bolgatty	-67.49600 *	10.18745	.001	-103.0435	-31.9485
		MS	-11.39600	14.48475	.927	-63.4991	40.7071
		Barmouth	-22.28400	20.97923	.819	-104.6203	60.0523
	BARMOUTH(S5)	Chitoor	39.86600	19.43627	.382	-46.5383	126.2703
		Bolgatty	45.21200	20.47390	.311	-128.2628	37.8388
		MS	10.88800	22.91734	.987	-72.1180	93.8940
		Thevara	22.28400	20.97923	.819	-60.0523	104.6203
D	CHITOOR (S1)	Bolgatty	13.15800	20.17920	.958	-74.5274	100.8434
		MS	7.47600	21.24992	.996	-77.3818	92.3338
		Thevara	-3.23000	22.89254	1.000	-87.7460	81.2860
		Barmouth	.20000	23.99541	1.000	-85.7306	86.1306
	BOLGATTY(S2)	Chittor	-13.15800	20.17920	.958	-100.8434	74.5274
		MS	-5.68200	7.91735	.944	-36.6552	25.2912
		Thevara	-16.38800	11.62729	.650	-64.8153	32.0393
		Barmouth	-12.95800	13.67131	.866	-70.8747	44.9587
	MS (S3)	Chittor	-7.47600	21.24992	.996	-92.3338	77.3818
		Bolgatty	5.68200	7.91735	.944	-25.2912	36.6552
		Thevara	-10.70600	13.39974	.923	-58.8902	37.4782
		Barmouth	-7.27600	15.20737	.987	-63.6871	49.1351
	THEVARA (S4)	Chittor	3.23000	22.89254	1.000	-81.2860	87.7460
		Bolgatty	16.38800	11.62729	.650	-32.0393	64.8153
		MS	10.70600	13.39974	.923	-37.4782	58.8902
		Barmouth	3.43000	17.42910	1.000	-57.2113	64.0713
	BARMOUTH(S5)	Chittor	-.20000	23.99541	1.000	-86.1306	85.7306
		Bolgatty	12.95800	13.67131	.866	-44.9587	70.8747
		MS	7.27600	15.20737	.987	-49.1351	63.6871
		Thevara	-3.43000	17.42910	1.000	-64.0713	57.2113

*. The mean difference is significant at the .05 level.

Table 4.2 Anova for distribution of virulence factor genes in *E. coli* isolates in five different stations set at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHITTOR	Between Groups	782.238	4	195.560	1.140	.375
	Within Groups	2573.211	15	171.547		
	Total	3355.450	19			
BOLGATTY	Between Groups	1371.771	4	342.943	2.222	.116
	Within Groups	2314.966	15	154.331		
	Total	3686.737	19			
MS	Between Groups	783.946	4	195.986	4.978	.009
	Within Groups	590.545	15	39.370		
	Total	1374.491	19			
THEVARA	Between Groups	2029.261	4	507.315	1.484	.256
	Within Groups	5126.595	15	341.773		
	Total	7155.856	19			
BARMOUTH	Between Groups	210.560	4	52.640	1.397	.282
	Within Groups	565.026	15	37.668		
	Total	775.586	19			

Table 4.3 Anova for distribution of virulence factor genes within different phylogenetic groups *E. coli* isolates in five different stations set at Cochin estuary

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHITOOR	Between Groups	932.223	3	310.741	2.052	.147
	Within Groups	2423.226	16	151.452		
	Total	3355.450	19			
BOLGATTY	Between Groups	24213.660	3	8071.220	110.230	.000
	Within Groups	1171.546	16	73.222		
	Total	25385.206	19			
MS	Between Groups	1372.819	3	457.606	1.467	.261
	Within Groups	4991.376	16	311.961		
	Total	6364.195	19			
THEVARA	Between Groups	1591.329	3	530.443	1.573	.235
	Within Groups	5394.114	16	337.132		
	Total	6985.443	19			
BARMOUTH	Between Groups	1499.800	3	499.933	.529	.669
	Within Groups	15109.689	16	944.356		
	Total	16609.489	19			

Table 4.4 Anova for distribution of virulence factor genes in *E. coli* isolates from different sources

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CLINICAL	Between Groups	18325.099	4	4581.275	9.252	.001
	Within Groups	7427.748	15	495.183		
	Total	25752.847	19			
SEAFOOD	Between Groups	66.406	4	16.602	.607	.664
	Within Groups	410.156	15	27.344		
	Total	476.563	19			
ESTUARY	Between Groups	814.379	4	203.595	2.532	.084
	Within Groups	1206.312	15	80.421		
	Total	2020.691	19			

Table 4.5 Games-Howell analysis of distribution of virulence factor genes in *E. coli* isolates from clinical sources

Games-Howell

(I) GENES	(J) GENES	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
papAH	*papC*	-19.89750	17.39227	.781	-91.1144	51.3194
	sfa	19.89250	9.65322	.368	-20.8204	60.6054
	iut	-60.10750*	12.72535	.018	-107.9582	-12.2568
	kpsMT	-51.77250	16.83747	.128	-119.9631	16.4181
papC	*papAH*	19.89750	17.39227	.781	-51.3194	91.1144
	sfa	39.79000	15.73492	.265	-35.6114	115.1914
	iut	-40.21000	17.78589	.292	-111.4960	31.0760
	kpsMT	-31.87500	20.92615	.585	-110.4207	46.6707
sfa	*papAH*	-19.89250	9.65322	.368	-60.6054	20.8204
	papC	-39.79000	15.73492	.265	-115.1914	35.6114
	iut	-80.00000*	10.34559	.006	-124.6246	-35.3754
	kpsMT	-71.66500	15.11943	.051	-143.5693	.2393
iut	*papAH*	60.10750*	12.72535	.018	12.2568	107.9582
	papC	40.21000	17.78589	.292	-31.0760	111.4960
	sfa	80.00000*	10.34559	.006	35.3754	124.6246
	kpsMT	8.33500	17.24376	.986	-60.0748	76.7448
kpsMT	*papAH*	51.77250	16.83747	.128	-16.4181	119.9631
	papC	31.87500	20.92615	.585	-46.6707	110.4207
	sfa	71.66500	15.11943	.051	-.2393	143.5693
	iut	-8.33500	17.24376	.986	-76.7448	60.0748

*. The mean difference is significant at the .05 level.

Table 4.6a Anova for distribution of virulence factor genes in different phylogenetic groups of *E. coli* isolates in clinical, seafood and estuarine sources

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CLINICAL	Between Groups	22016.672	3	7338.891	162.903	.000
	Within Groups	720.811	16	45.051		
	Total	22737.482	19			
SEAFOOD	Between Groups	13500.000	3	4500.000	6.000	.006
	Within Groups	12000.000	16	750.000		
	Total	25500.000	19			
ESTUARY	Between Groups	16122.197	3	5374.066	24.846	.000
	Within Groups	3460.717	16	216.295		
	Total	19582.914	19			

Table 4.6b Games-Howell analysis of distribution of virulence factor genes in different phylogenetic groups of *E. coli* isolates in clinical and estuarine sources

Games-Howell

Dependent Variable	(I) phylo	(J) phylo	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CLINICAL	A	B1	6.83200	2.88964	.196	-3.5983	17.2623
		B2	-72.56000*	5.51397	.000	-91.4103	-53.7097
		D	4.71800	3.40468	.542	-6.3238	15.7598
	B1	A	-6.83200	2.88964	.196	-17.2623	3.5983
		B2	-79.39200*	4.94456	.000	-98.6132	-60.1708
		D	-2.11400	2.37417	.810	-10.3277	6.0997
	B2	A	72.56000*	5.51397	.000	53.7097	91.4103
		B1	79.39200*	4.94456	.000	60.1708	98.6132
		D	77.27800*	5.26218	.000	58.4973	96.0587
	D	A	-4.71800	3.40468	.542	-15.7598	6.3238
		B1	2.11400	2.37417	.810	-6.0997	10.3277
		B2	-77.27800*	5.26218	.000	-96.0587	-58.4973
ESTUARY	A	B1	-.61200	3.93208	.999	-13.2245	12.0005
		B2	-68.30200*	11.16616	.009	-111.4121	-25.1919
		D	-9.21400	6.85929	.576	-33.8547	15.4267
	B1	A	.61200	3.93208	.999	-12.0005	13.2245
		B2	-67.69000*	11.22435	.009	-110.6630	-24.7170
		D	-8.60200	6.95361	.630	-33.2143	16.0103
	B2	A	68.30200*	11.16616	.009	25.1919	111.4121
		B1	67.69000*	11.22435	.009	24.7170	110.6630
		D	59.08800*	12.55287	.011	16.5528	101.6232
	D	A	9.21400	6.85929	.576	-15.4267	33.8547
		B1	8.60200	6.95361	.630	-16.0103	33.2143
		B2	-59.08800*	12.55287	.011	-101.6232	-16.5528

*. The mean difference is significant at the .05 level.

Table 4.7 Correlation between virulence factor genes, antibiotic resistance in *E. coli* isolates from Cochin estuary

		papAH	papC	sfa/focDE	iutA	kpsMTII	Amp	ctx	cx	Cpd	caz	ctr	cxm	C	Cip	cot	Gen	Na	S	Te	Tr
papAH	Pearson Correlation	1	.095	-.407	.604	.561	.345	.439	.484	.610	.588	.951 *	.369	.875	.831	-.222	.882	.890	.421	.988 *	-.228
	Sig. (2-tailed)		.905	.593	.396	.439	.655	.561	.516	.390	.412	.049	.631	.125	.169	.778	.118	.110	.579	.014	.772
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
papC	Pearson Correlation	.095	1	.349	-.680	-.726	.967 *	-.634	.883	.366	-.737	.257	-.570	-.379	.617	.100	.113	.208	.729	.393	-.653
	Sig. (2-tailed)	.905		.651	.320	.274	.033	.366	.117	.634	.263	.743	.430	.621	.383	.900	.887	.792	.271	.607	.347
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sfa/focDE	Pearson Correlation	-.407	.349	1	-.778	-.312	.240	.042	-.071	-.716	-.433	-.106	.194	-.649	-.239	.937	.040	.047	-.366	-.263	.445
	Sig. (2-tailed)	.593	.651		.222	.688	.760	.958	.929	.284	.567	.894	.806	.351	.761	.063	.960	.953	.634	.737	.555
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
iutA	Pearson Correlation	.604	-.680	-.778	1	.840	-.492	.595	-.259	.337	.903	.373	.496	.912	.141	-.589	.373	.317	-.066	.547	.124
	Sig. (2-tailed)	.396	.320	.222		.160	.508	.405	.741	.663	.097	.627	.504	.088	.859	.491	.627	.683	.934	.453	.876
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
kpsMTII	Pearson Correlation	.561	-.726	-.312	.840	1	-.536	.936	-.452	-.106	.962 **	.472	.872	.818	.907	-.039	.598	.520	-.463	.599	.572
	Sig. (2-tailed)	.439	.274	.688	.160		.464	.064	.548	.894	.038	.528	.128	.182	.093	.961	.402	.480	.537	.401	.428
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Amp	Pearson Correlation	.345	.967 *	.240	-.492	-.536	1	-.475	.961 *	.506	-.541	.491	-.430	-.135	.791	.056	.341	.432	.764	.343	-.670
	Sig. (2-tailed)	.655	.033	.760	.508	.464		.525	.049	.494	.459	.509	.570	.865	.209	.944	.659	.568	.236	.657	.330
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
ctx	Pearson Correlation	.439	-.634	.042	.595	.936	-.475	1	-.501	-.379	.883	.457	.988 *	.620	-.981	.388	.644	.564	-.630	.532	.757
	Sig. (2-tailed)	.561	.366	.958	.405	.064	.525		.499	.621	.117	.543	.012	.380	.019	.612	.356	.436	.370	.468	.243
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cx	Pearson Correlation	.484	.883	-.071	-.259	-.452	.951 *	-.501	1	.747	-.419	.538	-.503	.067	.889	-.240	.338	.429	.928	.436	-.829
	Sig. (2-tailed)	.516	.117	.929	.741	.548	.049	.499		.253	.581	.462	.497	.933	.111	.760	.662	.571	.072	.564	.171
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cpd	Pearson Correlation	.610	.366	-.716	.337	-.106	.506	-.379	.747	1	-.005	.447	-.481	.479	.781	-.793	.210	.268	.567	.480	-.872
	Sig. (2-tailed)	.390	.634	.284	.663	.894	.494	.621	.253		.995	.553	.519	.521	.219	.207	.790	.732	.093	.520	.128
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
caz	Pearson Correlation	.588	-.737	-.433	.903	.962 **	-.541	.883	-.419	-.005	1	.462	.891	.865	.939	-.091	.562	.467	-.387	.504	.482
	Sig. (2-tailed)	.412	.263	.567	.097	.038	.459	.117	.581	.995		.538	.109	.135	.061	.909	.438	.533	.613	.396	.518
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
ctr	Pearson Correlation	.951 *	.257	-.106	.373	.472	.491	.457	.538	.447	.462	1	.433	.719	.845	.062	.966 *	.982 *	.363	.961 *	-.134
	Sig. (2-tailed)	.049	.743	.894	.627	.528	.509	.543	.462	.553	.538		.567	.281	.155	.938	.034	.018	.637	.039	.866
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cxm	Pearson Correlation	.369	-.570	.194	.496	.872	-.430	.988 *	-.503	-.481	.801	.433	1	.510	-.116	.524	.638	.561	-.677	.482	.821
	Sig. (2-tailed)	.631	.430	.806	.504	.128	.570	.012	.497	.519	.199	.567		.490	.884	.476	.362	.439	.323	.518	.179
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C	Pearson Correlation	.875	-.379	-.649	.912	.818	-.135	.620	.067	.479	.865	.719	.510	1	.495	-.382	.696	.663	-.127	.341	.000
	Sig. (2-tailed)	.125	.621	.351	.088	.182	.865	.380	.933	.521	.135	.281	.490		.505	.618	.304	.337	.873	.659	1.000
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cip	Pearson Correlation	.831	.617	-.239	.141	.907	-.791	.981	-.889	.781	.939	.845	-.116	.495	1	-.248	.686	.748	.803	.798	-.636
	Sig. (2-tailed)	.169	.383	.761	.859	.093	.209	.019	.111	.219	.061	.155	.884	.505		.752	.314	.252	.197	.202	.364
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cot	Pearson Correlation	-.222	.100	.937	-.509	-.039	.056	.388	-.240	-.793	-.091	.062	.524	-.382	-.248	1	.261	.241	-.576	-.957	.679
	Sig. (2-tailed)	.778	.900	.063	.491	.961	.944	.612	.760	.207	.909	.938	.476	.618	.752		.739	.759	.424	.043	.321
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Gen	Pearson Correlation	.882	.113	.040	.373	.598	.341	.644	.338	.210	.562	.966 *	.638	.696	.686	.261	1	.995 **	.117	.949	.122
	Sig. (2-tailed)	.118	.887	.960	.627	.402	.659	.356	.662	.790	.438	.034	.362	.304	.314	.739		.005	.883	.051	.878
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Na	Pearson Correlation	.890	.208	.047	.317	.520	.432	.564	.429	.268	.467	.982 *	.561	.663	.748	.241	.995 **	1	.204	.951 *	.037
	Sig. (2-tailed)	.110	.792	.953	.683	.480	.568	.436	.571	.732	.533	.018	.439	.337	.252	.759	.005		.796	.049	.963
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
S	Pearson Correlation	.421	.729	-.366	-.066	-.463	.764	-.630	.928	.567	-.367	.363	-.677	.127	.803	-.576	.117	.204	1	.316	-.970
	Sig. (2-tailed)	.579	.271	.634	.934	.537	.236	.370	.072	.093	.633	.637	.323	.873	.197	.424	.883	.796		.684	.030
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Te	Pearson Correlation	.988 *	.053	-.263	.547	.599	.343	.532	.436	.480	.604	.981 *	.482	.841	.796	-.057	.949	.951 *	.316	1	-.102
	Sig. (2-tailed)	.014	.947	.737	.453	.401	.657	.468	.564	.520	.396	.019	.518	.159	.204	.943	.051	.049	.684		.898
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Tr	Pearson Correlation	-.228	-.653	.445	.124	.572	-.670	.757	-.829	-.872	.482	-.134	.821	.000	-.636	.679	.122	.037	-.970 *	-.102	1
	Sig. (2-tailed)	.772	.347	.555	.876	.428	.330	.243	.171	.128	.518	.866	.179	1.000	.364	.321	.878	.963	.030	.898	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.8 Correlation between virulence factor genes, antibiotic resistance genes in *E. coli* isolates from Cochin estuary

		papAH	papC	sfa	iut	kps	tem	ctxm	sul1	sul2	tetA	tetB	str	aph	Cat1	dhfr1	dhfr7	Class 1	int 1
papAH	Pearson Correlation	1	.911	1.000**	.804	.607	.444	.711	-.188	-.271	.308	.245	.045	.245	1.000**	1.000**	1.000**	-.188	.826
	Sig. (2-tailed)		.089	.000	.196	.393	.556	.289	.812	.729	.692	.755	.955	.755	.000	.000	.000	.812	.174
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
papC	Pearson Correlation	.911	1	.911	.967*	.879	.443	.925	-.360	-.491	.098	-.069	-.213	-.069	.911	.911	.911	-.360	.975*
	Sig. (2-tailed)	.089		.089	.033	.121	.557	.075	.640	.509	.902	.931	.787	.931	.089	.089	.089	.640	.025
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sfa	Pearson Correlation	1.000**	.911	1	.804	.607	.444	.711	-.188	-.271	.308	.245	.045	.245	1.000**	1.000**	1.000**	-.188	.826
	Sig. (2-tailed)	.000	.089		.196	.393	.556	.289	.812	.729	.692	.755	.955	.755	.000	.000	.000	.812	.174
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
iut	Pearson Correlation	.804	.967*	.804	1	.954*	.259	.990*	-.264	-.426	.139	-.094	-.189	-.094	.804	.804	.804	-.264	.999**
	Sig. (2-tailed)	.196	.033	.196		.046	.741	.010	.736	.574	.861	.906	.820	.906	.196	.196	.196	.736	.001
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
kps	Pearson Correlation	.607	.879	.607	.954*	1	.257	.982*	-.391	-.557	-.081	-.345	-.380	-.345	.607	.607	.607	-.391	.943
	Sig. (2-tailed)	.393	.121	.393	.046		.743	.018	.609	.443	.919	.655	.620	.655	.393	.393	.393	.609	.057
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
tem	Pearson Correlation	.444	.443	.444	.259	.257	1	.197	-.900	-.852	-.654	-.548	-.739	-.548	.444	.444	.444	-.900	.271
	Sig. (2-tailed)	.556	.557	.556	.741	.743		.803	.100	.148	.346	.452	.261	.452	.556	.556	.556	.100	.729
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
ctxm	Pearson Correlation	.711	.925	.711	.990*	.982*	.197	1	-.264	-.436	.093	-.167	-.229	-.167	.711	.711	.711	-.264	.984*
	Sig. (2-tailed)	.289	.075	.289	.010	.018	.803		.736	.564	.907	.833	.780	.833	.289	.289	.289	.736	.016
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sul1	Pearson Correlation	-.188	-.360	-.188	-.264	-.391	-.900	-.264	1	.982*	.877	.854	.957*	.854	-.188	-.188	-.188	1.000**	-.260
	Sig. (2-tailed)	.812	.640	.812	.736	.609	.100	.736		.018	.123	.146	.043	.146	.812	.812	.812	.000	.740
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sul2	Pearson Correlation	-.271	-.491	-.271	-.426	-.557	-.852	-.436	.982*	1	.818	.856	.949	.856	-.271	-.271	-.271	.982*	-.419
	Sig. (2-tailed)	.729	.509	.729	.574	.443	.148	.564	.018		.182	.144	.051	.144	.729	.729	.729	.018	.581
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
tetA	Pearson Correlation	.308	.098	.308	.139	-.081	-.654	.093	.877	.818	1	.947	.949	.947	.308	.308	.308	.877	.153
	Sig. (2-tailed)	.692	.902	.692	.861	.919	.346	.907	.123	.182		.053	.051	.053	.692	.692	.692	.123	.847
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
tetB	Pearson Correlation	.245	-.069	.245	-.094	-.345	-.548	-.167	.854	.856	.947	1	.968*	1.000**	.245	.245	.245	.854	-.071
	Sig. (2-tailed)	.755	.931	.755	.906	.655	.452	.833	.146	.144	.053		.032	.000	.755	.755	.755	.146	.929
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
str	Pearson Correlation	.045	-.213	.045	-.189	-.380	-.739	-.229	.957*	.949	.949	.968*	1	.968*	.045	.045	.045	.957*	-.165
	Sig. (2-tailed)	.955	.787	.955	.820	.620	.261	.780	.043	.051	.051	.032		.032	.955	.955	.955	.043	.835
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
aph	Pearson Correlation	.245	-.069	.245	-.094	-.345	-.548	-.167	.854	.856	.947	1.000**	.968*	1	.245	.245	.245	.854	-.071
	Sig. (2-tailed)	.755	.931	.755	.906	.655	.452	.833	.146	.144	.053	.000	.032		.755	.755	.755	.146	.929
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cat1	Pearson Correlation	1.000**	.911	1.000**	.804	.607	.444	.711	-.188	-.271	.308	.245	.045	.245	1	1.000**	1.000**	-.188	.826
	Sig. (2-tailed)	.000	.089	.000	.196	.393	.556	.289	.812	.729	.692	.755	.955	.755		.000	.000	.812	.174
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfr1	Pearson Correlation	1.000**	.911	1.000**	.804	.607	.444	.711	-.188	-.271	.308	.245	.045	.245	1.000**	1	1.000**	-.188	.826
	Sig. (2-tailed)	.000	.089	.000	.196	.393	.556	.289	.812	.729	.692	.755	.955	.755	.000		.000	.812	.174
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfr7	Pearson Correlation	1.000**	.911	1.000**	.804	.607	.444	.711	-.188	-.271	.308	.245	.045	.245	1.000**	1.000**	1	-.188	.826
	Sig. (2-tailed)	.000	.089	.000	.196	.393	.556	.289	.812	.729	.692	.755	.955	.755	.000	.000		.812	.174
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Class 1	Pearson Correlation	-.188	-.360	-.188	-.264	-.391	-.900	-.264	1.000**	.982*	.877	.854	.957*	.854	-.188	-.188	-.188	1	-.260
	Sig. (2-tailed)	.812	.640	.812	.736	.609	.100	.736	.000	.018	.123	.146	.043	.146	.812	.812	.812		.740
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
int 1	Pearson Correlation	.826	.975*	.826	.999**	.943	.271	.984*	-.260	-.419	.153	-.071	-.165	-.071	.826	.826	.826	-.260	1
	Sig. (2-tailed)	.174	.025	.174	.001	.057	.729	.016	.740	.581	.847	.929	.835	.929	.174	.174	.174	.740	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.9 Correlation between virulence factor genes, antibiotic resistance in *E. coli* isolates of clinical sources

Correlations

		papAH	papC	afa/focDE	iutA	kpsMTII	Amp	ctx	cx	Cpd	caz	ctr	cxm	C	Cip	cot	Gen	Na	S	Te	Tr
papAH	Pearson Correlation	1	.095	-.407	.604	.561	.345	.439	.484	.610	.588	.951 *	.369	.875	.831	-.222	.882	.890	.421	.986 *	-.228
	Sig. (2-tailed)		.905	.593	.396	.439	.655	.561	.516	.390	.412	.049	.631	.125	.169	.778	.118	.110	.579	.014	.772
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
papC	Pearson Correlation	.095	1	.349	-.680	-.726	.967 *	-.534	.883	.386	.737	.257	-.578	-.375	.617	.100	.113	.208	.729	.093	-.663
	Sig. (2-tailed)	.905		.651	.320	.274	.033	.466	.117	.615	.263	.743	.422	.625	.383	.900	.887	.792	.271	.907	.337
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
afa/focDE	Pearson Correlation	-.407	.349	1	-.778	-.312	.240	.342	-.071	-.716	-.433	-.105	.194	-.645	-.239	.937	.040	.047	-.386	-.263	.445
	Sig. (2-tailed)	.593	.651		.222	.688	.760	.658	.929	.284	.567	.894	.806	.355	.761	.063	.960	.953	.614	.737	.555
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
iutA	Pearson Correlation	.604	-.680	-.778	1	.840	-.492	.595	-.259	.337	.903	.373	.466	.912	.141	-.509	.373	.317	-.086	.547	.124
	Sig. (2-tailed)	.396	.320	.222		.160	.508	.405	.741	.663	.097	.627	.534	.088	.859	.491	.627	.683	.914	.453	.876
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
kpsMTII	Pearson Correlation	.561	-.726	-.312	.840	1	-.535	.936	-.452	-.108	.992 **	.472	.872	.818	.007	.039	.598	.520	-.463	.599	.572
	Sig. (2-tailed)	.439	.274	.688	.160		.464	.064	.548	.892	.008	.528	.128	.182	.993	.961	.402	.480	.537	.401	.428
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Amp	Pearson Correlation	.345	.967 *	.240	-.492	-.535	1	-.475	.951 *	.506	-.541	.491	-.430	-.135	.791	.056	.341	.432	.784	.343	-.670
	Sig. (2-tailed)	.655	.033	.760	.508	.464		.525	.049	.494	.459	.509	.570	.866	.209	.944	.659	.568	.216	.657	.330
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
ctx	Pearson Correlation	.439	-.634	.042	.595	.936	-.475	1	-.501	-.379	.883	.457	.988 *	.620	-.081	.388	.644	.564	-.630	.532	.767
	Sig. (2-tailed)	.561	.366	.958	.405	.064	.525		.499	.621	.117	.543	.012	.380	.919	.612	.356	.436	.370	.468	.233
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cx	Pearson Correlation	.484	.883	-.071	-.259	-.452	.951 *	-.501	1	.747	-.419	.538	-.503	.867	.889	-.240	.338	.429	.928	.436	-.829
	Sig. (2-tailed)	.516	.117	.929	.741	.548	.049	.499		.253	.581	.462	.497	.133	.111	.760	.662	.571	.072	.564	.171
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cpd	Pearson Correlation	.610	.386	-.716	.337	-.108	.506	-.379	.747	1	.005	.447	-.481	.475	.781	-.793	.210	.268	.907	.480	-.877
	Sig. (2-tailed)	.390	.615	.284	.663	.892	.494	.621	.253		.995	.553	.519	.521	.219	.207	.790	.732	.093	.520	.123
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
caz	Pearson Correlation	.588	-.737	-.433	.903	.992 **	-.541	.883	-.419	-.005	1	.462	.801	.866	.039	-.091	.562	.487	-.387	.604	.482
	Sig. (2-tailed)	.412	.263	.567	.097	.008	.459	.117	.581	.995		.538	.199	.134	.961	.909	.438	.513	.613	.396	.518
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
ctr	Pearson Correlation	.951 *	.257	-.105	.373	.472	.491	.457	.538	.447	.462	1	.433	.715	.645	.062	.966 *	.982 *	.363	.981 *	-.134
	Sig. (2-tailed)	.049	.743	.894	.627	.528	.509	.543	.462	.553	.538		.567	.285	.355	.938	.034	.018	.637	.019	.866
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cxm	Pearson Correlation	.369	-.578	.194	.466	.872	-.430	.988 *	-.503	-.481	.801	.433	1	.510	-.116	.524	.638	.561	-.577	.482	.821
	Sig. (2-tailed)	.631	.422	.806	.534	.128	.570	.012	.497	.519	.199	.567		.490	.884	.476	.362	.439	.323	.518	.179
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C	Pearson Correlation	.875	-.375	-.645	.912	.818	-.135	.620	.067	.479	.865	.715	.510	1	.495	-.382	.696	.663	.127	.841	.000
	Sig. (2-tailed)	.125	.625	.355	.088	.182	.865	.380	.933	.521	.135	.285	.490		.505	.618	.304	.337	.873	.159	1.000
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Cip	Pearson Correlation	.831	.617	-.239	.141	.007	.791	-.081	.889	.781	.039	.645	-.116	.495	1	-.248	.686	.748	.803	.796	-.636
	Sig. (2-tailed)	.169	.383	.761	.859	.993	.209	.919	.111	.219	.961	.355	.884	.505		.752	.314	.252	.197	.204	.364
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cot	Pearson Correlation	-.222	.100	.937	-.509	.039	.056	.388	-.240	-.793	-.091	.062	.524	-.382	-.248	1	.261	.241	-.576	-.057	.679
	Sig. (2-tailed)	.778	.900	.063	.491	.961	.944	.612	.760	.207	.909	.938	.476	.618	.752		.739	.759	.424	.943	.321
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Gen	Pearson Correlation	.882	.113	.040	.373	.598	.341	.644	.338	.210	.562	.965 *	.638	.696	.686	.261	1	.995 **	.117	.949	.122
	Sig. (2-tailed)	.118	.887	.960	.627	.402	.659	.356	.662	.790	.438	.034	.362	.304	.314	.739		.005	.883	.051	.878
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Na	Pearson Correlation	.890	.208	.047	.317	.520	.432	.564	.429	.268	.487	.982 *	.561	.662	.748	.241	.995 **	1	.204	.951 *	.037
	Sig. (2-tailed)	.110	.792	.953	.683	.480	.568	.436	.571	.732	.513	.018	.439	.337	.252	.759	.005		.796	.049	.963
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
S	Pearson Correlation	.421	.729	-.386	-.086	-.463	.784	-.530	.928	.907	-.387	.363	-.577	.127	.803	-.576	.117	.204	1	.316	-.970 *
	Sig. (2-tailed)	.579	.271	.614	.914	.537	.216	.370	.072	.093	.613	.637	.423	.873	.197	.424	.883	.796		.684	.030
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Te	Pearson Correlation	.986 *	.693	-.263	.547	.599	.343	.532	.436	.480	.604	.981 *	.482	.841	.796	-.057	.949	.951 *	.316	1	-.102
	Sig. (2-tailed)	.014	.307	.737	.453	.401	.657	.468	.564	.520	.396	.019	.518	.159	.204	.943	.051	.049	.684		.898
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Tr	Pearson Correlation	-.228	-.663	.445	.124	.572	-.670	.767	-.829	-.877	.482	-.134	.821	.000	-.636	.679	.122	.037	-.970 *	-.102	1
	Sig. (2-tailed)	.772	.337	.555	.876	.428	.330	.233	.171	.123	.518	.866	.179	1.000	.364	.321	.878	.963	.030	.898	
	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

* Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4.10 Correlation between virulence factor genes, antibiotic resistance genes in *E. coli* isolates from clinical sources

		papAH	papC	sfa/focDE	iutA	kpsMTII	tsm	chxm	sul 1	sul2	tetA	tetB	strA	aph	cat1	dhfr Ia	Dhfr 7	class 1	int 1
papAH	Pearson Correlation	1	.095	-.407	.604	.561	.579	.900	-.089	.938	.987 *	.913	.766	-.859	.859	.999 **	.999 **	.883	.834
	Sig. (2-tailed)		.905	.593	.396	.439	.421	.100	.911	.062	.013	.087	.234	.131	.141	.001	.001	.117	.166
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
papC	Pearson Correlation	.095	1	.349	-.680	-.726	-.622	-.329	-.066	.370	.195	.492	.479	-.220	-.387	.114	.133	-.286	-.228
	Sig. (2-tailed)	.905		.651	.320	.274	.378	.671	.934	.630	.805	.508	.521	.780	.613	.886	.867	.714	.772
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sfa/focDE	Pearson Correlation	-.407	.349	1	-.773	-.312	-.872	-.424	.835	-.107	-.260	-.184	-.563	.693	-.709	-.437	-.397	-.265	-.083
	Sig. (2-tailed)	.593	.651		.227	.688	.128	.576	.165	.893	.740	.816	.437	.307	.309	.563	.603	.734	.917
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
iutA	Pearson Correlation	.604	-.680	-.773	1	.840	.986 *	.815	-.304	.299	.484	.236	.307	-.552	.927	.603	.577	.723	.599
	Sig. (2-tailed)	.396	.320	.227		.160	.014	.185	.696	.701	.516	.764	.693	.448	.073	.397	.423	.277	.401
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
kpsMTII	Pearson Correlation	.561	-.726	-.312	.840	1	.736	.865	.262	.359	.507	.203	-.023	-.252	.796	.533	.529	.863	.833
	Sig. (2-tailed)	.439	.274	.688	.160		.264	.135	.738	.641	.493	.797	.977	.748	.204	.467	.471	.137	.167
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
tsm	Pearson Correlation	.579	-.622	-.872	.986 *	.736	1	.747	-.460	.261	.446	.235	.389	-.623	.908	.586	.554	.634	.488
	Sig. (2-tailed)	.421	.378	.128	.014	.264		.253	.540	.739	.554	.765	.611	.377	.092	.414	.446	.366	.512
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
chxm	Pearson Correlation	.900	-.329	-.424	.815	.865	.747	1	.070	.753	.862	.656	.456	-.665	.943	.886	.883	.965 *	.938
	Sig. (2-tailed)	.100	.671	.576	.185	.135	.253		.930	.247	.138	.344	.544	.335	.057	.114	.117	.014	.062
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sul 1	Pearson Correlation	-.089	-.066	.835	-.304	.262	-.460	.070	1	.399	.029	-.069	-.586	.558	-.251	-.136	-.097	.229	.397
	Sig. (2-tailed)	.911	.934	.165	.696	.738	.540	.930		.901	.971	.931	.414	.442	.749	.864	.903	.771	.603
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
sul2	Pearson Correlation	.938	.370	-.107	.299	.359	.261	.753	.099	1	.980 *	.978 *	.734	-.750	.633	.933	.947	.781	.787
	Sig. (2-tailed)	.062	.630	.893	.701	.641	.739	.247	.901		.020	.022	.266	.240	.367	.067	.053	.219	.213
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
tetA	Pearson Correlation	.987 *	.195	-.260	.484	.507	.446	.862	.029	.980 *	1	.947	.736	-.813	.775	.982 *	.990 *	.869	.849
	Sig. (2-tailed)	.013	.805	.740	.516	.493	.554	.138	.971	.020		.053	.264	.187	.225	.018	.010	.131	.151
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
tetB	Pearson Correlation	.913	.492	-.184	.236	.203	.235	.656	-.069	.978 *	.947	1	.847	-.833	.585	.918	.927	.665	.652
	Sig. (2-tailed)	.087	.508	.816	.764	.797	.765	.344	.931	.022	.053		.153	.167	.415	.082	.073	.335	.348
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
strA	Pearson Correlation	.766	.479	-.563	.307	-.023	.389	.456	-.586	.734	.736	.847	1	-.958 *	.566	.795	.784	.381	.286
	Sig. (2-tailed)	.234	.521	.437	.693	.977	.611	.544	.414	.266	.264	.153		.042	.434	.205	.216	.619	.714
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
aph	Pearson Correlation	-.859	-.220	.693	-.552	-.252	-.623	-.665	.558	-.750	-.813	-.833	-.958 *	1	-.777	-.892	-.876	-.579	-.466
	Sig. (2-tailed)	.131	.780	.307	.448	.748	.377	.335	.442	.240	.187	.167	.042		.223	.108	.124	.421	.534
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
cat1	Pearson Correlation	.859	-.387	-.709	.927	.796	.908	.943	-.251	.633	.775	.585	.566	-.777	1	.858	.841	.874	.770
	Sig. (2-tailed)	.141	.613	.309	.073	.204	.092	.057	.749	.367	.225	.415	.434	.223		.142	.159	.126	.230
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfr Ia	Pearson Correlation	.999 **	.114	-.437	.603	.533	.586	.886	-.136	.933	.982 *	.918	.795	-.892	.858	1	.999 **	.862	.807
	Sig. (2-tailed)	.001	.886	.563	.397	.467	.414	.114	.864	.067	.018	.082	.205	.108	.142		.001	.138	.193
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
dhfr7	Pearson Correlation	.999 **	.133	-.397	.577	.529	.554	.883	-.097	.947	.990 *	.927	.784	-.876	.841	.999 **	1	.865	.819
	Sig. (2-tailed)	.001	.867	.603	.423	.471	.446	.117	.903	.053	.010	.073	.216	.124	.159	.001		.134	.181
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
class 1	Pearson Correlation	.883	-.286	-.266	.723	.863	.634	.966 *	.229	.781	.869	.665	.381	-.579	.874	.862	.866	1	.983 *
	Sig. (2-tailed)	.117	.714	.734	.277	.137	.366	.014	.771	.219	.131	.335	.619	.421	.126	.138	.134		.017
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
int 1	Pearson Correlation	.834	-.228	-.083	.599	.833	.488	.938	.397	.787	.849	.652	.286	-.466	.770	.807	.819	.983 *	1
	Sig. (2-tailed)	.166	.772	.917	.401	.167	.512	.062	.603	.213	.151	.348	.714	.534	.230	.193	.181	.017	
	NI	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix -5

Table 5.1 One-sample kolmogorov-smirnov test for antibiotic resistance genes of strain CUSMBES11

One-Sample Kolmogorov-Smirnov Test

		blaTEM	blaCTXM	sul1	sul2	ietA	tetB	strA	aphA2	cat1	dhfrIa	dhfrVII	class 1 int	int1	int3
N		4	4	4	4	4	4	4	4	4	4	4	4	4	4
Normal Parameters [a,b]	Mean	25.2275	27.7450	25.8100	28.2575	26.2725	25.4400	27.6125	29.7125	28.2025	27.7375	26.0200	27.0850	27.6725	26.8250
	Std. Deviation	.79672	.52748	1.22692	.91678	.86079	.70588	1.14270	.29341	.38379	.52614	2.36027	1.49627	.89571	.22293
Most Extreme Differences	Absolute	.384	.237	.276	.294	.228	.155	.248	.301	.340	.357	.155	.260	.256	.312
	Positive	.272	.237	.242	.289	.228	.141	.248	.266	.227	.245	.136	.260	.256	.312
	Negative	-.384	-.192	-.276	-.294	-.206	-.155	-.205	-.301	-.340	-.357	-.155	-.190	-.179	-.243
Kolmogorov-Smirnov Z		.768	.475	.552	.588	.456	.310	.495	.601	.680	.713	.310	.519	.513	.625
Asymp. Sig. (2-tailed)		.598	.978	.921	.879	.985	1.000	.967	.863	.744	.689	1.000	.950	.955	.830

a. Test distribution is Normal.

b. Calculated from data.

Table 5.2 One-sample kolmogorov-smirnov test for house-keeping genes and virulence genes of strain CUSMBES11

One-Sample Kolmogorov-Smirnov Test

		uid	frr	papAH	papC	sfa/focDE	iutA	kpsMT II	stx1
N		4	4	4	4	4	4	4	4
Normal Parameters[a,b]	Mean	26.7900	21.3225	24.1975	27.6975	24.9650	27.7625	24.4600	28.1325
	Std. Deviation	2.94015	1.33463	.51578	.93863	1.17105	2.06494	.81866	1.59892
Most Extreme Differences	Absolute	.237	.263	.252	.329	.164	.288	.308	.281
	Positive	.164	.197	.209	.329	.164	.271	.308	.281
	Negative	-.237	-.263	-.252	-.229	-.144	-.288	-.190	-.193
Kolmogorov-Smirnov Z		.473	.527	.504	.658	.328	.575	.617	.563
Asymp. Sig. (2-tailed)		.979	.944	.961	.779	1.000	.896	.842	.909

a. Test distribution is Normal.

b. Calculated from data.

GENBANK SUBMISSIONS

Escherichia coli plasmid pCUSMBES10 beta-lactamase TEM1 (blaTEM-1) gene, partial cds

GenBank: JX964785.1

FASTAGraphics

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LOCUS       JX964785                411 bp    DNA     linear   BCT 20-NOV-2012
DEFINITION  Escherichia coli plasmid pCUSMBES10 beta-lactamase TEM1 (blaTEM-1)
            gene, partial cds.
ACCESSION   JX964785
VERSION     JX964785.1  GI:416951015
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 411)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Diversity of phylogenetic groups among Escherichia coli isolates
            carring blaTEM-1
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 411)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-SEP-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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                     /note="genotype: B2"
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     CDS             <1..>411
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                     /db_xref="GI:416951016"
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                     ELCSAAITMSDNTAANLLLTTIGGPKELTAFLHN"
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      121 agagttttcg ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg
      181 gtgcggtatt atcccgtgtt gacgccgggc aagagcaact cggtcgccgc atacactatt
      241 ctcagaatga cttggttgag tactcaccag tcacagaaaa gcatcttacg gatggcatga
      301 cagtaagaga attatgcagt gctgccataa ccatgagtga taacactgct gccaacttac
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Escherichia coli plasmid pCUSMBN7 beta-lactamase TEM1 (blaTEM-1) gene, partial cds

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GenBank: JX964786.1
FASTAGraphics
LOCUS       JX964786                452 bp    DNA     linear   BCT 20-NOV-2012
DEFINITION  Escherichia coli plasmid pCUSMBN7 beta-lactamase TEM1 (blaTEM-1)
            gene, partial cds.
ACCESSION   JX9647861
VERSION     JX964786.1  GI:416951019
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 452)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Diversity of phylogenetic groups among Escherichia coli isolates
            carring blaTEM-1
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 452)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-SEP-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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                     /serotype="UT"
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      121 agagttttcg ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg
      181 gtgcggtatt atcccgtgtt gacgccgggc aagagcaact cggtcgccgc atacactatt
      241 ctcagaatga cttggttgag tactcaccag tcacagaaaa gcatcttacg gatggcatga
      301 cagtaagaga attatgcagt gctgccataa ccatgagtga taacactgcg gccaacttac
      361 ttctgacaac gatcggagga ccgaaggagc taaccgcttt tttgcacaac atgggggatc
      421 atgtaactcg ccttgatcgt tgggaaccgg ag
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Escherichia coli strain CUSMBB4 16S ribosomal RNA gene, partial sequence

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GenBank:        JX183937.1
LOCUS           JX183937 920 bp DNA linear BCT 06-SEP-2012
DEFINITION      Escherichia coli strain CUSMBB4 16S ribosomal RNA gene, partial sequence.
ACCESSION       JX183937
VERSION         JX183937.1 GI:402169608
KEYWORDS .
SOURCE          Escherichia coli
ORGANISM        Escherichia coli
                Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
                Enterobacteriaceae; Escherichia.
REFERENCE     1 (bases 1 to 920)
AUTHORS         Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
TITLE           Molecular epidemiology of diarrheagenic Escherichia coli
JOURNAL         Unpublished
REFERENCE       2 (bases 1 to 920)
AUTHORS         Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
TITLE           Direct Submission
JOURNAL         Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
                and Biochemistry, Cochin University of Science and Technology, School of
                Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala682016,India
FEATURES      Location/Qualifiers
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121 atactggcaa gcttgagtct cgtagagggg ggtagaattc caggtgtagc ggtgaaatgc
181 gtagagatct ggaggaatac cggtggcgaa ggcggccccc tggacgaaga ctgacgctca
241 ggtgcgaaag cgtggggagc aaacaggatt agataccctg gtagtccacg ccgtaaacga
301 tgtcgacttg gaggttgtgc ccttgaggcg tggcttccgg agctaacgcg ttaagtcgac
361 cgcctgggga gtacggccgc aaggttaaaa ctcaaatgaa ttgacggggg cccgcacaag
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781 gtccggattg gagtctgcaa ctcgactcca tgaagtcgga atcgctagta atcgtggatc
841 agaatgccac ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac accatgggag
901 tgggttgcaa aagaagtagg
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Escherichia coli strain CUSMBC1 16S ribosomal RNA gene, partial sequence

GenBank: JX183938.1

FASTAGraphics

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LOCUS       JX183938                875 bp    DNA     linear   BCT 06-SEP-2012
DEFINITION  Escherichia coli strain CUSMBC1 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   JX183938
VERSION     JX183938.1  GI:402169609
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (sites)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 875)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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                     /strain="CUSMBC1"
                     /db_xref="taxon:562"
                     /note="clinical isolate"
     rRNA            <1..>875
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      121 ggtgaaatgc gtagagatct ggaggaatac cggtggcgaa ggcggccccc tggacgaaga
      181 ctgacgctca ggtgcgaaag cgtggggagc aaacaggatt agataccctg gtagtccacg
      241 ccgtaaacga tgtcgacttg gaggttgtgc ccttgaggcg tggcttccgg agctaacgcg
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      361 cccgcacaag cggtggagca tgtggtttaa ttcgatgcaa cgcgaagaac cttacctggt
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Escherichia coli strain CUSMBES1 16S ribosomal RNA gene, partial sequence

GenBank: JX183939.1

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DEFINITION     Escherichia coli strain CUSMBES1 16S ribosomal RNA gene, partial
sequence.
ACCESSION      JX183939
VERSION JX183939.1 GI:402169610
KEYWORDS .
SOURCE         Escherichia coli
ORGANISM       Escherichia coli
               Bacteria; Proteobacteria; Gammaproteobacteria;
Enterobacteriales;
               Enterobacteriaceae; Escherichia.
REFERENCE 1    (bases 1 to 401)
AUTHORS        Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
TITLE          Molecular epidemiology of diarrheagenic Escherichia coli
JOURNAL        Unpublished
REFERENCE 2    (bases 1 to 401)
AUTHORS        Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
TITLE          Direct Submission
JOURNAL        Submitted      (18-JUN-2012) Department of Marine Biology,
Microbiology
               and Biochemistry, Cochin University of Science and Technology,
               School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
                       682016, India
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121 tgccagtgat aaactggagg aaggtgggga tgacgtcaag tcatcatggc ccttacgacc
181 agggctacac acgtgctaca atggcgcata caaagagaag cgacctcgcg agagcaagcg
241 gacctcataa agtgcgtcgt agtccggatt ggagtctgca actcgactcc atgaagtcgg
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Escherichia coli strain CUSMBES9 16S ribosomal RNA gene, partial sequence

GenBank: JX183940.1

FASTAGraphics

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DEFINITION  Escherichia coli strain CUSMBES9 16S ribosomal RNA gene, partial
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ACCESSION   JX183940
VERSION     JX183940.1  GI:402169611
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 981)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 981)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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      661 ccgggaactc aaaggagact gccagtgata aactggagga aggtggggat gacgtcaagt
      721 catcatggcc cttacgacca gggctacaca cgtgctacaa tggcgcatac aaagagaagc
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      841 ctcgactcca tgaagtcgga atcgctagta atcgtggatc agaatgccac ggtgaatacg
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Escherichia coli strain CUSMBES10 16S ribosomal RNA gene, partial sequence

GenBank: JX183941.1

FASTAGraphics

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DEFINITION  Escherichia coli strain CUSMBES10 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   JX183941
VERSION     JX183941.1  GI:402169612
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 715)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 715)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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      121 cgacgatccc tagctggtct gagaggatga ccagccacac tggaactgag acacggtcca
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Escherichia coli strain CUSMBES11a 16S ribosomal RNA gene, partial sequence

GenBank: JX183942.1

FASTAGraphics

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DEFINITION  Escherichia coli strain CUSMBES11a 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   JX183942
VERSION     JX183942.1  GI:402169613
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 987)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 987)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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Escherichia coli strain CUSMBES11b 16S ribosomal RNA gene, partial sequence

GenBank: JX183943.1

FASTAGraphics

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  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 912)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 912)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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Escherichia coli strain CUSMBES12 16S ribosomal RNA gene, partial sequence

GenBank: JX183944.1

FASTAGraphics

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DEFINITION  Escherichia coli strain CUSMBES12 16S ribosomal RNA gene, partial
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VERSION     JX183944.1  GI:402169615
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SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
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REFERENCE   1  (bases 1 to 718)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 718)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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Escherichia coli strain CUSMBES23 16S ribosomal RNA gene, partial sequence

GenBank: JX183945.1

FASTAGraphics

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DEFINITION  Escherichia coli strain CUSMBES23 16S ribosomal RNA gene, partial
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VERSION     JX183945.1  GI:402169616
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 870)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 870)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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      721 tctgcaactc gactccatga agtcggaatc gctagtaatc gtggatcaga atgccacggt
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Escherichia coli strain CUSMBES44 16S ribosomal RNA gene, partial sequence

GenBank: JX183946.1

FASTAGraphics

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DEFINITION  Escherichia coli strain CUSMBES44 16S ribosomal RNA gene, partial
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VERSION     JX183946.1  GI:402169617
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 847)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 847)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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Escherichia coli strain CUSMBN2 16S ribosomal RNA gene, partial sequence

GenBank: JX183947.1

FASTAGraphics

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            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 996)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Molecular epidemiology of diarrheagenic Escherichia coli
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 996)
  AUTHORS   Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
            682016, India
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Escherichia coli strain CUSMBN7 16S ribosomal RNA gene, partial sequence

GenBank: JX183948.1

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VERSION        JX183948.1 GI:402169619
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SOURCE  Escherichia coli
ORGANISM       Escherichia coli
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               Enterobacteriaceae; Escherichia.
REFERENCE 1    (bases 1 to 926)
AUTHORS  Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
TITLE          Molecular epidemiology of diarrheagenic Escherichia coli
JOURNAL        Unpublished
REFERENCE 2    (bases 1 to 926)
AUTHORS  Divya,P.S., Manjusha,S.M. and Hatha,A.A.M.
TITLE          Direct Submission
JOURNAL        Submitted (18-JUN-2012) Department of Marine Biology, Microbiology
               and Biochemistry, Cochin University of Science and Technology,
               School of Marine Sciences, Near Fine Arts Avenue, Cochin, Kerala
               682016, India
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Publications

[1]. Sukumaran, D.P., Durairaj, S., Hatha, A.A.M., 2012. Antibiotic resistance of *Escherichia coli* serotypes from Cochin estuary. *Interdisciplinary Perspectives on Infectious Diseases*. Article ID 124879, doi:10.1155/2012/124879.

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Proceedings of National/International Conferences

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[2]. Divya, P.S., Sudha, S., Hatha, A.A.M., 2010. Diversity and antibiotic resistance of diarrhegenic *Escherichia coli* in the Cochin backwaters. Green Path to Sustainability - Prospects and Challenges. ISBN 978-81-907269-9-3. 40-43.

[3]. Divya, P.S., Chandran, A., Sudha, S., Hatha, A.A.M., 2011. Tropical estuary as a reservoir for diarrheagenic *Escherichia coli* - an emerging pathogen of global importance. Proceedings of the National Sympoisum - Emerging trends in Biotechnology. ISBN no. 978-93-80095-30-1.

[4]. Soumya, K.B., Divya, P.S., Hatha, A.A.M., 2013. Microbial source traking (MST): an emerging field to identify the source of faecal contamination. Environmental Microbiology:Techniques & Applications. ISBN 978-81-923850 - 7-5. 25-48.

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Interdisciplinary Perspectives on Infectious Diseases
Volume 2012, Article ID 124879, 7 pages
doi:10.1155/2012/124879

Research Article

Antibiotic Resistance of *Escherichia coli* Serotypes from Cochin Estuary

Divya P. Sukumaran,[1] Srinivasan Durairaj,[2,3] and Mohamed Hatha Abdulla[1,4]

[1] *Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Lakeside Campus, Cochin 682 016, India*
[2] *Mathematics & Sciences Division, Richland Community College, One College Park, Decatur, IL 62521, USA*
[3] *MacMurray College, 447 East College Avenue, Jacksonville, IL 62650, USA*
[4] *Environment Genomics Laboratory, Civil and Environmental Engineering, Michigan State University, East Lansing, MI 48824, USA*

Correspondence should be addressed to Mohamed Hatha Abdulla, mohamedhatha@gmail.com

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This study aimed at detecting the prevalence of antibiotic-resistant serotypes of *Escherichia coli* in Cochin estuary, India. *E. coli* strains were isolated during the period January 2010–December 2011 from five different stations set at Cochin estuary. Water samples from five different stations in Cochin estuary were collected on a monthly basis for a period of two years. Isolates were serotyped, antibiogram-phenotyped for twelve antimicrobial agents, and genotyped by polymerase chain reaction for *uid* gene that codes for β-D-glucuronidase. These *E. coli* strains from Cochin estuary were tested against twelve antibiotics to determine the prevalence of multiple antibiotic resistance among them. The results revealed that more than 53.33% of the isolates were multiple antibiotic resistant. Thirteen isolates showed resistance to sulphonamides and two of them contained the *sul* 1 gene. Class 1 integrons were detected in two *E. coli* strains which were resistant to more than seven antibiotics. In the present study, O serotyping, antibiotic sensitivity, and polymerase chain reaction were employed with the purpose of establishing the present distribution of multiple antibiotic-resistant serotypes, associated with *E. coli* isolated from different parts of Cochin estuary.

1. Introduction

The emergence of *Escherichia coli* isolates with multiple antibiotic-resistant phenotypes, involving coresistance to four or more unrelated families of antibiotics, has been previously reported and is considered a serious health concern [1–3]. Antimicrobials are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food producing animals. Many findings suggest that inadequate selection and abuse of antimicrobials may lead to resistance in various bacteria and make the treatment of bacterial infections more difficult [4]. Antimicrobial agents can be found in sewage effluents, particularly in places where these drugs are extensively used, such as hospitals, pharmaceutical production plants, and around farms where animal feed contains these agents. It has been suggested that resistance in bacterial populations may spread from one ecosystem to another [5]. The wild dissemination of antimicrobial resistance among bacterial populations is an increasing problem worldwide.

Antimicrobial resistance in *E. coli* has been reported worldwide. Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents, including fluoroquinolones [6]. Sulfamethoxazole in combination with trimethoprim (cotrimoxazole) is still commonly used in human medicine for the treatment of urinary tract infections [7]. Resistance to at least two classes of antimicrobial agents in *E. coli* is nowadays an ordinary finding in human and veterinary medicine and has an increasing impact on available therapeutic options.

Serotyping of the antigens is a very useful method for detecting pathogenic *E. coli* strains in clinical specimens, foods, and environmental samples and for understanding the epidemiology of the pathogen [8]. According to the modified Kauffman scheme, *E. coli* is serotyped on the basis of its O

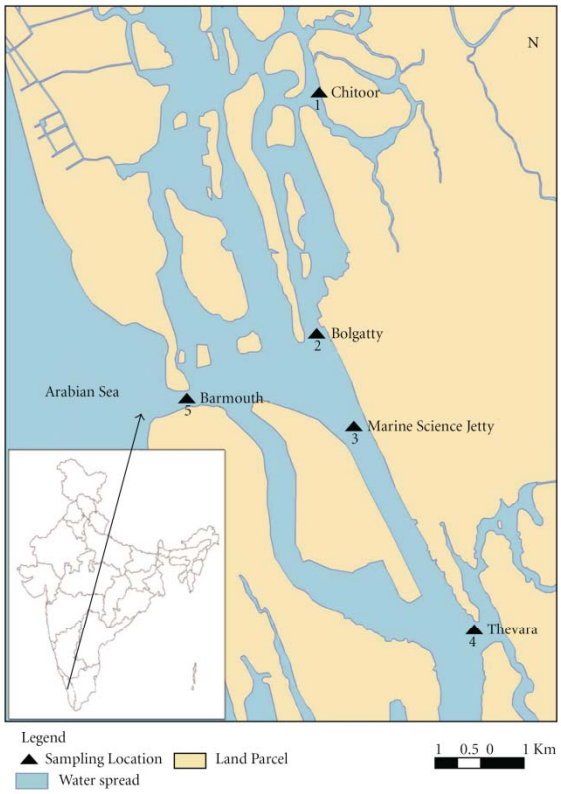

FIGURE 1

(somatic), H (flagellar), and K (capsular) surface antigen profiles [9]. A total of 170 different O antigens, each defining a serogroup, are recognized currently. The O antigen, as part of the LPS (Lipopolysaccharide) in the outer membrane of Gram-negative bacteria, is a major target of both the immune system and bacteriophages and plays an important role in the bacterium-host interplay. It is one of the most variable cell constituents and also plays an important role in virulence [10].

Cochin estuary, a part of Vembanad Lake, an important Ramsar site supports good shellfish and finfish fishery. Cochin estuary, a typical tropical estuary, had undergone considerable pollution in the last decade resulting mainly from the development of satellite townships all across the estuary. The development is mostly in the housing sector as there is great demand for water front apartments. Most of the markets situating along the coasts of the estuary release their waste water directly into natural water body. *E. coli* being a typical faecal indicator organism offers an excellent means to look at the organic pollution that is taking place in the Cochin estuary. Since the estuary supports good shellfish and finfish resources, it forms the lifeline of large number of people directly and indirectly depending on it for fishing, recreation, and transportation. The increasing prevalence of pathogenic *E. coli* with multiple antibiotic resistance could have a bearing on fish and shellfish harvested from the estuary which indirectly affect the health of people. The present study has been carried out with an objective of estimating the diversity of *Escherichia coli* serotypes from Cochin estuary and the risk posed by them is evaluated in terms of antibiotic resistance.

2. Materials and Methods

2.1. Description of the Study Area. The water samples were collected from five different stations along Cochin estuary (Figure 1). The stations were selected based on their closeness

to satellite townships and waste inputs. Two of the stations such as Chittoor (station 1) and Thevara (station 4) were fixed upstream, two in the central part of the estuary, namely, Bolgatty (station 2) and Marine Science Jetty (station 3), and one at the Barmouth (station 5). The sampling stations were fixed in and around Cochin estuary as they were suspected to receive high levels of sewage inputs.

2.2. Collection of Samples. The water samples were collected monthly from five different stations along Cochin estuary for a period of two years from January 2010 to December 2011. The water samples were collected in sterile plastic bottles (Tarson, India) one foot below the surface to get a better representation of the water column. Water samples were transported to the laboratory in an ice box and subjected to bacteriological examination within four hours of collection.

2.3. Isolation, Identification, and Serotyping of E. coli. Samples were analysed for faecal coliforms by most probable number method. The most probable number (MPN) load of faecal coliform bacteria was determined by three-tube dilution method using MacConkey broth as medium. Ten mL, 1 mL, and 0.1 mL of water samples were inoculated into respective dilution tubes containing inverted Durham's tubes. Inoculated tubes were incubated at 37°C for 24 hours and observed for growth and gas production. For isolation of *E. coli*, one loopfuls from positive MacConkey broth tube cultures were streaked onto Eosin methylene blue (EMB) (Hi-Media, India) plates and incubated at 37°C for 24 Hours. After incubation, plates were examined, typical *E. coli*-like colonies were selected and subcultured. All isolates were submitted to a biochemical screening which included the Indole test, Methyl Red test, Voges-Proskauer test, and Citrate utilization (IMViC) test. The cultures giving + + − − reaction were confirmed as *E. coli*. Confirmed *E. coli* cultures were serotyped at National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli, Himachal Pradesh, India.

2.4. Isolation of DNA from E. coli. DNA from the bacterial genome was extracted as per standard Proteinase-K digestion method [11]. Bacterial cultures were suspended in Luria Bertani broth (Hi-media, India) and incubated in an orbital incubator (Orbitek, India) at 37°C, 110 rpm for 12-hours. The 12-hour old bacterial cells were pelleted at 15000 g for 10 minutes and then suspended in TEN (Tris-HCl (pH 7.2), 10 mM EDTA, 250 mM NaCl) buffer having 1% sodium dodecyl sulphate (Hi-Media, India). Proteinase-K (GeNei, India) was then added to a final concentration of 100 μg/mL and mixed gently. The suspension was incubated at 37°C for 60 min. DNA obtained by sequential phenol-chloroform and chloroform-isoamyl alcohol extractions was precipitated by adding 2.5 volumes of absolute ethanol, and DNA was suspended in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA-pH 7.5). DNA was checked for purity by Agarose gel electrophoresis.

2.5. Polymerase Chain Reaction (PCR) for Detection of uid A Gene in E. coli. The polymerase chain reaction (PCR) was used to detect the presence of the *uid* gene, which codes for the β-D-glucuronidase enzyme. A 147 bp coding region of the *E. coli uid* gene was amplified by PCR, using the 20 and 21-mer primers UAL-754 (5′-AAAACGGCAAGAAAA-AGCAG-3′) and UAR-900 (5′-ACGCGTGGTTACAGT-CTTGCG-3′) [12]. The optimized protocol was carried out with a PCR mix of 25 μL that contained 2.5 mM $MgCl_2$, 2.5 μL of Taq buffer (Tris (pH 9.0) at 25°C, KCl, and Triton X-100), 2.5 mM each of dNTP mixture, 1 pmol/μL of each of the primers, 1 U of Taq polymerase (GeNei, India), and 1 μL of the DNA template. Amplification was performed with a thermal cycler programmed for 1 cycle of 2 min at 94°C; 25 cycles of 1 min at 94°C, 1.5 min at 58°C, 2 min at 72°C; 1 cycle of 5 min at 72°C. PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei, India), and visualized by Gel Documentation System (Bio-Rad Gel Doc EZ Imager, USA).

2.6. Antimicrobial Susceptibility Testing. Antibiograms and their interpretation were made using the disk diffusion method [13], following the Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. All *E. coli* isolates were examined for resistance to ampicillin (Amp, 10 mcg), amikacin (Ak, 30 mcg), ceftriaxone (Ctr, 30 mcg), chloramphenicol (C, 30 mcg), ciprofloxacin (Cip, 5 mcg), cotrimoxazole (Cot, 25 mcg), gentamicin (Gen, 10 mcg), kanamycin (K, 30 mcg), nalidixic acid (Na, 30 mcg), streptomycin (S, 10 mcg), tetracycline (Te, 30 mcg), and trimethoprim (Tr, 5 mcg).

2.7. PCR Detection of sul 1 Gene. The presence of *sul 1* gene was detected using the PCR method described by Marynard et al. [3]. The primers used for *sul 1* gene were 5′-TTC-GGCATTCTGAATCTCAC-3′ and 5′-ATGATCTAACCC-TCGGTCTC-3′. Amplified PCR products were separated using 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei, India), and visualized by Gel Documentation System (Bio-Rad Gel Doc EZ Imager, USA).

2.8. Detection of Class 1 Integrons. The presence of class 1 integrons was detected using the PCR method described by Levesque et al. [15]. Class 1 integrons were amplified using degenerate primers 5′ CS (5′-GGC ATC CAA GCA GCA AG-3′) and 3′ CS (5′-AAG CAG ACT TGA CCT GA-3′). PCR products were then electrophoresed on a 1.5% agarose gel (Hi-Media, India), stained with ethidium bromide (GeNei, India), and visualized by Gel Documentation system (Bio-Rad Gel Doc EZ Imager, USA).

3. Results

Serotyping of the 75 *E. coli* isolates that were from Cochin estuary revealed that they belonged to 25 different serotypes (Table 1). Twelve strains of *E. coli* were rough and hence not typable. All isolates were confirmed by molecular level

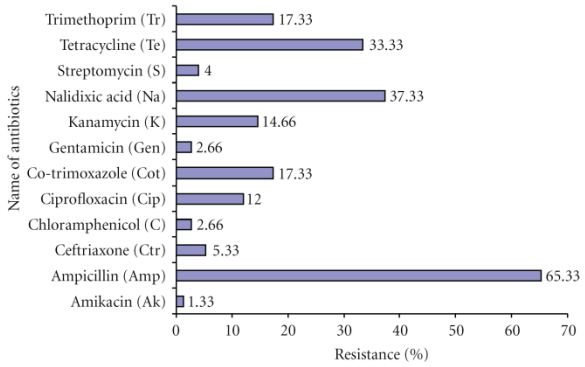

Figure 2: Percentage of antibiotic resistance among *Escherichia coli* strains from Cochin estuary during the study period (January 2010–December 2011).

Table 1: Prevalence of different serotypes of *E. coli* in Cochin estuary during the study period January 2010–December 2011.

E. coli serotypes	Percentag of incidence	*E. coli* serotypes	Percentage of incidence
O4	12	O5	8
O60	8	R (Rough)	6.6
O41	5.3	O59	4
O1	4	O22	2.6
O21	2.6	O102	2.6
O103	2.6	O116	2.6
O69	2.6	O91	2.6
O157	1.3	O34	1.3
O35	1.3	O37	1.3
O20	1.3	O141	1.3
O104	1.3	O49	1.3
O148	1.3	O64	1.3
O153	1.3	UT (untypable)	16

identification by polymerase chain reaction with primers UAL-754 and UAR-900 to amplify the amino coding region of *uid A* produced amplified DNA bands of 147 bp for all *E. coli* isolates. Stations close to the city such as Bolghatty and Off Marine Science Jetty yielded more diverse serotypes of *E. coli*.

The results (Table 1) revealed remarkable diversity of *E. coli* strains in the system. The diversity of *E. coli* serotypes was higher at station 2 (32%) followed by station 3 (22%), station 5 (17%), station 4 (15%), and station 1 (13%). *E. coli* serotypes such as O4, O5, O60 were isolated with high frequency, whereas O157, O153, O148 were encountered very rarely. The serotypes commonly associated with pathogenic strains, such as O5, O4, and O41, were found significantly high at station 2 (Bolgatty) (Table 2).

Percentage of antibiotic resistance of *E. coli* strains isolated from Cochin estuary is given in Figure 2. Most of the *E. coli* strains were found to be resistant to ampicillin (65.33%) followed by nalidixic acid (37.33%), tetracycline (33.33%), cotrimoxazole (17%), trimethoprim (17%), kanamycin (14%), and ciprofloxacin (12%). The least resistance was detected against ceftriaxone (5.33%), streptomycin (4%), chloramphenicol (2.66%), gentamicin (2.66%), and amikacin (1.33%). The *E. coli* strains showing resistance to five or more antimicrobial agents belongs to serotypes O5, O4, O60, O69, O1, O157, O20, O153, O22, and O34. Nearly 60% and 50% of *E. coli* isolates encountered at stations 3 and 2, respectively, were resistant to more than five antibiotics. While 18% of *E. coli* isolates recovered from station 4 were resistant to more than five antibiotics, none of the isolates from station 5 was resistant to more than five antibiotics. One *E. coli* isolate recovered from Chittor (station 1) was resistant against more than five antibiotics.

A total of 24 *E. coli* strains were isolated from station 2 (Bolgatty). All isolates were susceptible to amikacin, chloramphenicol, gentamicin, and streptomycin. About 29% of *E. coli* strains isolated from Bolgatty showed resistance against the sulphonamides tested. The *E. coli* strains isolated from Off Marine Science Jetty were resistant to ampicillin (76%); some were resistant to tetracycline (41%), nalidixic acid (41%), kanamycin (35%), and ciprofloxacin (17%). One *E. coli* isolate was resistant to cotrimoxazole and trimethoprim, and another isolate was resistant to chloramphenicol. A total of 11 strains were recovered from station 4 (Thevara). All *E. coli* were susceptible to amikacin, chloramphenicol, ciprofloxacin, gentamicin, and streptomycin. Almost 81% of the isolates showed ampicillin resistance and 27% isolates exhibited tetracycline resistance. While all isolates were susceptible to amikacin, ceftriaxone, chloramphenicol, ciprofloxacin, cotrimoxazole, gentamicin, kanamycin, streptomycin, and trimethoprim, resistance to ampicillin, nalidixic acid, and tetracycline ranged from 15% to 30%.

Table 2: Spatial distribution of various serotypes of *E. coli* in Cochin estuary during the study period (January 2010–December 2011).

Station/name	*E. coli* serotypes encountered
(1) Chittor	O60 (1), O22 (1), O59 (1), O1 (2), O91 (2), O21 (1), R (2)
(2) Bolgatty*	O59 (1), O157 (1), O41 (2), O4 (7), O5 (4), O60 (1), O69 (2), O21 (1), O37 (1), O34 (1), R (1), UT (2)
(3) Off Marine Science Jetty*	O35 (1), O22 (1), O20 (1), O141 (1), O116 (1), O41 (1), O148 (1), O153 (1), O60 (1), R (1), UT (7)
(4) Thevara	O59 (1), O49 (1), O41 (2), O4 (1), O116 (1), O60 (3), R (1), UT (1)
(5) Barmouth	O1 (1), O4 (1), O5 (2), O64 (1), O102 (2), O103 (2), O104 (1), UT (2)

* Stations close to the Cochin city.
**Values in parenthesis indicate number of isolates of each serotype.

Of 75 strains of *E. coli*, 13 were resistant to sulphonamides. Of these, 2 were positive in PCR for *sul 1* gene (Figure 3). Both isolates were highly resistant to sulphonamides; one strain (ES11) was also resistant to ampicillin, ciprofloxacin, ceftriaxone, cotrimoxazole, gentamicin, kanamycin, nalidixic acid, tetracycline, and trimethoprim; the other (ES44) was also resistant to ampicillin, ciprofloxacin, cotrimoxazole, nalidixic acid, streptomycin, tetracycline, and trimethoprim. Figure 4 shows the PCR amplification of the class 1 integrons from environmental isolates. Amplicons with the sizes of 1.6 Kb, 500 bp, 250 bp, 150 bp were obtained in ES11 while ES44 gave the product of 1.6 Kb size. These results showed that the strains tested contain an integron which possessed one or more inserted genes, suggesting the presence of multiresistance integrons in these strains.

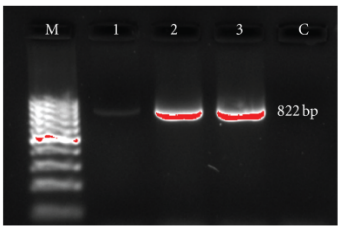

Figure 3: PCR detection of the *sul 1* gene in *E. coli* isolates. Lanes: M, 100 bp ladder; 1, clinical strain; 2, ES11; 3, ES44; C, negative control.

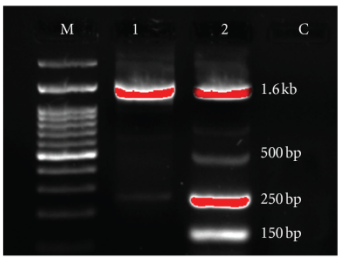

Figure 4: PCR detection of the class 1 integron in *E. coli* isolates. Lanes: M, 100 bp ladder; 1, ES44; 2, ES11; C, negative control.

4. Discussion

E. coli causes a wide variety of intestinal diseases. Diarrheagenic *E. coli* is classified into six categories: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [9]. Bej et al. [12] suggested that a PCR-based method for *uid A* gene is more sensitive in detecting *E. coli* isolates from water samples. The *uid* gene was detected in all *E. coli* isolates. According to the result of serotyping, O4 (12%) was predominant, followed by O5 (8%), O60 (8%), R (6.6%), O41 (5.3%), O59, O1 (4%), O22, O21, O102, O103, O116, O69, O91 (2.6%), and O157, O34, O35, O37, O20, O141, O103, O104, O49, O148, O64, O153 (1.3%). When an analysis of the geographic distribution of *E. coli* O157 was done by Sehgal et al. [16], it was observed to be widely distributed in all parts of India showing wide prevalence of this strain in almost all regions of the country and 1.1% of O157 isolates were from Kerala. Though food- and waterborne diarrheal diseases are very common in the study area, the investigations are usually limited to characterisation of *E. coli* alone. Serotyping of the strains is not undertaken by most clinics. Hence it is difficult to comment on the prevalence of these strains from the clinical samples in Cochin region.

Perelle et al. [17] reported that contamination by the pathogenic *E. coli* serotypes, including O103, O157, and O145, represents a major public health concern. The isolation of a great number of strains with the serotypes O5, O1, and O104 was a reason of concern because they have been recognised as pathogenic *E. coli* serotypes. Conventional serotyping methods for *E. coli* somatic and flagellar antigens are still important technique in many laboratories for diagnosis and surveillance [18]. Furthermore, serologic antigens are not directly involved in virulence but can provide important information about the circulating serotypes in the communities and in outbreaks [9].

The interesting observation was that more diverse serotypes were isolated from the station near to Cochin city, which suggests the possible release of these organisms through hospital waste from many of the hospitals in and around Cochin City. Hospital wastewater is often contaminated by antimicrobial agents, which even in subinhibitory concentrations may promote selection and survival of resistant strains [19, 20]. These serotypes may represent a significant risk to human to acquire severe infections and might emerge as a major public health problem in our country.

A total of 75 *E. coli* strains isolated from five different stations in Cochin estuary were tested against 12 different antibiotics. Though there was spatial variation in the antibiotic resistance pattern, all the *E. coli* strains were resistant to two or more antimicrobials in different combinations. In general, the resistance to sulphonamides is increasing in incidence among *E. coli* serotypes from the study area. Previous studies from our research group [21] also showed high prevalence of antibiotic resistance in *E. coli* isolates from same region, although resistance levels were higher than the ones obtained in the present study. Antimicrobial drug therapy is recommended when diarrhoeal disease is severe to reduce duration of the symptoms. Concerns are increasing in studies reporting high levels of resistance to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole among diarrhoea-associated bacteria [22]. *Enterobacteriaceae* showing resistance to nalidixic acid and susceptibility to ciprofloxacin presents a mutation in the A subunit of the DNA-Gyrase, usually, at a position equivalent to amino acid 83 in *E. coli* [23–25].

Different use patterns of antimicrobial agents are expected to have some impact on the distribution of antimicrobial resistance phenotypes [26] and possibly of resistant determinants. The result of the antibiotic resistance analysis revealed that more than 53.33% of *E. coli* were multiple antibiotic resistant. Detection of an isolate (ES11) simultaneously resistant to 10 antimicrobials is a concern, as it may pose risk to public health where these isolate to gain to the food chain. Serotypes showing multidrug-resistance to five or more antibiotics have been reported [27]. Integron-positive strains showed resistance against different groups antimicrobial agents (cephalosporins, aminoglycosides, quinolones, sulphonamides, tetracycline) tested. Isolates ES11 and ES44 were screened for class 1 integrons. Both of the isolates carried class 1 integrons and sul I gene, which is consistent with the presence of sul-associated class 1 integrons [28]. In *Escherichia coli*, trimethoprim-sulfamethoxazole resistance often correlates with the presence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes in integrons [29, 30]. Multiple antibiotic resistance may be acquired through mobile genetic elements such as plasmids, transposons, and class 1 integrons [27, 31]. Integrons play an essential role in facilitating the transfer of the resistance genes, contributing to the creation of multidrug-resistant phenotype [32].

We conclude that the high prevalence of different serotypes and multidrug resistance detected in our study is a matter of concern, since there is a large reservoir of antibiotic resistance genes within the community, and that the resistance genes and plasmid-encoded virulent genes were easily transferable to other strains. Pathogen cycling through food is very common and fish and shellfish that harbour these diarrheagenic strains might pose potential health risk to consumer. Cochin estuary supports good shellfish and finfish fishery which is exploited by local fishermen for livelihood. Shellfish being a filter feeder tend to concentrate bacteria in their body and lack of depuration practices in the study area adds guaranty to the problem of shellfish-borne food poisoning. The present study contributes to understanding the prevalence of different serotypes of *E. coli* and their antibiotic resistance patterns. In conclusion, we would like to highlight that the diversity of *E. coli* serotypes in the Cochin estuary has increased considerably when compared to our previous studies [21] which might be due to large-scale influx of organic wastes into the estuary from the satellite townships along the banks of Cochin estuary. It may also be worthwhile for the clinical laboratories of this locality to further characterise the *E. coli* serotypes isolated from diarrheal patients so as to get a clear picture of the emergence of pathogenic strains of *E. coli* in the study area. Future experimental studies to assess the efficiency of antimicrobial resistance transfer using different donor and recipient *E. coli* combinations would be of value to improve our understanding of how resistance genes may flow across strains.

Acknowledgments

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