Adaptation and survival of *Escherichia coli* in tropical aquatic environments

A thesis submitted to the University of Dhaka in fulfillment of the requirements for the award of the degree of Doctor of Philosophy.

by

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Declaration

This is to certify that the thesis entitled "Adaptation and survival of *Escherichia coli* in tropical aquatic environments" contains the findings of Ms. Selina Akter's PhD research works. She has fulfilled the ordinance and regulations required for the degree of Doctor of Philosophy (PhD) from the Department of Microbiology, University of Dhaka, Bangladesh.

It may be mentioned here that based on her thesis work, one research paper has already been published in a peer reviewed international journal and two more papers are in the pipeline to be published soon.

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ABSTRACT

About 3.2 billion episodes of diarrhea, with 1.15 million estimated deaths annually occur in children in South and South-East Asia. *Escherichia coli*, the widely used water quality indicator, is one of the prominent waterborne pathogens causing a variety of gastrointestinal and extra-intestinal infections. However, information about the influence of seasonal factors and its prevalence and persistence in tropical surface waters is limited.

To monitor *E. coli* in tropical aquatic systems, 46 sampling sites were selected covering major natural water bodies in Bangladesh. To enhance *E. coli* recovery from the environment, enrichment techniques were designed. DNA extracted directly from water filtrates, pre-enrichment and two enrichment cultures were assessed for distribution of *E. coli* virulotypes and -outbreak strains, for three successive seasons. *fliC*-PCR followed by Southern blot hybridization was used to track *E. coli*. Multiplex PCR was used to assess nine virulence-associated genes of diarrheagenic *E. coli*. Genes associated with enterotoxigenic (ETEC), enteropathogenic (EPEC), and enterohaemorrhagic (EHEC) virulotypes were detected consistently in this study, but that of enteroinvasive (EIEC) and enteroaggregative (EAEC) virulotypes could be traced only occasionally. ETEC was the most prevalent virulotype detected followed by EPEC. However, the EIEC and EAEC virulotypes could not be detected in winter and in the rainy seasons, respectively. Specific regional distribution patterns of different *E. coli* virulotypes and their temporal fluctuations were identified. Prevalence of cytolethal distending toxin (Cdt)-genes harbored by *E. coli* was also identified by PCR targeting five different *E. coli-cdt* alleles (*cdt I* to *V*). *cdt-IA* was the most prevalent gene detected. Significant influence of salinity was observed on the prevalence of different *cdt*-alleles. Correlations of the occurrence of *cdt*-alleles with diarrheagenic *E. coli* virulotypes could not be established. *E. coli* H-types (based on the flagellar antigen) known to be associated with outbreaks, were also monitored using *fliC*-sequence genotyping. H4 was the most prevalent H-type detected followed by H10 in this study. This method could help in emergency epidemiological investigations and to track mutated or emerging H-types. These approaches have potential as a methodological platform for seasonal risk assessment and to identify vulnerable areas of the country prone to *E. coli*-associated outbreaks.

This study also encompasses investigations into the adaptation and survival potential of *E. coli* in tropical waters. Water microcosm study with genetically marked strains revealed significant bacterial association with the freshwater fish, *Heteropneustes fossilis*. Surface water film, sediments, as well as phytoplankton rhizoids also contained a high proportion of *E. coli* test organisms as compared to bulk water. No signs of colonization by the test strains were found in the fish gut except for one specific environmental isolate, termed DUM10, isolated from a pond at the University of Dhaka campus. Fish intestinal content harbored the *E. coli* DUM10 with a load of 2.0×10^6 cfu/g, indicating colonization and active growth of the bacterium in the fish gut. This finding points toward fish as a possible *E. coli* reservoir in aquatic systems. Measurement of hydrophobicity, biofilm formation and detection of adhesion-related genes in the strains could not be correlated with the colonization of the fish gut. In both sterile marine and fresh water microcosms experiments, the *E. coli* strains, isolated from a sea beach at the Bay of Bengal, steadily maintained their survivability up to 53days. The generation time of these isolates was lower in marine media as compared to freshwater media. These isolates, when grown in media with different salt concentrations (0.85% to 10%), showed a characteristic aggregation pattern and biofilm formation. The isolates were viable and showed motility in medium containing as high as 10% sea salt. The high salt tolerance, extended survivability and faster growth rate in marine water indicate an adaptation of the isolates to the marine ecosystems.

This study also aimed to screen the environmental *E. coli* isolates for cross-reaction with *Shigella*-specific antisera and to assess their potential as probiotics or vaccine agents against shigellosis. Sixty nine *E. coli* isolates were shown to cross-react with polyvalent and monovalent antisera specific for all the four *Shigella* serogroups. Some isolates cross-reacted with multiple antisera concurrently. Although none of the isolates were found PCR-positive for any of *E. coli* and *Shigella* spp.-specific virulence genes, further studies are required to assess their potential for development of safe and effective probiotics or vaccine candidates to combat shigellosis.

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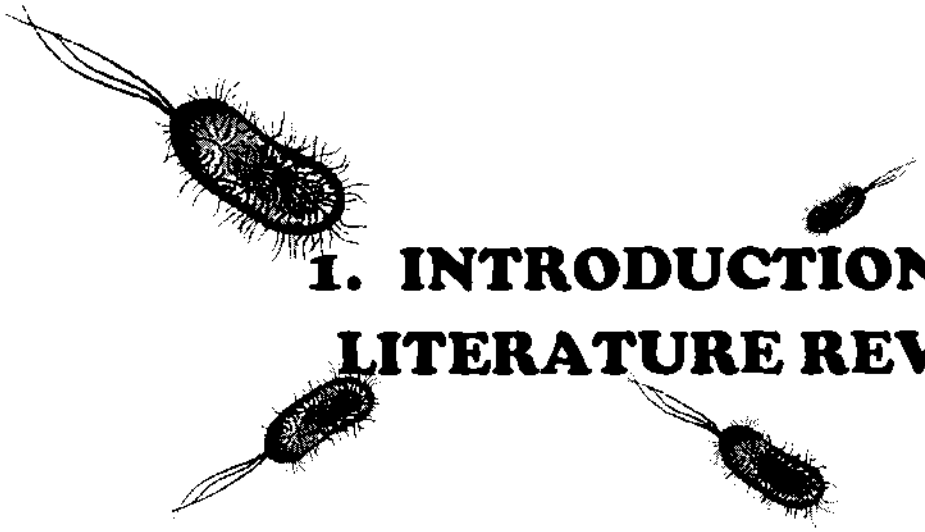
LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µl	Microliter
µg	Microgram
AAF	aggregative adherence fimbriae
AIEC	adherent invasive *E. coli*
A/E	attaching and effacing
AODC	acridine orange direct counting
APHA	American public health association
ARP	actin-related protein
APEC	avian pathogenic *E. coli*
ATCC	American type culture collection
BSR	BLAST Score Ratio
BFP	bundle-forming pilus
BBB	blood–brain barrier
BST	Bacterial source tracking
bp	Base pair
CDC	centre for disease control
CDEC	cell-detaching *E. coli*
Cdt	Cytolethal distending toxin
CNF	cytotoxic necrotizing factor
CEACAM	carcinoembryonic-antigen-related cell-adhesion molecule
CF	colonization factor
cm	Centimeter
CFs	colonization factors
CFTR	cystic fibrosis transmembrane conductance regulator
CTAB	Cetyl trimethyle ammonium bromide
d	Days
DAEC	Diffusely adherent *E. coli*
DAF	decay-accelerating factor
DIG	Digoxigenin
dNTP	Deoxyribonucleotide triphosphate
DGGE	Denaturing Gradient Gel Electrophoresis
EAEC	Enteroaggregative *E. coli*
EcN	*E. coli* strain Nissle
EDTA	Ethylene diamine Tetra-Acetic Acid
EIEC	enteroinvasive *E. coli*
ECP	*E. coli* common pilus
EHEC	enterohemorrhagic *E. coli*
ETEC	enterotoxigenic *E. coli*
EPEC	enteropathogenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
et al.	and others
FC/FS	fecal coliform/fecal streptococci
g	Gram
h	Hour
HCP	haemorrhagic coli pilus
HGT	Horizontal gene transfer
HlyA	haemolysin A
HUS	hemolytic-uremic syndrome
IBCs	intracellular bacterial communities
kbp	Kilobase paie
kDa	Kilo Dalton

L	Liter
LH-PCR	length heterogeneity PCR
LPS	Lipopolysaccahride
M	Molar
Mda	Mega Dalton
mL	milliliter
min	minute
mM	Millimole
MLST	Multi locus sequence typing
N	normal
NBTC	Nitoblue tetrazolium chloride
nm	Nanometer
NMEC	neonatal meningitis *E. coli*
NTEC	necrotoxigenic *E. coli*
N-WASP	Neural Wiskott–Aldrich Syndrome Protein
OD	Optical Density
OmpA	outer-membrane protein A
ONPG	ortho-nitrophenyl galactoside
PAGE	Polyacrylamide gel electrophoresis
PAIs	pathogenicity islands
PCR	Polymerase chain reaction
pH	negative logarithm of hydrogen ion concentration
QIRs	quiescent intracellular reservoirs
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	Rotetion per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
sp.	Species (singular)
spp.	Species (plural)
sec	Second
STEC	Shiga toxin producing *E. coli*
SGT1	sodium-D-glucose cotransporter 1
SPATE	serine protease autotransporter of the Enterobacteriaceae
TAE	Tris-Acetate EDTA
TUG	truly unique genes
tTJs	tight junctions
USEPA	United States Environmental Protection Agency
UPEC	Uropathogenic *E. coli*
UTIs	urinary tract infections
V	Volt
VBNC	Viable but non-culturable
Vol.	Volume
w/v	Weight per volume
WHO	World Health Organization



1. INTRODUCTION and LITERATURE REVIEW

According to the WHO and UNICEF (2012), regions with the lowest coverage of improved sanitation in 2006 included Southern (33%) and Eastern Asia (65%). A systematic review (Fischer Walker *et al.*, 2012) revealed that there are 5 billion episodes of diarrhea in children annually, with 3.2 billion cases in these regions. Although diarrheagenic agents are known to spread through food and water contaminated by feces, limited information is available on climatic factors or the occurrence and prevalence in surface waters that are used for drinking, bathing, cooking and washing in developing countries (Rowland, 1986). This study approaches to have an insight in the prevalence of *Escherichia coli*, widely used fecal indicator and one of the major diarrheal agents, in a tropical region and its potential for adaptation and survival strategies in aquatic systems.

1.1 INTRODUCTION

According to centre for disease control (CDC, 2004), an estimated four billion episodes of diarrhea resulted in an approximately two million deaths annually, mostly among children. Waterborne bacterial infections may account for as many as half of these episodes and deaths each year. The episodes have also emerged as a major diarrheal disease in Bangladesh. Several studies suggest that diarrheal disease accounts for about 20% of all deaths (Kosek *et al.*, 2003). Investigations into the microbial water quality in developing countries have primarily focused on drinking water. This is because there are nearly 1.1 billion people in the world lacking access to safe drinking water, living in developing countries (WHO, 2009). Global estimates indicate that each year, more than 120 million cases of gastrointestinal diseases are caused by swimming and bathing (Shuval, 2003). In recent years, there has been an increasing concern about the sanitary quality of beach sand and its potential influence on water quality of intertidal zone and on public health (Whitman and Nevers, 2003; Yamahara *et al.*, 2007; Heaney *et al.*, 2009; Whitman *et al.*, 2009). Potential sources of these bacteria may include zoonotic sources (Standridge *et al.*, 1979; Benoit *et al.*, 1993; Wyer *et al.*, 1995; Alderisio and DeLuca, 1999), diffuse sources located directly on aquatic systems (Weiskel *et al.*, 1996; Roll and Fujioka, 1997; Boehm *et al.*, 2002; Dwight *et al.*, 2002), the shedding through swimming-related activities coupled with interactions between water and beach sand (Papadakis *et al.*, 1997; Elmir *et al.*, 2007), aquatic macrophytes (Whitman and Nevers, 2003; Englebert *et al.*, 2008; Ishii and Sadowsky, 2008) and marsh vegetation (Anderson *et al.*, 1997; Grant *et al.*, 2001). Microbial occurrence, survival and growth in natural environments are influenced by various physical, chemical, and biological factors. Within aquatic systems, physical and biological factors control the dynamics of the bacterial flux (Whitman and Nevers, 2003; Ge *et al.*, 2010).

Escherichia coli is a member of the predominant enteric bacteria that is widely distributed in the intestines of human and other warm-blooded animals. Although found in all sorts of environment, *E. coli* strains are usually harmless; even their presence in the human intestine is necessary for normal health. Some strains help synthesize K and B vitamins and act as probiotics. In 1982, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination based on the premise that *E. coli* is abundant in human and animal feces and not usually found in other niches (Feng and Hartman, 1982; Feng *et al.*, 2002). However, now it has been discovered that *E. coli* has adapted in soil, sediment and aquatic environment quite well particularly in tropical countries. Adaptation of *E. coli* in marine environment creates skepticism about the credibility of this organism as an indicator. Elevated survival and growth rate in sea water have prompted concern that environmental reservoirs of fecal indicator bacteria disrupt the correlation between indicator organisms, pathogens and human health risks. As a consequence of adaptive evolution, microbes develop and adapt a variety of traits for adhesion and successful colonization, survival, and growth in a variety of natural environments. The survivability of *E. coli* in surface waters is influenced by water salinity, temperature, sunlight, predation and competition for available nutrients (Fujioka *et al.*, 1981; McCambridge and McMeekin, 1981). Solar inactivation is an important mechanism for natural reduction of indicator bacteria in water (Fujioka *et al.*, 1981; Whitman *et al.*, 2004). In seawater, in presence of sunlight, 90% of the fecal coliforms are inactivated within 3 hours, whereas, in the absence of sunlight, these bacteria survive for days and even for weeks. Among the abiotic factors, water salinity has a significant influence on bacterial survival. Generally, lower salinity leads to a longer survival time

(Sinton *et al.*, 2002); salinity limits *E. coli* survival affecting glucose uptake, which resulting in rapid cell death (Carlucci and Pramer, 1960; Barcina *et al.*, 1990). But *E. coli* strains have been reported to selectively adapt to the salinity of seawater, remaining in a culturable state for long time (Byrd and Colwell, 1993). *E. coli,* generally considered as neutrophiles, can also grow in moderate acidic or alkaline conditions (Hughes *et al.*, 2007). *E. coli* survives at a pH as low as 2.0 (Gorden and Small, 1993), and as high as 10.0 (Small *et al.*, 1994) for several hours but the range of pH values under which growth is possible is significantly smaller. In estuarine ecosystem, the survival of fecal coliforms were found to be negatively correlated with increasing water temperature (Faust *et al.*, 1975). Study on *E. coli* O157:H7 serotype in water microcosms found that the bacteria survived for more than 12 weeks at 8°C and for 7 to 12 weeks at 25°C, depending on the water source. In addition, significant levels of viable bacteria were detected after samples could no longer be cultured on traditional media, which also indicate the possibility of these cells to enter into VBNC states (Wang and Doyle, 1998). As a secondary habitat in the environment, *E. coli* remain in stressed and starved conditions compared to its primary habitat, intestine. Reduction of bacterial recovery was interpreted as a form of cellular damage that reduced the culturability (Harris, 1963; Ray, 1989) and such damaged bacteria became more fastidious in their nutritional requirements for subsequent growth (Wright, 1955).

Predation and competition are perhaps the two biotic factors that most strongly influence the long-term survival and growth of *E. coli* in the environment (Korhonen and Martikainen, 1991). Free and suspended bacteria in water are more likely to be affected by competition and predation compared to attached ones (McCambridge and McMeekin, 1981). In seawater, these bacteria are subject to higher rate of predation than in freshwater (Jannasch, 1968). Competition and antagonism were less important in controlling growth (Marino and Gannon, 1991); however, competition for growth limiting nutrients can greatly influence the growth of *E. coli* in soil (Byappanahalli and Fujioka, 2004) and saline ecosystems (Carlucci and Pramer, 1960). The competition with the natural microbial flora of the water and soil may have a primary impact on the reduction in numbers of *E. coli* (Flint, 1987; Cooley *et al.*, 2003). However, the growth requirements of *E. coli* are relatively simple compared to other enteric members and it has the ability to synthesize complex building blocks from inorganic nutrients and glucose (Andrews, 1991). Thus *E. coli* could be assumed to outcompete its relatives for growth and survival under nutrient deficient environment. In natural microbial ecosystem, such as soil or aquatic environment, adherence of a cell to a solid surface confers several competitive advantages and provides the cells with constant carbon and energy sources (Boland *et al.*, 2000). Attachment, colonization and adaptation of enteric *E. coli* to natural surfaces like clay, sand, soil, sediment or aquatic biota might have far reaching implications. In this respect, role of *E. coli*, which has already been adapted to the environment, as a reliable indicator particularly for tropical countries requires to be reassessed.

Although most strains of *E. coli* are regarded as harmless, virulent *E. coli* strains are now considered as major diarrhoeagenic pathogens and are classified into several virulotypes according to their virulence mechanisms (Scott and Kaper, 1994). There is evidence that pathogenic *E. coli* is well-adapted for aquatic survival. The first widespread outbreak of *E. coli* O157:H7 in the USA was actually associated with a groundwater source (Swerdlow *et al.*, 1992). Other outbreaks have also been tied to recreational waters, which is a strong indicator that this pathogen may survive in freshwater environments (Leclercq *et al.*, 2002; Muniesa *et al.*, 2006). These studies contribute to the

growing evidence that certain environments support indigenous populations of *E. coli* (Fujioka *et al.*, 1998; Winfield and Groisman, 2003) with a belief that populations of these bacteria may grow and establish themselves in the environment. These findings have led several researchers to propose that environmental fecal indicator bacteria should be considered as part of the natural biota (Fujioka and Byappanahalli, 2003; Lasalde *et al.*, 2005).

There are many tracking methods practiced in water safety assay. Multiplex PCR amplification has been adopted to detect almost all of these virulotypes in a single reaction (Aranda *et al.*, 2004), which can be a good approach for environmental monitoring of *E. coli* virulotypes in aquatic systems, to observe the distribution of different *E. coli* virulotypes in surface water, to assess the seasonal risk for communities that are vulnerable to waterborne diseases as well in understanding the environmental factors driving these fluctuations. Cytolethal distending toxins (Cdt), acquired by a group of unrelated Gram-negative bacteria (Cortes-Bratti *et al.*, 2001), has been reported in *E. coli* as well. The *cdt*-allelic cluster was found in most of shiga toxin producing *E. coli* (STEC) O157:H^- strains (Janka *et al.*, 2003), in enteropathogenic *E. coli* (EPEC), *E. coli* causing extraintestinal infections, and animal pathogenic *E. coli*. However, infection with Cdt-producing *E. coli* has already been reported in Bangladesh (Albert *et al.*, 1996; Talukder *et al.*, 2008) and thus should not be neglected in tropical countries. *E. coli* strains causing diseases in humans are associated with certain serotypes characterized by their O (lipopolysaccharide) -H (flagellar) antigens (Orskov *et al.*, 1990; Whittam *et al.*, 1993). Thus, investigation of serotypes is important in investigations of outbreaks and for studying reservoirs, spread and emergence. But high number of O- and H-types (Wang *et al.*, 2003), as well as frequent serological cross-reactions between *E. coli* surface antigens, spontaneously agglutinating strains and nonmotile (NM) strains limits the use of serotyping as a routine method for nonspecialized laboratories (Scheutz *et al.*, 2004). The *fliC* gene, specifying structural subunit of H-antigen (Macnab, 1996), has been successfully used for polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) genotyping (Fields *et al.*, 1997; Machado *et al.*, 2000) of both motile and nonmotile strains. It might be advantageous over classical H-serotyping and could be useful for epidemiological investigations and studies on the emergence of new EPEC and STEC clones.

E. coli strain Nissle (EcN) is the most studied probiotic strain (Dezfulian *et. al.*, 2008). With antagonistic activity against enteric pathogens, this strain has been proposed for treatment of acute diarrhea in infants. On the other hand, shigellosis is endemic throughout the world caused by *Shigella* spp., which is categorized as a priority waterborne disease by world health organization (WHO, 2001). It has long been reported that *E. coli* gave serological cross-reactivity with polyclonal group-specific *Shigella* antisera (Lefebvre *et. al*, 1995). Thus, cross-reactive *E. coli* strains could have potential to be a vaccine or probiotics against shigellosis.

1.2.1 The bacterium *Escherichia coli*

1.2.1.1 Historical background

Escherichia coli was first described by the German pediatrician, Theodore Escherich in 1885. He termed this organism as *Bacterium coli*, to indicate its universal occurrence in the colon of healthy individuals. After much controversy, it was renamed and is now placed in the family Enterobacteriaceae (Janda and Abbott, 2006). Escherich showed that the organism was pathogenic if injected into rabbits, but he remained uncommitted on the subject of its pathogenicity for humans (Robins-Browne, 1987). Over the past hundred years, *E. coli* has been studied to such an extent that it is now the most thoroughly understood free-living form so far.

1.2.1.2 Taxonomy and classification

Taxonomically *E. coli* is the member of the family Enterobacteriaceae (Janda and Abbott, 2006). The classification scheme of *E. coli* is as follows:

Phylum: Proteobacteria
Class: Gamma Proteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Escherichia*
Species: *Escherichia coli*

1.2.1.3 *E. coli* pan genome

This section is based on the observation of Rasko *et al.*, (2008). A total of 17 *E. coli* isolates were included in the study representing a broad sampling of the diverse pathogens of this species. A similar methodology to Tettelin (2005) was used in the determination of the pan-genome. The trend is the continual addition of new genes with each newly sequenced genome and as such, the pan-genome of *E. coli* is considered to be open. The open pan genome indicates that the species is still evolving by gene acquisition and diversification. The *E. coli* pan-genome contains a reservoir of greater than 13,000 genes. The complete sequence of a commensal *E. coli* HS strain has revealed significant genome mosaicism between pathogen and commensal. This has significant implications on the evolution of pathogenic *E. coli* in members of the commensal microflora, which may act as genetic sinks. The interaction of "precursor" pathogen species with commensals may allow the development of fully pathogenic isolates via horizontal transfer (Rasko *et al.*, 2008).

1.2.1.3.1. Conserved core genome

Comparative genome analysis preformed with the 17 genomes (Rasko *et al.*, 2008) provided with a glimpse of the genetic diversity within the *E. coli* species. The isolates had an average genome size of 5020 ± 446 genes (mean ± sd). Using the BLAST Score Ratio (BSR) analyses (Rasko *et al.*, 2005) "conserved core" genome size was calculated to be 2344 ± 43 genes. The exponential decay model predicted that the number of core genes in the *E. coli* is approximately 2200. Examination of the functional annotation of these genes suggests that the conserved core genes are mostly of core metabolic processes. Previous comparative genome microarray hybridization based methods using

22 isolates with an incomplete array dataset indicated that the "conserved core" of *E. coli* genome was approximately 2800 genes (Fukiya *et al.*, 2004). Chen *et al.*, (2006), using bioinformatics, determine that the core genome size of *E. coli* was 2865 genes, which was derived from a dataset of the genomic content of seven isolates, two of which were *Shigella flexneri*.

1.2.1.3.2. Unique gene

The pan-genome analysis also identified truly unique genes (TUG), i.e. the genes present only in the reference genome and not in any of the other genomes. The number of TUG ranges from ~20 in the laboratory adapted isolates to greater than 300 depending on the genome used as the reference. The mean number of TUG found within this *E. coli* genome dataset is 176 ± 95. The large deviation to the mean is indicative of the high degree of variation within *E. coli*, as well as the fact that not all isolates have similar clinical presentations. The majority of the TUGs do not contain functional annotation and as such they may represent novel biosynthetic or pathogenic features. Uropathogenic *E. coli* (UPEC) isolates contain the greatest number of unique genes, which anticipated that they contain a greater amount of genetic diversity mostly conveyed by multiple pathogenicity and/or genomic islands (Welch *et al.*, 2002; Dobrindt *et al.*, 2003; Lloyd *et al.*, 2007). Enteroaggregative *E. coli* (EAEC) was identified as a group with unexpectedly significant diversity. Most typical EAEC isolates are known to contain a large virulence plasmid (Harrington *et al.*, 2006; Huang *et al.*, 2006). However, the large numbers of TUG in each EAEC isolate suggests that chromosomal differences may also play a role in pathogenesis. The identification of unique genes in each isolate may not always represent the virulence factors of the group, as these genes could be shared with isolates of similar clinical origin.

1.2.1.3.3. Pathovar specific gene content

The 17 *E. coli* genomes, observed by Rasko *et al.*, (2008), contained at least one genome sequence from each of the *E. coli* pathovars. But surprisingly there are fewer pathovar-specific genes than anticipated. Only the enterohemorrhagic *E. coli* (EHEC) genomes contain a significant proportion of pathovar-specific genes. Of those genes unique to EHEC, 43% are associated with prophage and phage elements, confirming that the phage have played a significant role in the evolution of this pathovar (Wick *et al.*, 2005; Steele *et al.*, 2007; Zhang *et al.*, 2007). In contrast, eleven or fewer pathovar-specific genes could be identified in the commensal, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) or EAEC groups. This suggests that while these isolates are grouped as pathovars, there is significant genetic heterogeneity within these groups and is most likely a reflection on how these isolates cause disease. The extraintestinal pathogenic *E. coli* (ExPEC) genomes share a significant level of pathovar similarity suggesting that outside of the gastrointestinal tract *E. coli* is utilizing common molecular mechanisms. One exception to this observation was the avian pathogenic *E. coli* (APEC) isolate, which shares 3 genes with the other ExPEC isolates (Johnson *et al.*, 2005, 2007). This would indicate that the APEC strain contains a distinct genetic repertoire for colonization and infection of avian species that is not required for the infection of humans. In general, these comparisons suggest that *E. coli* pathovars are not distinct on the molecular level, with the exception of EHEC and ExPEC to a lesser extent, or that *E. coli*, as a pathogen, is a "generalist" containing the genetic potential to colonize and infect humans (Rasko *et al.*, 2008).

1.2.1.4 Biochemical properties of *E. coli*

The major set of phenotypic characteristics defining the Enterobacteriaceae family include the following: short Gram-negative rods, facultative anaerobic but preferring aerobic metabolism, lack cytochrome-C oxidase and are catalase positive, most can reduce nitrates, do not require Na^+ for growth, can ferment D-glucose producing lactic acid and other products. They contain enterobacterial common antigens, and have a mol% G+C content of 38 to 60 (Janda and Abbott, 2006). Most *E. coli* ferment lactose, approximately 10% are late lactose fermenter and a few of them are non-lactose fermenter. They are positive for methyl red test and negative for Voges-Proskauer test. They produce indole and do not utilize citrate as the sole carbon source. Isolates of this species usually do not produce extracellular DNase, H_2S, phenylalanine deaminase and urease and do not grow in inositol and KCN containing media. Typical gas production from glucose, indole, lysine, arabinose, mannitol, ortho-nitrophenyl galactoside (ONPG), trehalose, lysine and xylose is found in this species (Farmer and Kelly, 1992).

1.2.1.5 *E. coli* groups and types

E. coli can be sub-classified into three groups: commensal, diarrheagenic and extraintestinal. The commensal *E. coli* is the nonpathogenic and often considered to be the normal inhabitant of the gastrointestinal tract of warm-blooded animals. The second group can cause infection in the gastrointestinal tract and the third is the group capable of infecting sites other than intestine (Carrero-Colon *et al.*, 2011).

1.2.1.5.1. Virulotypes

Virulent *E. coli* strains are now considered as one of the major diarrheagenic pathogens, and are classified into several virulotypes according to their virulence mechanisms (Scott and Kaper, 1994). There are certain strains capable to colonizing host sites other than GI tract, commonly named as ExPEC strains and attributed to a range of mild to severe fatal complications of diseases in human. A detail story of the pathogenic strains of *E. coli* has been provided in Section 1.2.2.

1.2.1.5.2. Serotypes

The O-antigen is a part of the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria. O-antigens are present in both pathogenic and nonpathogenic *E. coli* strains but some are most commonly found in pathogenic clones (Nataro and Kaper, 1998). The O-antigen is a major target of both the immune system and bacteriophages, and plays an important role in the bacterium–host interplay. *E. coli* strains causing diseases in humans are associated with certain serotypes characterized by their O and H (flagellar) -antigens and sometimes with K-antigen (capsular). It has been shown that the major clonal types of EPEC and STEC could be linked to distinct O:H serotypes of their representative strains (Orskov *et al.*, 1990; Whittam *et al.*, 1993). At least 166 antigen types have been recognized in *E. coli* and 33 in *Shigella* spp. (Lior, 1994). Thirteen of those were common to both based on cross-reactions summarized by Ewing (1986) and chemical structural data (Parolis *et al.*, 1997), which indicates the close relationship between *Shigella* spp. and *E. coli*.

1.2.2 Pathogenesis of *E. coli*

E. coli is a remarkable and diverse organism. Certain isolates have been implicated in a wide range of diseases that affect either animals or humans worldwide (Croxen and Finlay, 2010). This normally harmless commensal can become a highly adapted pathogen capable of causing a range of diseases, from gastroenteritis to extraintestinal infections of the urinary tract, bloodstream and central nervous system (Croxen and Finlay, 2010). The subset of genes involved in the subversion of host responses and hijacking of host cell machinery makes each pathovar distinct. To date, eight pathovars and their mechanisms of disease have been extensively studied. These pathovars can be broadly classified as either diarrheagenic *E. coli* or extraintestinal *E. coli* (ExPEC) (Kaper *et al.*, 2004). Six pathovars: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC; including *Shigella*), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) are diarrheagenic, and two pathovars: uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the most common ExPEC isolates. Other pathovars have been identified, but their mechanisms of pathogenesis are not as well defined. In addition to the eight main *E. coli* pathovars, necrotoxigenic *E. coli* (NTEC), cell-detaching *E. coli* (CDEC) and adherent invasive *E. coli* (AIEC) have also been reported.

1.2.2.1 Intestinal pathogenic *E. coli*

1.2.2.1.1 Enteropathogenic *E. coli* (EPEC)

EPEC is a major cause of potentially fatal diarrhea in infants in developing countries (Kaper *et al.*, 2004). It is an attaching and effacing (A/E) pathogens that efface the microvilli and subvert host cell actin to form pedestals beneath the attachment site (Frankel and Phillips, 2008).

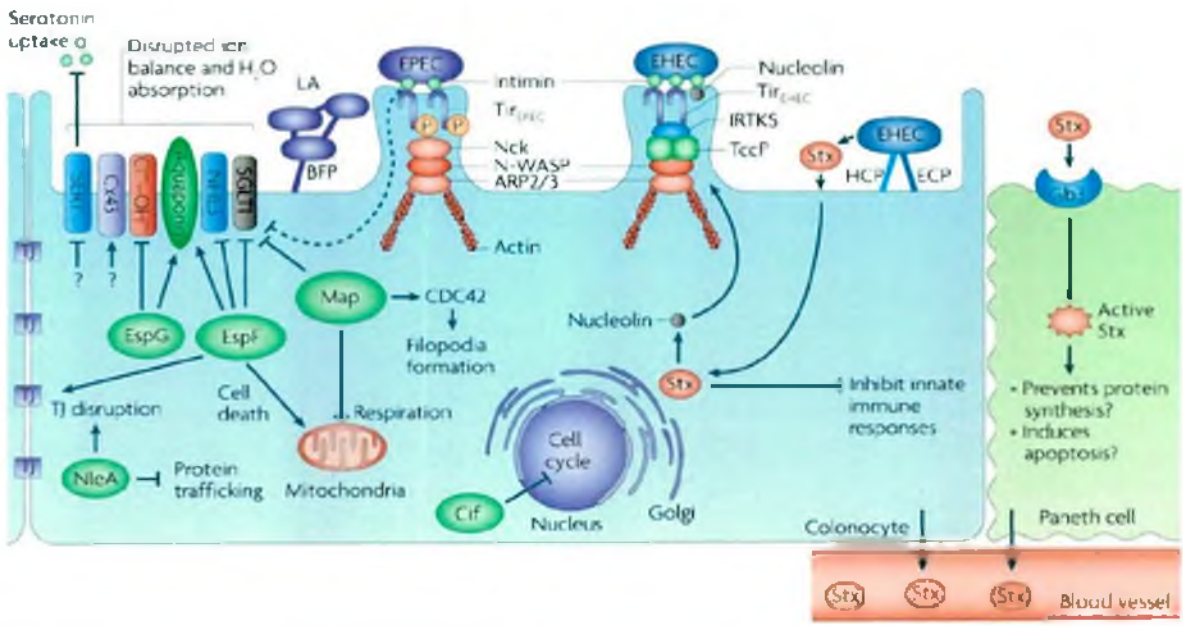


Fig. 1.1 The mechanism of pathogenesis of EPEC and EHEC. The figure has been adopted from Croxen and Finlay (2010).

(Additional abbreviations used in the figure are CDC42, cell division control protein 42; Cx43, connexin 43 (or GJA1); Cif, cycle-inhibiting factor; Map, mitochondrial-associated protein; NHE3, Na^+–H^+ exchanger 3; NleA, non-LEE-encoded effector A (or, EspI); SERT, serotonin transporter; TJ, tight junctions).

Effectors secreted by the type III secretion system can affect Cl^-–OH^- and Na^+–H^+ exchanger activity, mislocalize aquaporins and inhibit sodium-D-glucose cotransporter 1 (SGT1). EPEC attaches to the small bowel through the bundle-forming pilus (BFP), forming localized adhesions (LA). Intimate attachment is mediated by the interaction between intimin and the translocated intimin receptor (Tir). Tir is phosphorylated by host tyrosine kinases, and phosphorylated Tir recruits Nck, which activates neural Wiskott–Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (ARP 2/3) complex to mediate actin rearrangements and pedestal formation. Using the locus of the enterocyte effacement-encoded type III secretion system, a large repertoire of effector proteins is injected into the host cell, subverting host cell pathways (Fig. 1.1).

1.2.2.1.2 *Enterohaemorrhagic E. coli* (EHEC)

EHEC is a highly infectious pathogen that colonizes the distal ileum and large bowel in humans and is often the causative agent of outbreaks of severe gastroenteritis in developed countries. *E. coli* O157:H7 has been considered the main cause of the human diseases in both infants and adults e.g. hemorrhagic colitis (Padhye and Doyle, 1992), and further complications can lead to the potentially fatal hemolytic-uremic syndrome (HUS) (Griffin and Tauxe, 1991), and thrombotic thrombocytopenic purpura (Machin, 1984). Cattle are a key reservoir for EHEC. It is also an attaching and effacing (A/E) pathogen. The mechanism of pedestal formation by EHEC is slightly different from that used by EPEC. Tir is not phosphorylated, and pedestal formation is Nck-independent. The actin rearrangements that are necessary for pedestal formation are mediated by Tir cytoskeleton-coupling protein (TccP; also known as $EspF_U$), which is linked to Tir through the host protein insulin receptor tyrosine kinase substrate (IRTKS; also known as BAIAP2L1) and interacts with N-WASP to activate the ARP2/3 complex. In addition to this intimate attachment, EHEC attaches to the large bowel through the *E. coli* common pilus (ECP) and the haemorrhagic coli pilus (HCP). EHEC injects many of the same effectors as EPEC into the host cell to manipulate host processes (Fig. 1.1). In addition, Shiga toxin (Stx; also known as verocytotoxin) is released following phage-mediated lysis in response to stress, further contributing to disease. EHEC lacks a secretory mechanism for Stx, so the release of Stx occurs through lambdoid phage-mediated lysis in response to DNA damage and the SOS response (Toshima *et al.*, 2007); antibiotic therapy should therefore be discouraged, as the toxin would be released. Globotriaosylceramides (Gb3s) on Paneth cells in the human intestinal mucosa act as receptors for Stx (Fig. 1.1).

1.2.2.1.3 Enterotoxigenic *E. coli* (ETEC)

ETEC, the most common cause of traveler's diarrhea, can have fatal consequences for children less than 5 years of age and infants. In humans, ETEC caused disease results in the greatest number of *E. coli* related deaths per year, exceeding 800,000 worldwide (Turner *et al.*, 2006). However, the vast majority of these cases occur in underdeveloped countries. ETEC is also important in the farming industry, as post-weaning piglets are highly susceptible to infection (Nataro and Kaper, 1998). ETEC become anchored to enterocytes of the small bowel through colonization factors (CFs) and an adhesin that is found at the tip of the flagella (EtpA). Tighter adherence is mediated through Tia and TibA. Two toxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), are secreted and cause diarrhea through cyclic AMP and cyclic GMP mediated activation of cystic fibrosis transmembrane conductance regulator (CFTR). Other virulence factors have also been shown to be secreted by ETEC (Turner *et al.*, 2006), e.g. EatA, a serine protease autotransporter that may

accelerate fluid build-up, CylA, a pore-forming cytotoxin, and *E. coli* ST1 (EAST1), which may have similar functions to STa (Fig. 1.2).

1.2.2.1.4 Enteroagreggative *E. coli* (EAEC)

Although EAEC is considered to be an emerging pathogen, is the second most common cause of traveler's diarrhea after ETEC in both developed and developing countries. EAEC is also becoming commonly recognized as a cause of endemic and epidemic diarrhea worldwide. Diarrhea caused by EAEC is often watery, but it can be accompanied by mucus or blood. EAEC colonization can occur in the mucosa of both the small and large bowels, which can lead to mild inflammation in the colon (Nataro and Kaper, 1998). EAEC attaches to enterocytes in both the small and large bowels through aggregative adherence fimbriae (AAF) that stimulate a strong interleukin-8 (IL-8) response, allowing biofilms to form on the surface of cells. Plasmid-encoded toxin (Pet) is a serine protease autotransporter of the Enterobacteriaceae (SPATE) that targets α-fodrin (also known as SPTAN1), which disrupts the actin cytoskeleton and induces exfoliation (Fig. 1.2).

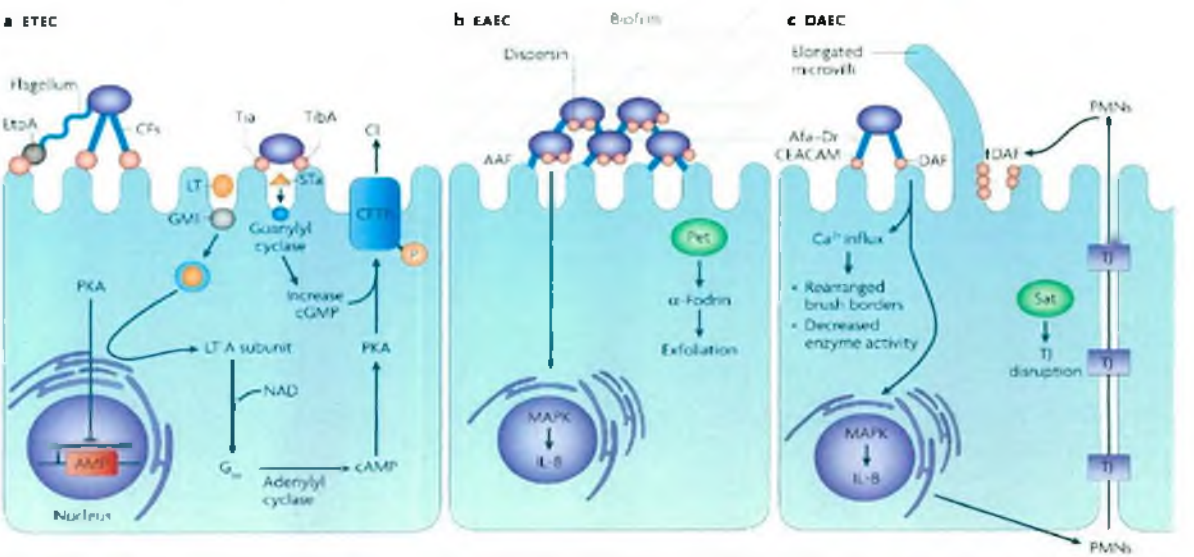


Fig. 1.2 The mechanism of pathogenesis of ETEC, EAEC and DAEC. The figure has been adopted from Croxen and Finlay (2010).

(Additional abbreviations used in the figure are AMP, antimicrobial peptides; $G_{\alpha s}$, stimulatory guanylyl-nucleotide-binding (G) protein α-subunit; MAPK, mitogen-activated protein kinase; PKA, protein kinase A).

1.2.2.1.5 Diffusely adherent *E. coli* (DAEC)

DAEC colonize the small bowel and have been implicated in diarrhea in children between the ages of 18 months and 5 years, as well as in recurring urinary tract infections (UTIs) in adults (Servin, 2005). DAEC forms a diffuse attaching pattern on enterocytes of the small bowel, which is mediated through afimbrial (Afa) and fimbrial adhesins, which are collectively known as Afa–Dr fimbriae. Most Afa–Dr fimbriae bind to complement decay-accelerating factor (DAF); a subset of Afa–Dr fimbriae bind to receptors in the carcinoembryonic-antigen-related cell-adhesion molecule (CEACAM) family. The autotransported toxin Sat has been implicated in lesions of tight junctions (TJs) in Afa–Dr-expressing DAEC, as well as in increased permeability. Polymorphonuclear leukocyte (PMN) infiltration increases surface localization of DAF (Fig. 1.2).

1.2.2.1.6 Enteroinvasive *E. coli* (EIEC)

EIEC pathovar, similar to *Shigella* spp. differs from the other *E. coli* pathovars, because it includes obligate intracellular bacteria that have neither flagella nor adherence factors. Virulence is largely due to a 220 kb plasmid that encodes a T3SS that is required for invasion, cell survival and apoptosis of macrophages (Ogawa *et al.*, 2008; Schroeder and Hilbi, 2008). EIEC or *Shigella* gain access to the submucosa through microfold (M) cells and, following replication in macrophages, invade the basolateral side of colonocytes; all of these processes are achieved by effectors that are secreted into host cells by the type III secretion system. Once in the colonocyte cytoplasm, more effectors are injected to hijack host machinery, prevent detection by the host immune system and promote cell-to-cell dissemination of the bacterium (Fig. 1.3).

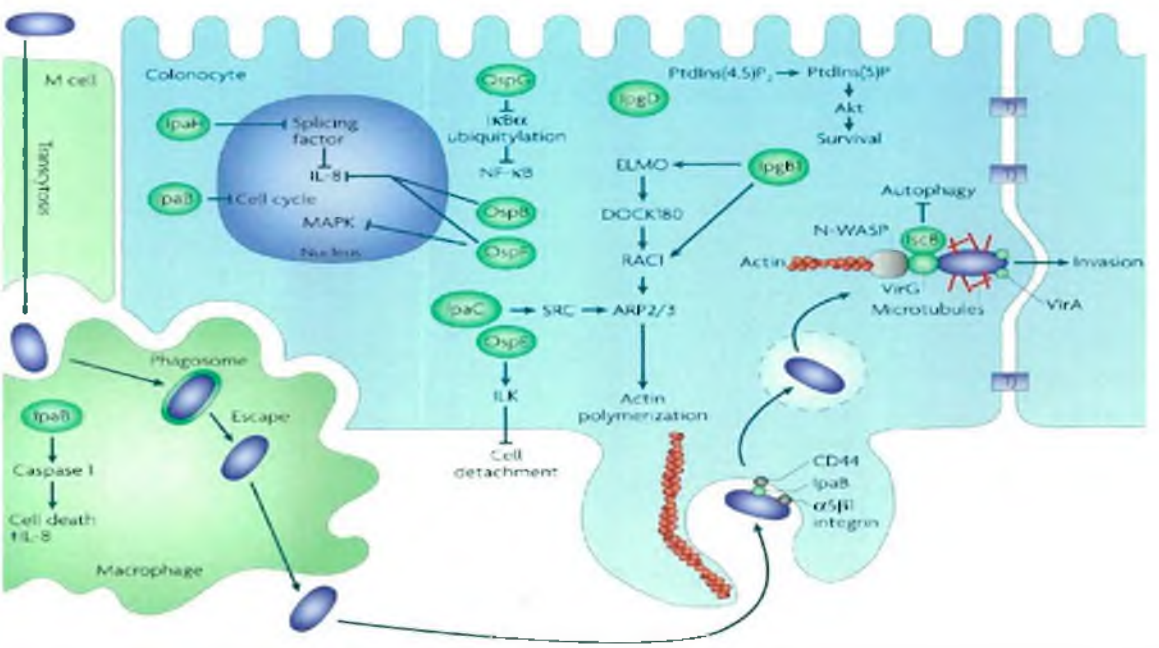


Fig. 1.3 The mechanism of pathogenesis of EIEC. The figure has been adopted from Croxen and Finlay (2010).

(Additional abbreviations used in the figure are ARP2/3, actin-related protein 2/3; DOCK180, dedicator of cytokinesis protein 1; ELMO, engulfment and cell motility; IκBα, inhibitor of NF-κB subunit-α; IL-8, interleukin-8; ILK, integrin-linked kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; N-WASP, neural Wiskott–Aldrich syndrome protein; PtdIns(5)P, phosphatidylinositol-5-phosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; TJ, tight junctions).

1.2.2.2 Extra-intestinal pathogenic *E. coli*

ExPEC strains were defined by Johnson *et al.*, (2003) as *E. coli* isolates containing two or more of the following virulence markers: *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *sfa/foc* (S and F1C fimbriae subunits), *afa/dra* (Dr-antigen-binding adhesins), *kpsMT* II (group 2 capsular polysaccharide units), and *iutA* (aerobactin receptor). Other virulence markers may also be associated with ExPEC status (Johnson and Russo, 2005).

1.2.2.2.1 Uropathogenic *E. coli* (UPEC)

UPEC has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness (Alteri *et al.*, 2009). The ability to ascend the urinary tract from the urethra to the bladder and kidneys reflects exceptional mechanisms for organ tropism, evading innate immunity and avoiding clearance by urination. Highly regulated virulence factors contribute to this complex pathogenesis, including multiple pili, secreted toxins e.g., Sat and Vat, multiple iron acquisition systems and a polysaccharide capsule (Wiles *et al.*, 2008). UPEC attaches to the uroepithelium through type 1 pili, which bind the receptors uroplakin Ia and IIIa; this binding stimulates unknown signalling pathways (indicated by the question mark) that mediate invasion and apoptosis. Binding of type 1 pili to α3β1 integrins also mediates internalization of the bacteria into superficial facet cells to form intracellular bacterial communities (IBCs) or pods. Sublytic concentrations of the pore-forming haemolysin A (HlyA) toxin can inhibit the activation of Akt proteins and leads to host cell apoptosis and exfoliation. Exfoliation of the uroepithelium exposes the underlying transition cells for further UPEC invasion, and the bacteria can reside in these cells as quiescent intracellular reservoirs (QIRs) that may be involved in recurrent infections. UTIs that are left untreated can disseminate to the kidney in an ascending progression of disease. Ascension to the kidney is mediated by type 1 pili and motility. Motility was shown to permit the ascension from the bladder to the kidney (Lane *et al.*, 2007). UPEC isolates that are associated with pyelonephritis often express the P fimbriae that adhere to kidney epithelial cells (Kaper *et al.*, 2004) (Fig. 1.4).

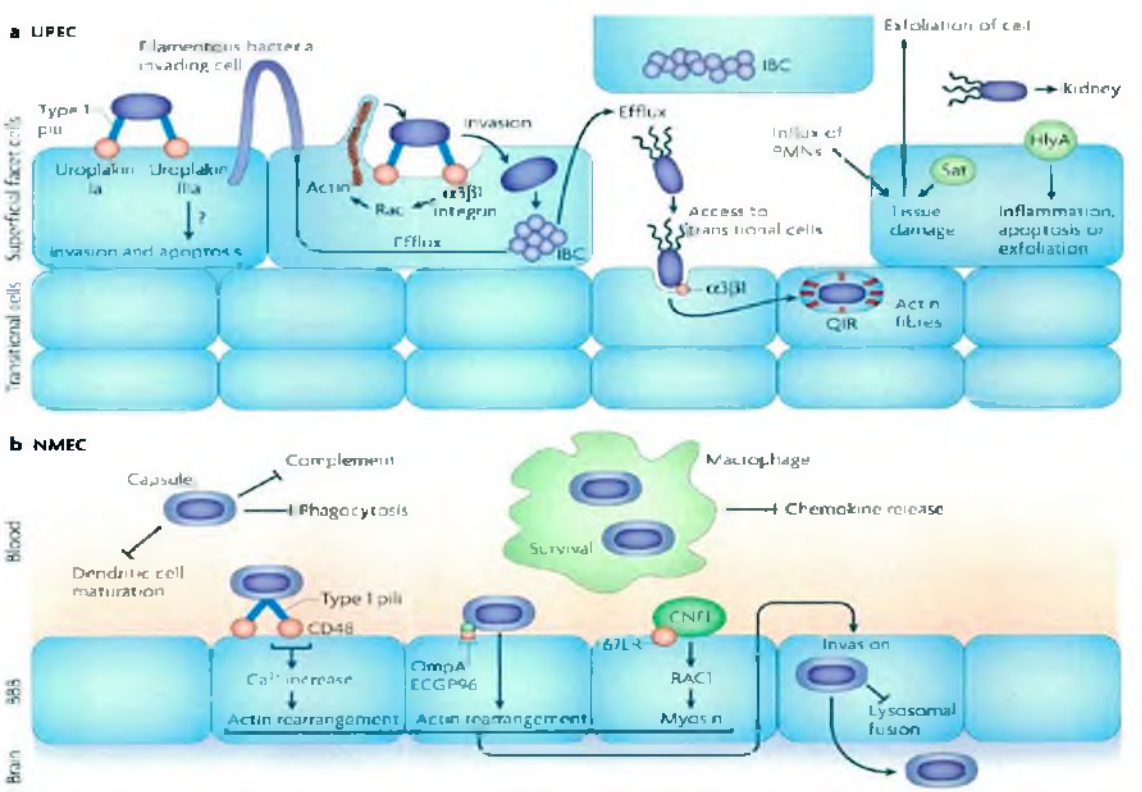


Fig. 1.4 The mechanism of pathogenesis of UPEC and NMEC. The figure has been adopted from Croxen and Finlay (2010).

(Additional abbreviations used in the figure are PMN, polymorphonuclear leukocyte; Sat, secreted autotransporter toxin; Vat, vacuolating autotransporter toxin).

1.2.2.2.2 Neonatal Meningitis *E. coli* (NMEC)

NMEC is a common inhabitant of the gastrointestinal tract, is the most frequent cause of Gram-negative-associated meningitis in newborns. Fatality rates can approach 40% (Kaper *et al.*, 2004), and survivors are usually burdened with severe neurological sequelae. The pathogenesis of NMEC is complex, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood–brain barrier into the central nervous system, which leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid. NMEC is protected from the host immune response by its K1 capsule and outer-membrane protein A (OmpA). Invasion into macrophages may provide a replicative niche for high bacteraemia, allowing the generation of sufficient bacteria to cross the blood–brain barrier (BBB) into the central nervous system. Attachment of NMEC is mediated by type 1 pili binding to CD48 and OmpA binding to ECGP96. Invasion involves cytotoxic necrotizing factor 1 (CNF1) binding to 67 kDa laminin receptor (67LR; also known as RPSA, as well as type1 pili and OmpA binding their receptors (Fig. 1.4).

1.2.2.2.3 Cytolethal Distending toxin producing *E. coli* (CDTEC)

Cytolethal distending toxins have been reported to be acquired by a group of unrelated Gram-negative bacteria (Cortes-Bratti *et al.*, 2001). Cdt effects on arrest of the eukaryotic cell cycle in the G_1 or G_2 phase (Pickett *et al.*, 1994; Peres *et al.*, 1997; Cortes-Bratti *et al.*, 2001; Lara-Tejero and Galan, 2001), which characteristically distends cell morphology leading to cell death (Peres *et al.*, 1997; Lara-Tejero and Galan, 2000, 2001; Cortes-Bratti *et al.*, 2001). Cdt is tripartite toxin encoded by three adjacent or slightly overlapping genes named as *cdtA*, *cdtB*, and *cdtC* (Pickett *et al.*, 1994; Scott and Kaper, 1994; Peres *et al.*, 1997; Lara-Tejero and Galan, 2001; Bielaszewska *et al.*, 2004). Within the Cdt holotoxin, CdtB is the enzymatically active subunit having DNase I-like activity (Lara-Tejero and Galan, 2000, 2001) that arrests the cell cycle inflicting DNA damage. The CdtA and CdtC polypeptides constitute the heterodimeric subunit (Lara-Tejero and Galan, 2001; Lee *et al.*, 2003) that is required for Cdt binding to target cells (Lee *et al.*, 2003) and for intracellular delivery of CdtB (Lara-Tejero and Galan, 2001). It was first identified in culture supernatants from clinical isolates of *E. coli* and *Campylobacter* spp. (Johnson and Lior, 1988). Currently, it is established that Cdt is also produced by *Haemophilus ducreyi* (Cope *et al.*, 1997), *Actinobacillus actinomycetemcomitans* (Sugai *et al.*, 1998), *Helicobacter hepaticus* and *H. pullorum* (Young *et al.*, 2000). *Salmonella enterica* serovar contains a *cdtB* gene in its genome, but no *cdtA* or *cdtC* genes (Parkhill *et al.*, 2001).

Five different *cdt* alleles have been reported in *E. coli* encoding Cdt-I (Scott and Kaper, 1994), Cdt-II (Pickett *et al.*, 1994), Cdt-III (Peres *et al.*, 1997), Cdt-IV (Toth *et al.*, 2003) and Cdt-V (Janka *et al.*, 2003) respectively. The fifth *cdt* allelic cluster was found in most of sorbitol-fermenting STEC O157:H^- strain (Janka *et al.*, 2003) and the others were reported in enteropathogenic *E. coli* (EPEC), *E. coli* causing extraintestinal infections, and animal pathogenic *E. coli*. Although, Cdt infer pathogenesis in 'chancroid', a sexually transmitted disease characterized by genital ulcers caused by *H. ducreyi* (Stevens *et al.*, 1999; Lewis *et al.*, 2001; Young *et al.*, 2001) and in localized-aggressive-periodontitis, endocarditis, meningitis, osteomyelitis and glomerulonephritis (Collazos *et al.*, 1999; Slots and Ting, 1999; Henderson *et al.*, 2003) caused by *A. actinomycetemcomitans*; only a low percentage of *E. coli* isolates taken from diarrheal stools strains are positive for *cdt* genes, although the percentage is higher than that in non-diarrheic isolates (Albert *et al.*, 1996; da Silva and da Silva Leite, 2002; Marques *et al.*, 2003). These studies do not show a strong association of Cdt with

diarrheal disease. However, there are reasons for suspecting an underestimate in detection of *cdt* genes, as not all *E. coli* Cdt variants, such as the IV (Toth *et al.*, 2003) and V (Janka *et al.*, 2003) would have been tested in these early studies. Cdt producing *E. coli* infection has already been reported in Bangladesh (Albert *et al.*, 1996; Talukder *et al.*, 2008).

1.2.2.2.4 Necrotoxigenic *E. coli* (NTEC)

NTEC can be isolated from human extra intestinal infections such as urinary tract infections. NTEC secretes two cytotoxic necrotizing factors (CFN1 and CNF2), as well as cytolethal distending toxin. Cell-detaching *E. coli* (CDEC), which secretes CNF1 and a haemolysin, may be associated with diarrhea in children (Kaper *et al.*, 2004).

1.2.2.2.5 Adherent invasive *E. coli* (AIEC)

Perhaps the most interesting pathovar is AIEC as it has been implicated in 36% of ileal Crohn's disease (Barnich *et al.*, 2007; Carvalho *et al.*, 2009). AIEC adheres to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) in the ileum, which is over-expressed in patients who are predisposed to Crohn's disease. Adherence of AIEC also increases the expression of CEACAM6 through the stimulation of tumor necrosis factor and interferon-γ, which possibly promotes better colonization of the ileal mucosa and subsequent inflammation (Barnich *et al.*, 2007). Following adhesion, AIEC can invade the intestinal epithelium. Individuals with Crohn's disease are often unable to control bacterial infections, possibly owing to defects in autophagy (Lapaquette *et al.*, 2010), so AIEC can replicate quickly in epithelial cells and is inefficiently cleared by the innate immune response. AIEC can translocate through the intestinal epithelium into the lamina propria, where the bacteria interact and survive inside macrophages; they further exacerbate inflammation by stimulating tumour necrosis factor, eventually forming granulomas (Rolhion and Darfeuille-Michaud, 2007).

1.2.2.3 Evolution of pathovars

Horizontal gene transfer (HGT) is an important mechanism that rapidly disseminates new traits to recipient organisms. Acquiring these new traits is crucial in promoting the fitness and survival of a pathogen while it co-evolves with its host (Shames *et al.*, 2009). Large clusters of virulence genes, called pathogenicity islands (PAIs), can be found on plasmids or integrated into the chromosome in pathogenic bacteria. It is not surprising that many of the virulence traits that are present in *E. coli* are carried on PAIs as well as on plasmids and prophages. One theory is that multiple HGT events expose the bacteria to new selective pressures, which eventually select for more virulent organisms that become epidemic, such as EHEC and EIEC (Wirth *et al.*, 2006). It should be noted that the evolution of pathovars might not always occur in a lineage-specific manner; for example, EHEC virulence factors were found to have been acquired independently by different phylogenies of *E. coli* (Ogura *et al.*, 2009). Although the genomes of most pathogenic *E. coli* isolates can encode more than 5,000 genes, less than half of these genes make up the core genome (Rasko *et al.*, 2008; Touchon *et al.*, 2009). This allows for substantial genetic diversity and plasticity in pathogenic isolates. The distribution of virulence factors among UPEC isolates is heterogeneous, and no single factor has been implicated in uropathogenesis. Comparative genomics has identified 131 UPEC-specific genes (Lloyd *et al.*, 2007). As these genes are specific to UPEC isolates, they might constitute a common subset that contributes to virulence. Recently sequenced EPEC found to encode approximately 400 more genes than *E. coli* K-12 sub-strains, but around 650 fewer genes than EHEC O157:H7 and 770

fewer genes than UPEC (str. CFT073) (Iguchi *et al.*, 2009), suggesting that the repertoire of acquired virulence factors that are necessary to become EPEC might be substantially smaller than that required to create the other pathovars. Of the pathogenic *E. coli*, EIEC has endured the most recombination and has undergone patho-adaptation (Maurelli, 2007), including the loss of genetic material. HGT of the virulence plasmid pINV gave EIEC the ability to be invasive; however, a deletion, also referred to as black holes (Maurelli, 2007), in a region of the genome that contains the lysine decarboxylase gene (*cadA*), was necessary for full fitness and adaptation to an intracellular lifestyle. Both gene loss and gain have contributed to the divergence and emergence of a diverse set of *E. coli* pathovars.

1.2.3 Epidemiological features

1.2.3.1 Global scenario

Precise information on the health impact from *E. coli* is needed to adequately inform policy-makers how best to allocate resources. To date, however, no comprehensive information exists. In particular data on *E. coli* from developing countries, where populations are more exposed to contaminated environments, are scarce. In order to fill the current data vacuum, WHO and its partners launched the initiative to estimate the global burden of food-borne diseases. Three to five billion diarrheal illness cases and 5-10 million diarrhea-related deaths occur annually among the 3 billion people living in Africa, Asia, and Latin America (Lima and Guerrant, 1992). A systematic review by Fischer-Walker *et al.*, (2012) revealed that there are 5 billion episodes of diarrhea in children aged >5 annually, with 3.2 billion cases in South-East Asia. There were more than 1.15 million estimated deaths from diarrhea in South East Asia and Africa each year in children >5. Pathogens in the spotlight in these systematic reviews were the usual suspects, including *E. coli*, *Shigella* spp., *Vibrio cholerae*, *Campylobacter* spp. and *Salmonella enterica* serovers. The burden is not solely borne by people living in poverty, but with 455 million episodes of diarrhea each year in the USA and 419 million in Europe (Jones, 2009).

In North America, Japan and parts of Europe, most outbreaks are due to EHEC serotype O157:H7, whereas other serotypes are important health concerns in other developed countries (Kaper *et al.*, 2004). In the ten years following the 1982 outbreak, approximately thirty *E. coli* O157:H7 outbreaks were recorded in the United States (Griffin and Tauxe, 1991). Though, EHEC occurs in all countries, the incidence seems to vary between countries. In 2004 the number of laboratory confirmed cases in the European Union and Norway was 1.3 cases per 100000 populations while in the same year the incidence in the USA was 0.9 cases per 100000 people. These differences could be real or could simply reflect different capacities of surveillance systems to accurately collect and report cases (Stein, 2010). According to CDC (2012), the most recent *E. coli* associated outbreaks reported in 2012 is a multistate outbreak of shiga toxin-producing *E. coli* O26. In 2011, a total of four major multistate outbreaks were reported in CDC, three of which were *E. coli* O157:H7 associated infections linked to a romaine lettuce, another with Lebanon bologna and the third one associated with in-shell hazelnuts. A severe pandemic outbreak of shiga toxin-producing *E. coli* O104 (STEC O104:H4) got the most coverage in 2011, which was associated with travel to Germany. In 2010, two *E. coli* O157:H7 outbreaks were reported, one associated with cheeses and another one with beef. An *E. coli* O145 outbreak was reported to be associated with shredded romaine lettuce from a single processing facility. Between 2006 and 2009, several outbreaks were

reported and in all these cases, *E. coli* O157:H7 was detected only; 3 outbreaks in 2009 associated with beef; one outbreak in 2008 was also associated with beef; two outbreaks in 2007was associated with pizza and ground beef patties; one outbreak in 2006 associated with fresh spinach.

1.2.3.2 Bangladesh perspective

Diarrhea caused by ETEC is highly prevalent in young children in developing countries as well as in travelers to these areas (WHO, 1999). In Bangladesh, ETEC is a commonly isolated agent from patients seeking treatment in hospitals (Black *et al.*, 1981; Qadri *et al.*, 2000). Reports have suggested that ETEC, in addition to cholera, is a predominant cause of diarrhea in Bangladesh (Khan *et al.*, 1988; Faruque *et al.*, 1998). Flooding in Dhaka (capital of Bangladesh) in July 2004 caused a diarrheal epidemic. ETEC was almost as prevalent as *Vibrio cholerae* O1 in diarrheal stools. ETEC that produced heat-stable enterotoxin alone was most prevalent (Qadri *et al.*, 2005). During the peak period, >700 patients were attended per day, and the total number during the epidemic was >17,000. Bangladesh experienced severe flooding and diarrheal epidemics in 2007 also. A comparative study with flood data from 2007, 2004 and 1998 for diarrheal patients attending a hospital in Dhaka revealed a shifting in prevalence of major diarrheal pathogens in the region (Harris *et al.*, 2008). More severe dehydration was seen in 2007 compared with 2004 and 1998 ($P < 0.001$). In 2007, 51% of ETEC produced the heat labile toxin, 22% expressed the heat stable and 27% were ST/LT positive. The CS7 colonization factor (CF) was the most prevalent in 2007 (20% compared with 6% in 2004; $P = 0.05$). The findings demonstrate alterations in clinical features and phenotypic changes of major bacterial pathogens in the recent Bangladesh flood.

In India and Bangladesh, ETEC was first described extensively and was shown to be a cause of adult diarrhea resembling cholera in the severity of infection (Ryder *et al.*, 1976; Merson *et al.*, 1980). In a detailed investigation in children aged from 0 to 5 years in Bangladesh, 90% of cases of ETEC diarrhea reporting to the hospital were children aged from 3 months to 2 years (Qadri *et al.*, 2000). In an ongoing birth cohort study in Bangladesh, it was found to be the most common cause of diarrhea in children 0 to 2 years of age, accounting for 18% of all diarrheas. Limited epidemiological information is available for adults. In Bangladesh, ETEC follows a very characteristic biannual seasonality with two separate peaks, one at the beginning of the hot season, that is the spring, and another peak in the autumn months, just after the monsoons, but it remains endemic all the year round (Rowland, 1986; Khan *et al.*, 1988; Albert *et al.*, 1995; Qadri *et al.*, 2000). Surface water sources in Bangladesh, in both rural and urban areas, are highly contaminated with ETEC. In a study, ETEC strains were obtained from clinical samples as well as from ponds, rivers, and lakes around the clinical field site; 32% of water samples obtained from the surface water sources were contaminated with ETEC (Begum *et al.*, 2005). In a study with ETEC strains, isolated from diarrheal patients in Bangladesh from 1996 to 1998, O6 (n=28), O115 (n=20) and O128 (n=20) were found to be the prominent O-serotype (Ansaruzzaman *et al.*, 2007). STEC is not a common pathogen in Bangladesh neither among hospitalized patients with diarrhea nor among mild cases of diarrhea in the community. STEC was found in 9 out of 410 hospitalized children aged 2 to 5 years and 11 out of 160 diarrheal patients of same age in a rural slum of Dhaka city in Bangladesh (Islam *et al.*, 2007). Avian pathogenic *E. coli*, close to ExPEC, are commonly found in the poultry industries of Bangladesh. In a study, 101 pathogenic *E. coli* strains were isolated from 279 dead or sick broilers and layer hens. The high level of antibiotic resistance found among APEC isolates indicates that

widespread use of antibiotics as feed additives for growth promotion and disease prevention could have negative implications for human and animal health and the environment (Hasan *et al.*, 2009).

1.2.4. Adaptation and survival of *E. coli*

Understanding mechanisms and processes that generate biological diversity is a fundamental problem in evolutionary ecology. In the past decade, the theory of evolutionary branching and adaptive diversification has provided new perspectives for understanding the evolution of diversity caused by ecological interactions (Spencer *et al.*, 2008). Due to their high growth rate and large population size, microbes have a remarkable capacity to evolve and diversify by generation and spread of mutations that improve their fitness in a given environment (Elena and Lenski, 2003).

1.2.4.1. *E. coli* in human host

E. coli is one of the first colonizers of the human GI tract. At birth, the vaginally delivered neonate can be colonized with the bacteria via exposure to maternal flora and subsequently through exposure to other people (Adlerberth and Wold, 2009). By 12 months of age, 100% of infants are colonized by *E. coli*. It is found in mammalian feces in numbers as high as 10^9 per gram of feces and often comprises almost 1% of the total bacterial biomass. More importantly, *E. coli* are considered as a stable member of intestinal community, and they are not as sensitive to dietary considerations as bacteria in other genera (Leclerc *et al.*, 2001). Yet, the intestine is a complex and highly dynamic ecosystem composed of a large diversity of niches that vary in space and time, where bacteria confront a permanent adaptive challenge. Furthermore, intestinal bacteria must be able to hurdle between their hosts across the exterior environment and to switch between two entirely distinct natural environments (Giraud *et al.*, 2001). In *E. coli*, stress protection comes at the cost of nutritional competence through the regulation of membrane permeability (King *et al.*, 2004). In the gut environment, bacterial nutrient intake is likely to be sufficient even if permeability is restrained, so that the growth rate is not significantly affected (Ferenci, 2005). Intriguingly, certain *E. coli* (EHEC) can sense the hormones adrenaline and noradrenaline from host cells, as well as the quorum-sensing molecule from gastrointestinal cells, to regulate motility and receptor expression (Hughes and Sperandio, 2008). EHEC injects around twice as many effectors into host cells as EPEC, most of which are redundant (Tobe *et al.*, 2006). This redundancy may provide EHEC with an evolutionary advantage that allows it outcompete other bacteria. The initial attachment of EPEC to enterocytes in the small bowel is thought to involve the bundle-forming pili that are encoded on the EPEC adherence factor (EAF) plasmid (Hyland *et al.*, 2008). Recently, it was demonstrated that the *E. coli* common pilus may act in concert with bundle-forming pili to stabilize interactions between EPEC and host cells (Saldana *et al.*, 2009). Type IV pilus, also named as hemorrhagic coli pilus (Xicohtencatl-Cortes *et al.*, 2009) is involved in adherence and biofilm formation. Intimate attachment of both the EHEC and EPEC to host cells occurs through interactions between intimin and Tir (Robinson *et al.*, 2006). Cyclomodulin, produced by EPEC and EHEC, is able to block host eukaryotic cell-cycle progression (Marches *et al.*, 2003; Taieb *et al.*, 2006).

The mammalian immune response, maintenance of epithelial barrier functions, and cellular differentiation affect the growth and colonization of pathogenic bacteria. The gut microbiome is an intimate symbiosis with the host (Dinalo and Relman, 2009). Major factors affecting microbial number and composition along the length of the intestine appear to be age, diet, and host genotype

(van Der Wielen *et al.*, 2000; Apajalahti *et al.*, 2001; Hill *et al.*, 2005). The complements of genes represented in the gut microbiome is estimated to be 100 times the number of genes found in the host (Backhed *et al.*, 2005) and is enriched in genes for glycan, amino acid, and xenobiotic metabolism; methanogenesis; and synthesis of vitamins and isoprenoids (Gill *et al.*, 2006). Interestingly, motility and chemotaxis are two characteristics that seem to be underrepresented among gut microbiota (Turnbaugh *et al.*, 2009; Verberkmoes *et al.*, 2009).

1.2.4.2. *E. coli* in nonhuman host

E. coli is usually the first colonizer not only of human but of almost every mammalian newborn germ-free intestine (Gophna *et al.*, 2006; Palmer *et al.*, 2007). Both monogastric and polygastric livestock carry *E. coli*. In some cases, they are asymptomatic carriers of serotypes that are pathogenic to humans. Cattle, sheep, and goats are the most important reservoir for the EHEC, with some reports in swine as well as wild animals (Yoon and Hovde, 2008; La Ragione *et al.*, 2009). The Stx receptors found on Paneth cells in the human intestinal mucosa (Schuller *et al.*, 2007) and the surface of kidney epithelial cells (Nataro and Kaper, 1998) are absent in cattle, which may explain why EHEC colonization in cattle is asymptomatic (Pruimboom-Brees *et al.*, 2000). Household dogs, cats and cockatoos are also reservoirs of *E. coli*, including pathogenic types (Flammer and Drewes, 1988; Beutin, 1999; Damborg *et al.*, 2009). Various pathovars of *E. coli* can also be associated with a wide variety of serious illness in animals, including the GI infection accompanied by diarrhea in calves, lambs and piglets; mastitis in dairy cattle; and colibacillosis in poultry (Gyles, 1994). Gordon and Cowling (2003) included some key phenomena about *E. coli* reservoir, it stated that the species was more commonly detected in mammals, less frequently in birds, and infrequently in fish, frogs, and reptiles. It was less prevalent in carnivores than herbivores or omnivores. Proximity to humans increased the likelihood of *E. coli* being detected in wildlife. Animals living in desert climates were less likely to harbor *E. coli* than if they lived in temperate or tropical regions. Organisms with large body mass or with a GI tract that included a hindgut fermentation chamber had a higher prevalence of *E. coli* (Harwood *et al.*, 1999; Gopee *et al.*, 2000; Montgomery *et al.*, 2002; Santoro *et al.*, 2006). Animals living in Polar Regions also carry *E. coli* (Aguirre *et al.*, 2000; Lisle *et al.*, 2004; Buck *et al.*, 2006). Aquarium experiments with fish immersed in water containing varying densities of *E. coli* inoculums revealed that higher levels of water contamination resulted in higher levels of *E. coli* in gut contents (Hansen *et al.*, 2008) suggesting that fish can be considered as a vector for *E. coli* rather than a reservoir.

1.2.4.3. *E. coli* in host's extra-intestinal sites

Characterization of extra intestinal pathogenic *E. coli* (ExPEC) strains has shown that they harbor many similarities, despite their isolation from different host species, leading to the hypothesis that ExPEC may have zoonotic potential (Tivendale *et al.*, 2010). Similarities in the virulence traits found among avian pathogenic *E. coli* (APEC) and other ExPEC sub-pathotypes have led to the similar speculation (Johnson *et al.*, 2006, 2007, 2008, 2009; Moulin-Schouleur *et al.*, 2006; Manges *et al.*, 2007; Mellata *et al.*, 2009). At least one study has found avian isolates to be indistinguishable from human isolates (Johnson *et al.*, 2006). However, other studies showed that human ExPEC strains were clearly distinct from avian strains (Graziani *et al.*, 2009) and that the consumption of poultry or contact with poultry did not correlate with the colonization of APEC (Sannes *et al.*, 2008).

1.2.4.4. *E. coli* in the environment

1.2.4.4.1 Environmental source *of E. coli*

There are various point sources e.g. sewage outflows to be responsible for the fecal bacteria pollution in sea beaches but nonpoint sources are also involved including river outfalls (Nevers and Whitman, 2005), beach sands (Whitman and Nevers, 2003) and aquatic birds (Alderisio and DeLuca, 1999). In addition, hydrometeorological conditions, like precipitation accompanied by increased runoff and onshore wind can contribute to pollution. Studies across geographical areas covering tropical, sub-tropical and temperate locations have shown that populations of fecal indicator bacteria are commonly found in variety of habitates: rivers and streams in presumably pristine watersheds (Bermudez and Hazen, 1988; Fujioka *et al.*, 1988), stream banks and upland soils (Hardina and Fujioka, 1991; Fujioka *et al.*, 1998; Solo-Gabriele *et al.*, 2000), beach sands (Ghinsberg *et al.*, 1994) and plants and aquatic machophytes (Rivera *et al.*, 1988; Muller *et al.*, 2001; Whitman and Nevers, 2003). Fujioka and Byappanahalli (2001) observed that populations of fecal indicator bacteria have the genetic capability to colonize and adapt to become a minor but significant fraction of soil microflora in humid, warm climates. *E. coli* densities were found highest between summer and early fall and lowest in the winter in a temperate, boreal watershed of Lake Superior (Ishii *et al.*, 2006). *E. coli* densities also showed to have a cyclic pattern corresponding to the tidal cycles: the highest and lowest of *E. coli* densities were found during high and low tides respectively (Solo-Gabriele *et al.*, 2000). The soil environment may not select one or two dominant *E. coli* strains, instead numerous strains may have the ability to colonize, persist and adapt to the soil habitat to become part of the microflora (Fujioka and Byappanahalli, 2001). Several studies showed that both the fresh and marine water sediments harbor high densities of fecal indicator bacteria (Gerba and McLeod, 1976; Gary and Adams, 1985; Obiri-Danso and Jones, 2000; An *et al.*, 2002), with relative densities being several orders of magnitude higher than those in the overlying water (Burton *et al.*, 1987), which can be attributed to their better survival against environmental stresses, such as solar inactivation and predation (Ishii *et al.*, 2007). Potential sources may include beach-dwelling birds (Ishii *et al.*, 2007), the shedding of human derived bacteria during swimming or related activities coupled with bacterial flux or interaction between water of intertidal zone and sand, aquatic macrophytes (Englebert *et al.*, 2008) and marsh vegetation. *Cladophora* spp., a macrophytic green algae found in fresh and marine waters, harbores high levels of *E. coli* (Whitman *et al.*, 2003). Even the *E. coli* found to survive and persisted in sun-dried algal mats. In mesocosm study, *Cladophora* was able to prolong *E. coli* survival longer than in water. Fecal indicator bacteria normally retained in the upper layer of the soil depending on soil type, temperature, rate of water flow and other environmental variables (Bicudo and Goyal, 2003) and possessed potential to contaminate ground and surface water (Unc and Goss, 2004). The impact of wild animals on water quality is usually a more limited or a localized problem compared to migratory birds (Gabrey, 1996). Stream outfalls have been identified as significant sources of fecal bacteria to standing water ecosystems. Even during dry periods, these input sources provide a continuous background microbial loading and, during floods, their concentrations may increase several orders of magnitude because the river carries microbial pollution from the entire watershed (Whitman *et al.*, 2006; Nevers *et al.*, 2007). *E. coli* flows within and between stream and beach watersheds has been presented in the following conceptual diagram (Fig. 1.5).

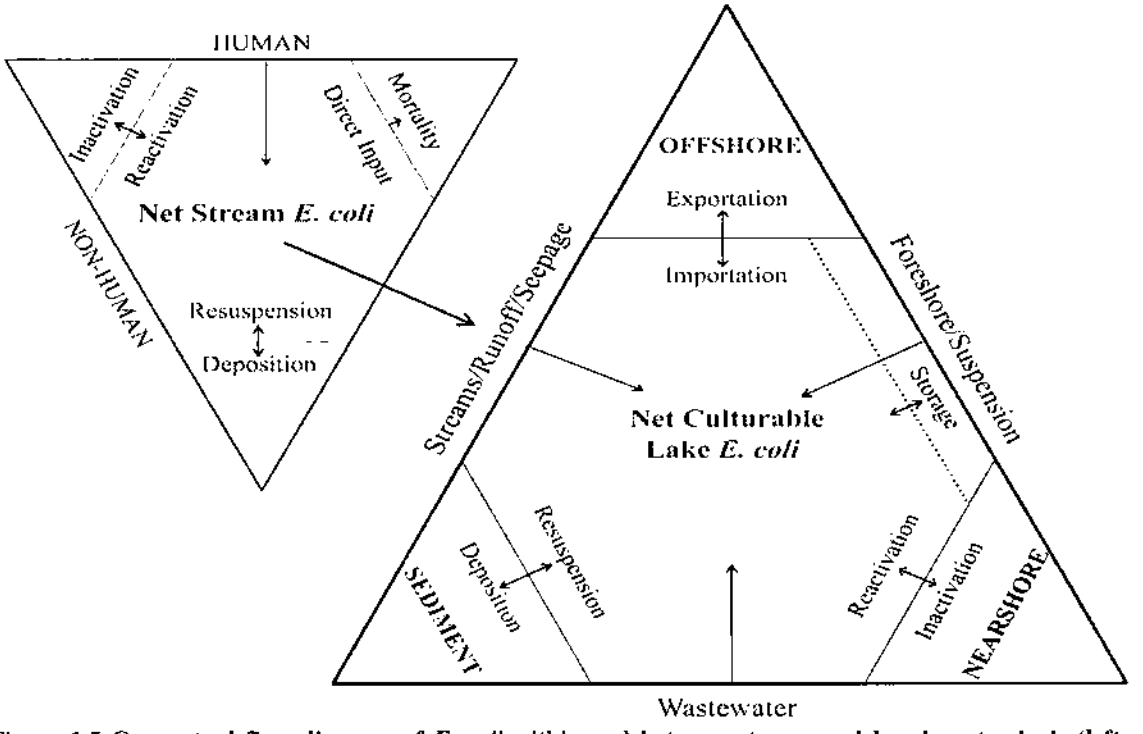


Figure 1.5 Conceptual flow diagram of *E. coli* within and between stream and beach watersheds (left and right triangles, respectively). For streams, the model partitions stream inputs as human or nonhuman; the latter might include bacterial inputs originating from growth within riparian soils and long-term storage. Within the stream, major processes include (i) inactivation and reactivation, (ii) deposition (temporary and permanent loss) and resuspension, and (iii) death and direct input (e.g., animal defecation and multiplication). Culturable bacteria are delivered from the stream to the beachshore as surface water or ground water. Internal input of the *E. coli* into the lake include foreshore bacteria resuspension (from storage, defecates and growth) and wastewater releases. Within the lake, there are dynamic interactions between inactivation/reactivation, deposition/resuspension, and offshore importation/exportation of bacteria. The whole beachshed system eventually yields the net culturable *E. coli* densities. The figure has been quoted from Whitman *et al.*, (2006).

Many human activities, such as dairy and poultry farming and grazing, can directly affect sediment quality by introducing fecal indicators into the system. Because of their close interaction with the surface water, sediments play a significant role in affecting water quality through resuspension by mechanical disturbances or hydrological processes (Goyal *et al.*, 1977; Crabill *et al.*, 1999; An *et al.*, 2002; Ferguson *et al.*, 2005). In large water bodies, more than 80% of indicator organisms were directly associated with suspended sediments (Sayler *et al.*, 1975; Jamieson *et al.*, 2005). Moreover, other human activities, such as dredging can also result in the release of significant amount of indicators organisms into the water (Grimes, 1975). Within aquatic systems, biological and physical factors control the dynamics of the bacterial flux (Whitman and Nevers, 2003). Survival is mediated by the ability of the fecal bacteria to recover once injured (Sinton *et al.*, 2002; Jungfer *et al.*, 2007) in environment and an apparent increase in the cell density may be caused by the increasing culturability over time. Thus the concentration of bacteria in water is really the net total of these inputs and losses. Wave action may influences the early colonization, homogenous distribution, and release of *E. coli* from beach sand into the water column (Ishii *et al.*, 2007). The fecal bacteria population may be attached to materials in biofilms or entrained within the matrix. The degree of these associations likely affects the transport of *E. coli* into the overlying water. Growth of *E. coli* within these microhabitats has been suggested (Byappanahalli *et al.*, 2003).

1.2.4.4.2 Prevalence of *E. coli* in the environment

Environmental prevalence and survival of *E. coli* is perhaps the most studied of all the enteric pathogens. *E. coli* have been detected in a variety of environments and geographical areas (Winfield and Groisman, 2003; Byappanahalli *et al.*, 2006). Concentrations in the coastal waters of southern Portugal ranged between 20 and 2.7×10^5 cfu/100 mL (Dionisio *et al.*, 2002) but in New Zealand, it was detected in low numbers in ground water (Savill *et al.*, 2001). A study on *E. coli* in a temperate forest soil environment found the organism in 88% of samples with a mean of 16 MPN/g; the result also indicated that *E. coli* was capable of surviving over winter in the soil (Byappanahalli *et al.*, 2006). In a study of river soil and sediments in Florida, *E. coli* was detected in significant numbers in the soil along the river, but very little in the soil more than 20 m from the bank (Desmarais *et al.*, 2002).

1.2.4.4.3 Survival of *E. coli* in the environment

The survival of *E. coli* in the environment is influenced by many factors including temperature, protozoa grazing, exposure to UV radiation, and the availability of nutrients. The survivality of *E. coli* in surface waters is affected by water salinity, temperature, sunlight, predation and competition for available nutrients (Fujioka *et al.*, 1981; McCambridge and McMeekin, 1981). Solar inactivation is an important mechanism for natural reduction of indicator bacteria in water (Fujioka *et al.*, 1981; Whitman *et al.*, 2004). In seawater, in presence of sunlight, 90% of the fecal coliforms are inactivated within 3 hours, whereas, in the absence of sunlight, these bacteria survive for days. Moisture has been shown to affect fecal indicator bacterial activity in soils. *E. coli* appears to be more sensitive to soil desiccation than enterococci (Byappanahalli *et al.*, 2006). No viable *E. coli* cells were found in soils when moisture dropped from 33% to 12% over 4 days; however they recovered within two days following rehydration (Byappanahalli and Fujioka, 2004). The organism can be harbored, survive, and potentially proliferate in other secondary hosts and environmental matrices (Anderson *et al.*, 2008). There is evidence that pathogenic *E. coli* is well-adapted for aquatic survival. The first widespread outbreak of *E. coli* O157:H7 in the USA was actually associated with a groundwater source (Swerdlow *et al.*, 1992). Other outbreaks of this serotype have been tied to recreational waters, which is a strong indicator that this pathogen may survive in freshwater environments (Leclercq *et al.*, 2002; Muniesa *et al.*, 2006). A review by Rozen and Belkin (2001) provides an in-depth discussion of factors affecting the survival of *E. coli* in seawater. They concluded that the organism is capable of extended survival in marine environments when available nutrients are available with limited exposure to sunlight. Low water temperature, favorable pH, and association of sediments are also conditions associated with extended survival (Rozen and Belkin, 2001). The serotype O157:H7 survived more than 15 days in seawater and able to multiply in sterilized water (Miyagi *et al.*, 2001). Of the enteric pathogens, *E. coli* O157:H7 is one of the best survivors. *E. coli* was found to survive longer in river sediments that overlying waters (Czajkowska *et al.*, 2005). *E. coli* population in marine sediments maintained stable levels for more than 2 months (Davies *et al.*, 1995). Moreover, the organism survived much longer in soil-water microcosm than in microcosm containing only water (Fish and Pettibone, 1995). One of the most interesting results of environmental surveys for *E. coli* is the growing belief that populations of these bacteria may grow and establish themselves in the environment. *E. coli* isolates taken from river water multiplied upon addition of sterile soil and then began to decrease after 2 days (Desmarais *et al.*, 2002). Another

study showed potential re-growth of *E. coli* in riverbank soils (Solo-Gabriele *et al.*, 2000). A laboratory mesocosm study demonstrated that *E. coli* and enterococci tended to die off in water, but survived and replicated in wet and dry sterile sand. No population growth was recorded in unsterilized sand, which the author attributed to competition and limitation of essential nutrients (Hartz *et al.*, 2008). But in other studies, *E. coli* was shown to survive in water containing a reasonable amount of microflora and nutrients at 15 to 18°C for approximately 4 to 12 weeks (Edberg *et al.*, 2000) Additional studies have also suggested that *E. coli* may be capable of proliferation in beach sand (Whitman and Nevers, 2003; Beversdorf *et al.*, 2007). These studies and others contribute to the growing evidence that certain environments support indigenous populations of *E. coli* (Fujioka *et al.*, 1998; Winfield and Groisman, 2003).

1.2.4.4.4 Adaptive mechanism of *E. coli* to extreme environments

Many environmental factors such as temperature, pH, moisture content, antimicrobial agents, and water activity affect the growth of bacteria in nature (Jay, 2000). As a consequence of adaptive evolution, natural populations of an organism under selective conditions change genetically and phenotypically after a number of generations in order to survive in that particular environment. The available data suggest that the selection for genotypes capable of survival in the environments (Lasalde *et al.*, 2005; Byappanahalli *et al.*, 2006), competitive interaction with other indigenous microflora, and selection of strains capable of utilizing different carbon substrates (Fujioka and Byappanahalli, 2001) influences survival of fecal indicator bacterial populations. These findings have led several researchers to propose that environmental fecal indicator bacteria should be considered as part of the natural biota (Fujioka and Byappanahalli, 2003; Lasalde *et al.*, 2005).

Predation and competition are the two biotic factors that most strongly influence the long term survival and growth of fecal indicator bacteria in the environment. An experiment of *E. coli*, in a small eutrophic lake, concluded that those two factors are affecting survival of the bacteria in aquatic environment (Korhonen and Martikainen, 1991). It has been demonstrated by Gonzalez *et al.*, (1992) that the predation of enteric bacteria was the dominating factor in their disappearance from marine and freshwater aquatic ecosystem. Moreover, many different studies showed that *E. coli* could survive longer in the absence of protozoa in estuarine and marine habitats (McCambridge and McMeekin, 1981; Sørensen *et al.*, 1999). Similar result has been observed in Rhodes and Kator's study (1988) that reduction of *E. coli* concentrations in estuarine waters were associated with increase in the density of microflagellates and predators. Free and suspended bacteria in water are more likely to be affected by competition and predation than attached one (McCambridge and McMeekin, 1981). In seawater or settled sediments, these bacteria are subject to higher rate of predation than in freshwater (Jannasch, 1968). The rate of predation is related to initial predator densities, and in some extent with solar radiation, which injure cells making them more susceptible to predation (McCambridge and McMeekin, 1981). Competition and antagonism were less important than predation in controlling growth (Marino and Gannon, 1991); however, competition for growth limiting nutrients can greatly influence the growth of *E. coli* in soil (Byappanahalli and Fujioka, 2004) and saline ecosystems (Carlucci and Pramer, 1960). In the presence of available nutrients leading to less competition and predation, these bacteria can readily grow (Solo-Gabriele *et al.*, 2000; Byappanahalli and Fujioka, 2004). It has found that the competition with the natural microbial flora of the water and soil may have a primary impact on the reduction in numbers of *E. coli* (Flint, 1987;

Cooley *et al.*, 2003). The growth requirements of *E. coli* are relatively simple than other enteric members and this bacterium has the ability to synthesize complex building blocks from simple inorganic nutrients and glucose (Andrews, 1991), thus *E. coli* can be assumed to outcompete its relatives in growth and survival under nutrient deficient environment.

Among the abiotic factors, water salinity has a significant influence on bacterial survival. Generally, lower salinity leads to a ionger survival time (Sinton *et al.*, 2002). The salinity of natural sea water limits *E. coli* survival, with higher salinities affecting glucose uptake, resulting in rapid cell death (Carlucci and Pramer, 1960; Barcina *et al.*, 1990). It has been reported that the higher the salinity, the greater the stress on *E. coli* survival (Anderson, 1979). The survival pattern and plasmid maintenance of was examined in an artificial seawater microcosm and found that the strains of *E. coli* were able to maintain a portion of cells in the culturable phase for at least 3 years in artificial seawater. It was concluded that cells of *E. coli* were selectively adapted to the salinity of seawater, remaining in a culturable state (Byrd and Colwell, 1993). In an experiment, *E. coli* cells were grown anaerobically in batch and chemostat reactors at different NaCl concentrations using glycerol as a carbon source and observed enzyme activities of the main metabolic pathways, external metabolites, ATP level, $NADH/NAD^+$ ratio and the expression level of the main genes related to stress response to characterize the metabolic state under the osmotic stress (Arense *et al.*, 2010). The results demonstrate that, under salt stress, *E. coli* enhances energy production by substrate-level phosphorylation (Pta–Ack pathway) and the anaplerotic function of the TCA cycle, in order to provide precursors for biosynthesis. The *ant* gene of *E. coli* have been found to be responsible for the salt adaptation (Padan *et al.*, 1989).

pH is a biologically significant environmental stress for enteric bacteria, such as *E. coli,* which are considered neutrophiles growing best at neutral pH, although they can also grow in moderate acid or base (Hughes *et al.*, 2007). Tolerance of even more extreme pH conditions is fundamental to the survival of enteric bacteria, as the mammalian intestinal tract subjects them to extreme acidity in the stomach before reaching the more alkaline intestine. With tolerance to pH as low as 2.0, some *E. coli* are able to colonize within a host with as few as 10 bacterial cells (Audia *et al.*, 2001) and their complex mechanisms for acid resistance have suggested that they may be partly acidophilic and partly neutrophilic (Foster, 2004). In addition, *E. coli* are also exposed to some alkaline conditions at the pancreatic duct (Evans *et al.*, 1988). There are reports of *E. coli* to survive at a pH as low as 2.0 (Gorden and Small, 1993), and as high as 10.0 (Small *et al.*, 1994) for several hours but the range of pH values under which growth is possible is significantly smaller. Three different mechanisms of acid resistance in *E. coli* have been described. The first mechanism is an RpoS-dependent oxidative or glucose-repressed system (Small *et al.*, 1994). The second mechanism is a GAD system that involves glutamate decarboxylase (Lin *et al.*, 1995; Hersh *et al.*, 1996; De Biase *et al.*, 1999); and the third mechanism is an ARG system that requires arginine decarboxylase activity (Lin *et al.*, 1995; Castanie-Cornet *et al.*, 1999). In the presence of glutamate or arginine, *E. coli* can reverse the electrical membrane potential to make the inside of the cell positively charged, the same strategy used by various acidophiles growing in extremely low pH environments (Richard and Foster, 2004). Alkaline stress resistance may involve different mechanisms, some suggestions are: a protein repair mechanism of L-isoaspartyl protein carboxyl methyltransferase (PCM) (Hicks *et al.*, 2005), topoisomerase I influence on RpoS-dependent and GadA and GadB systems (Stewart *et al.*, 2005), the decarboxylase activity linked to chloride ion exchange activities of ClC-ec1 (Gut *et al.*, 2006), or

the identification of alkaline resistance activity through ATP synthase and NhaA/MdfA antiporters (Padan *et al.*, 2005). As a pH stress becomes more extreme, adaptation becomes more rapid and extensive (Hughes *et al.*, 2007).

In an estuarine ecosystem, the survival of fecal coliforms were found to be negatively correlated with increasing water temperature (Faust *et al.*, 1975). In river water, *E. coli* survived for longer than 260 days in the absence of other microorganisms at natural temperature between 4 and 25°C. Study of O157:H7 serotype in water microcosms found that the bacteria survived for more than 12 weeks at 8°C and for 7 to 12 weeks at 25°C, depending on the water source. In addition, significant levels of viable bacteria were detected after samples could no longer be cultured on traditional media, which also indicate the possibility of these cell to enter VBNC states (Wang and Doyle, 1998). *E. coli* survived in soil for 12 to 100 days and river water for 6 to 12 days in another study, with wide variation in survival time dependent on temperature (Bogosian *et al.*, 1996). Heat stress in *E. coli* O157:H7 can be classified as heat shock or heat adaptation. Heat shock is a short period of exposure of a cell to temperatures above its normal growth maximum, and the response is associated with heat shock genes (*rpoH*). In a similar manner, heat adaptation is a long period of exposure of a cell to a temperature above its optimal range for growth and the response of the cell is associated with the *rpoS* gene and the alteration of the cell membrane. The alteration of membrane lipid composition plays an important role in bacterial response to heat stress, the so-called "homeoviscous adaptation" (Sinensky, 1974; Arneborg *et al.*, 1993). Heat-adapted *E. coli* O157:H7 strains were more heat resistant than control cells or heat-shocked cells.

1.2.4.4.5 VBNC state of *E. coli*

The term 'nonculturable' was invoked (Xu *et al.*, 1982) to describe starved but viable cells in a survival or dormant state of existence, observed to occur after an actively growing culture of *E. coli.* These cultures enumerated by acridine orange direct counting (AODC) were metabolically active even though they could not be recovered on any plating medium or broth (Kogure *et al.*, 1979). Hence, the phrase "viable but nonculturable (VBNC)" was coined to describe these bacteria that were apparently in a dormant state. As a secondary habitat, in the environment *E. coli* remain in stressed and starved conditions compared to its primary habitat, intestine. Reduction of bacterial recovery was interpreted as a form of cellular damage that reduced the culturability (Harris, 1963; Ray, 1989) and such damaged bacteria became more fastidious in their nutritional requirements for subsequent growth (Wright, 1955). Several enrichment media has been designed and used successfully, e.g. buffered peptone water alone or containing vancomycin, cefixime and cefsulodin (Foster *et al.*, 2003), peptone water containing 0.5% sodium thioglycolate (Sata *et al.*, 2003), minimal lactose enrichment broth (Shelton *et al.*, 2004) consisting the basal salts medium of Hylemon and Phibbs (Hylemon and Phibbs, 1972) (50 mM potassium phosphate, 15 mM ammonium and trace nutrients) modified by the addition of NaCl, bile salts and lactose.

1.2.4.5 Biofilm formation as a means of adaptation

In most ecological niches, bacterial interactions with a surface promote novel behaviors leading to the development of structured and heterogeneous, matrix-encased bacterial communities known as biofilms. Several key factors, including different extracellular appendages, are implicated in *E. coli* surface colonization and their expression and activity are finely regulated, both in space and time, to

ensure productive events leading to mature biofilm formation (Beloin *et al.*, 2008). Many *E. coli* isolates have the capacity to form biofilm structures in vivo and in vitro. Indeed, *E. coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract, where it thrives in an environment with structural characteristics of a multispecies biofilm (Costerton *et al.*, 1995; Probert and Gibson, 2002).

1.2.4.5.1 Pre-determinants of *E. coli* biofilm

Pratt and Kolter (1998) showed that motility itself, and not chemotaxis or direct surface contact by flagella, was required to form a biofilm. Genevaux and co-workers (1996) confirmed the study and, more recently, Wood and co-workers (2006) demonstrated that the biofilm formation capacities of *E. coli* K-12 were directly correlated with its motility. Although, it is not an absolute requirement, and nonmotile bacteria can still form biofilms under certain conditions, as shown for *E. coli* K-12 (Pratt and Kolter, 1999), and also for EAEC (Sheikh *et al.*, 2001). Even some reports demonstrated that neither flagellar motility is required for initial adhesion, nor is it required for biofilm development (Prigent-Combaret *et al.*, 2000). In *E. coli* strain bearing a conjugative plasmid, known as a strong adhesion factor (Ghigo, 2001), the presence of flagellum was dispensable for biofilm formation (Reisner *et al.*, 2003). In these cases, the expression of strong adhesion factors might replace flagellar movements during initial interactions between adhering bacteria and the surface (Pratt and Kolter, 1999; Geesey, 2001; Donlan, 2002).

Initial bacterial adhesion to abiotic surfaces is likely to be highly dependent on physicochemical and electrostatic interactions between the bacterial envelope itself and the substrate conditioned by the fluids to which it is exposed (Dunne, 2002). This reversible attachment is strongly influenced both by environmental conditions such as pH and the ionic force of the medium or the temperature (Fletcher, 1988; Danese *et al.*, 2000). The nature of the surface e.g. rugosity, increasing surface adhesion or hydrophobicity (Donlan, 2002), adsorption and desorption of nutrients at the surface are also important factors that may influence bacterial initial attachment both positively and negatively (Zobell, 1943; van Loosdrecht *et al.*, 1990). Type 1 fimbriae, curli, and conjugative pili have a role in strengthening the bacteria-to-surface interactions. Type 1 fimbriae, commonly expressed both by commensal and pathogenic *E. coli* isolates (Sauer *et al.*, 2000), can adhere to a variety of receptor molecules on eukaryotic cell surfaces (Duncan *et al.*, 2005). Along with their role in the formation of biofilm within the gut (Bollinger *et al.*, 2003, 2006; Orndorff *et al.*, 2004), type 1 fimbriae have reported to be critical for biofilm formation on abiotic surfaces (Harris *et al.*, 1990; Pratt and Kolter, 1998; Cookson *et al.*, 2002; Moreira *et al.*, 2003; Orndorff *et al.*, 2004). FimH adhesin may also have similar binding activity at abiotic surfaces (Pratt and Kolter, 1998). Type 1 fimbriae-mediated abiotic biofilm formation by K-12 *E. coli* MG1655strain might represent a strategy to escape bacteriophage attack (Lacqua *et al.*, 2006). Curli fimbriae in *E. coli* aggregate at the cell surface and have been demonstrated to attach to proteins of the extracellular matrix (Olsen *et al.*, 1989; Ben Nasr *et al.*, 1996), thus promoting adhesion of the bacteria to different human cells. In addition, they promote biofilm formation to abiotic surfaces both by facilitating initial cell to surface interactions and subsequent cell to cell interactions (Vidal *et al.*, 1998; Cookson *et al.*, 2002; Uhlich *et al.*, 2006). Although most laboratory *E. coli* K-12 strains are poor biofilm formers, the introduction, either artificially or naturally, in mixed *E. coli* communities of a conjugative plasmid in these strains induces formation of a thick mature biofilm (Ghigo, 2001; Reisner *et al.*, 2003; Reisner *et al.*, 2006).

The F-pilus promotes both initial adhesion and biofilm maturation through nonspecific attachment to abiotic surfaces and subsequent cell to cell contacts, which stabilize the structure of the biofilm (Ghigo, 2001; Molin and Tolker-Nielsen, 2003; Reisner *et al.*, 2003). Both conjugative and non-conjugative plasmids are likely to carry determinants for biofilm initiation and architecture, thus promoting biofilm development, which in turn affects the extent of plasmid-mediated horizontal gene transfer within the biofilm (Wuertz *et al.*, 2004). Ag43 is a surface autotransporter protein and does not seem to be involved in non-specific initial adhesion to abiotic surfaces, but rather, promotes cell to cell adhesion (Kjaergaard *et al.*, 2000). While, in liquid culture, this property leads to autoaggregation and clump formation, it also facilitates three-dimensional development of the biofilm (Owen *et al.*, 1996; Henderson *et al.*, 1997; Hasman *et al.*, 1999; Kjaergaard *et al.*, 2000). Correlation between an increased level of *flu* expression and capacity to form a biofilm on different abiotic surfaces (Danese *et al.*, 2000; Kjaergaard *et al.*, 2000; Beloin *et al.*, 2006) was demonstrated. Gene fusion studies suggest that the expression of up to 38% of the *E. coli* genome is affected by biofilm formation (Prigent-Combaret *et al.*, 1999). The virulence activator RfaH, has recently been linked to biofilm formation in *E. coli* (Beloin *et al.*, 2006), since RfaH-dependent biofilm formation and virulence gene expression are mutually exclusive processes. Signals leading to activation of stress responses often lead to biofilm formation which, in turn, can trigger induction of stress response mechanisms, suggesting direct cross-talk between the two cellular processes (Landini, 2009).

1.2.4.5.2 Biofilm matrices

One of the most distinctive features of biofilms is the presence of an extracellular matrix embedding the biofilm bacteria and determining mature biofilm architecture (Sutherland, 2001). Along with expression of proteinaceous adhesins, production of this matrix is essential for maturation of the biofilm structure. The biofilm matrix is a complex milieu essentially composed of water (97%), but it also includes exopolysaccharide polymers, proteins, nucleic acids, lipids/phospholipids, absorbed nutrients, and metabolites (Ghannoum and O'Toole, 2001). Although the matrix is a hallmark of bacterial biofilms, its role is not fully understood. The biofilm matrix offers a constantly hydrated viscous layer protecting embedded bacteria from desiccation or from host defenses by preventing recognition of biofilm bacteria by the immune system. The matrix may also play a significant protective role as a diffusion barrier and a sink for toxic molecules (antimicrobials, hydroxyl radicals, and superoxide anions). The biofilm matrix could also inhibit wash-out of enzymes, nutrients, or even signaling molecules that could then accumulate locally and create more favorable microenvironments within the biofilm (Redfield, 2002; Welch *et al.*, 2002). All these aspects of the putative roles of the matrix could contribute to development of phenotypic resistance of pathogenic *E. coli* biofilms and lead to persistent infections (Anderson *et al.*, 2003; Justice *et al.*, 2004). Several exopolysaccharides found in the *E. coli* biofilm matrix (cellulose, PGA, colanic acid) are key components of the biofilm matrix, while others such are lipopolysaccharides and capsular polysaccharides may not accumulate significantly in the matrix, but still play an important indirect role in biofilm formation. To date, three exopolysaccharides, β-1,6-N-acetyl-D-glucosamine polymer (PGA), colanic acid, and cellulose, have been detected in the biofilm matrix of *E. coli* and have been shown to be important for biofilm formation. Mutations affecting LPS synthesis have been shown to affect *E. coli* ability to adhere to abiotic surfaces (Genevaux *et al.*, 1999), suggesting a role of LPS in

adhesion processes. As seen for colanic acid and LPS, the *E. coli* capsule has also been shown to play an indirect role in biofilms by shielding of bacterial surface adhesin (Schembri *et al.*, 2004).

1.2.5 *E. coli* as an indicator

Indicators are 'living tracers' and should have similar behavioral characteristics and responses to environmental perturbations as pathogens (Armon and Kott, 1996). Fecal indicators have been defined as 'bacteria utilized to determine the sanitary and microbiological quality of water' (Haack *et al.*, 2009). Indicator bacteria must ideally meet a number of criteria in order to be useful robust indicators of fecal pollution (Buttiaux and Mossel, 1961; Yates, 2007).

In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination (Feng *et al.*, 2002). This was based on the premise that *E. coli* is abundant in human and animal feces and not usually found in other niches. Although the concept of using *E .coli* as an indirect indicator of health risk was sound, it was complicated in practice, due to the presence of other enteric bacteria like *Citrobacter, Klebsiella* and *Enterobacter* that are alomost similar to *E. coli* in phenotypic characteristics. As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas within 48 h at 35°C. In 1914, the U.S. Public Health Service adopted the enumeration of coliforms as a more convenient standard of sanitary significance. A test for this organism as a measure of drinking water potability was suggested in 1895 by T. Smith. In 1986, the United States Environmental Protection Agency produced guidelines recommending *E. coli* as an appropriate indicator to monitor recreational waters (USEPA, 1986) replacing the thermo-tolerant *E. coli* recommended in 1976. Now, *E. coli* has been recognized as the fecal indicator bacteria of choice for monitoring drinking water, and has adopted in WHO drinking water guidelines and in many national drinking water quality standards.

1.2.5.1 Controversy of using *E. coli* as Indicator

There have been several reports showing that *E. coli* are capable of surviving and growing in the environment (Davies *et al.*, 1995). This violates one of the major criteria applied to indicators, namely that a fecal indicator must not grow in the natural environment (Dufour, 1984). Moreover, there are reports that contamination can be introduced by birds and animals. Bird feces, for example, are rich in fecal bacteria (Oshiro and Fujioka, 1995). *E .coli* is capable of surviving and replicating in beach sand. So, sand may act as a reservoir of fecal bacteria and that, during strong weather or tidal events, these cells may be released into the water (Hartz *et al.*, 2008). Reports of high numbers of *E. coli* in sand (Bonilla *et al.*, 2007), their occurrence at locations remote from obvious sewage contamination and the notion that *E. coli* is growing in soils and sediments combine to potentially weaken the usefulness of *E. coli* as indicator for monitoring recreational water quality.

1.2.6 Tracking of *E. coli*

Bacterial source tracking (BST) or MST (microbial source tracking), includes several methodologies used to determine sources of bacteria (wildlife, humans, and domestic livestock) from environmental samples. The term was first coined by Hagedorn and Wiggins, which describes various sub-typing methods (Harwood, 2002). Methods for BST fall into three groups: molecular, biochemical, and

chemical. Currently, there is no standard method that has been adopted for source tracking. Source tracking of bacterial contamination in drinking water is a rapidly growing area of research and technology development.

1.2.6.1 Culture dependant tracking

In the past, fecal coliform/fecal streptococci (FC/FS) ratios have been used to assess the general source of nonpoint fecal pollution, with FC/FS >4 indicating humans, FC/FS between 0.1 and 0.6 indicating domestic animals, and FC/FS <0.1 indicating wild animals as the source (Geldreich, 1976). However, studies have found that the FC/FS ratio was difficult to use in agricultural settings (Howell *et al.*, 1996), and the American public health association (APHA) no longer recommends the use of the FC/FS ratio as a means of differentiating human and animal sources of pollution (APHA, 1998). Approved by the U.S. Environmental Protection Agency (USEPA, 1991), 4-methylumbelliferyl-β-D-glucuronide *E. coli* broth medium (EC-MUG) is an effective and rapid method for detection and verification of *E. coli* in food, water, and environmental samples. The enzyme ß-glucuronidase was recognized in about 96 to 97% *E. coli* (Hajna and Perry, 1943; Buehler *et al.*, 1949) that is capable of hydrolyzing MUG to yield a bluish fluorogenic product 4-methylumbelliferone that fluoresces under long wave UV light (366 nm) and can be easily visualized in the medium or around the colonies (Poelma *et al.*, 1987; Farnleitner *et al.*, 2001). This tracking has been used to detect *E. coli* in milk and dairy products, food and shellfish (Feng and Hartman, 1982; Watkins *et al.*, 1988), water and wastewater (Feng and Hartman, 1982; Clark *et al.*, 1991) and urine and other clinical samples (Chang *et al.*, 1989; Manafi and Kneifel, 1989). This technique is sensitive, selective, and specific and has high precision and accuracy rate.

1.2.6.2 Molecular tracking

The lack of culturability of *E. coli* possesses a potential public health challenge, because the methodologies accepted for detecting and counting *E. coli* rely mostly on culturing (Muela *et al.*, 2008). The use of culture independent method like PCR to measure the occurrence or concentration of fecal indicators is gaining widespread interest. The PCR can also be performed quantitatively in real-time to detect and quantify the number of copies of target DNA sequence in the original sample. Multiplex PCR detecting the virulence associated genes of *E. coli* pathovars can be an easier alternatives of isolation dependent tracking. Today, there are a number of different molecular and biochemical methods proposed for BST including: ribotyping (Parveen *et al.*, 1999; Carson *et al.*, 2001; Hager, 2001; Hartel *et al.*, 2002; Scott *et al.*, 2002; Simpson *et al.*, 2002), randomly amplified polymorphic DNA (RAPD) (Tynkkynen *et al.*, 1999), denaturing-gradient gel electrophoresis (DGGE) (Farnleitner *et al.*, 2000; Buchan *et al.*, 2001; Chee-Sanford *et al.*, 2001; Simpson *et al.*, 2002), repetitive DNA sequences (Rep-PCR) (Dombek *et al.*, 2000), length heterogeneity PCR (LH-PCR) (Suzuki *et al.*, 1998; Bernhard and Field, 2000), terminal restriction fragment length polymorphism analysis (T-RFLP) (Bernhard and Field, 2000), hostspecific 16S rDNA (Suzuki *et al.*, 1998; Bernhard and Field, 2000), toxin biomarkers (Hager, 2001; Olson *et al.*, 2002), reverse transcriptase PCR (Hager, 2001), phage analysis (Hager, 2001) and antibiotic resistance analyses (ARA) (Wiggins, 1996; Parveen *et al.*, 1997; Hagedorn *et al.*, 1999; Wiggins *et al.*, 1999; Harwood *et al.*, 2000; Hager, 2001). Several of the molecular and biochemical techniques have been applied or suggested for use in watershed studies (Simpson *et al.*, 2002).

1.2.6.3 New approaches to track *E. coli*

Serotyping, an important tool to identify and group *E. coli* strains, hence also provide source tracking in investigations of outbreaks, reservoirs, spread and emergence. The *fliC* gene has been successfully used for PCR/RFLP and sequence genotyping (Fields *et al.*, 1997; Machado *et al.*, 2000) of both motile and nonmotile strains might be advantageous over classical H serotyping.

Chen and his group (2010) used laser imaging and high-resolution optical scattering image analysis to produce their new detection method. One of the most important implications of the new work is that authorities might soon become able figure out which waters are infected more rapidly. This could help them develop relevant clean-up programs in due time, and thus decrease the health risk to the general population.

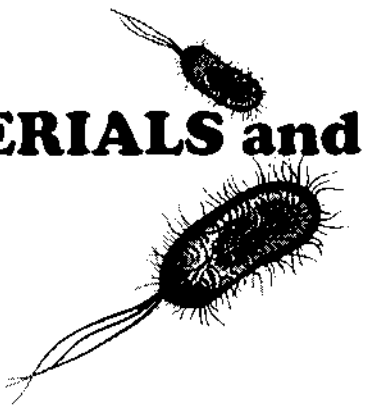
Escherichia coli is an indicator of fecal contamination based on the premise that *E. coli* is abundant in human and animal feces, is not usually found in other niches and has in general a limited survival time following discharge. *E. coli* has been detected in a variety of environments and geographical areas (Winfield and Groisman, 2003; Byappanahalli *et al.*, 2006) and there are several reports showing that *E. coli* are capable of surviving for extended periods and even multiplying in certain environments (Davies *et al.*, 1995). However, contamination can be introduced by zoonotic sources even in environments considered to be unaffected by human activity. This normally harmless commensal bacterium can become an aggressive pathogen, capable of causing a range of diseases, affecting either animals or humans worldwide (Croxen and Finlay, 2010). Precise information on the health impact from *E. coli* is needed to adequately inform policy-makers. Although *E. coli* is one of the most studied enteric pathogens, no comprehensive information exists to date about its environmental prevalence and survival in developing countries, where the human populations are more exposed to contaminated environments. The goal of the present study is to monitor *E. coli* pathovars in tropical aquatic systems covering a nationwide area using different tracking systems and to further assess novel tracking approaches using molecular techniques. Adaptive potential and survivability of environmental *E. coli* isolates have also been discussed, and, *E. coli* isolates possessing potential to be used as probiotics or vaccine agents against shigellosis have been characterized.

Specific objective of the study

- Collection of water samples from natural aquatic systems covering a large area in tropical region in three successive seasons to assess the water quality and tracking of *E. coli*.
- Designing of different pre-enrichment and enrichment media to resuscitate environmental *E. coli* isolates and molecular tracking of the species using *fliC* PCR and subsequent Southern blot analysis.
- Monitoring of five different diarrheagenic *E. coli* virulotypes in the water sample by multiplex PCR assay along with monitoring of comparatively new virulotype, cytolethal distending toxin producing-*E. coli* in the environmental water samples in three seasons. The study will serve to assess the temporal and regional occurrence and prevalence of these *E. coli* virulotypes in the aquatic systems.
- Use of *fliC*-sequence genotype to assess the prevalence of different *E. coli* H-types in the aquatic systems and among the environmental isolates, which would facilitate investigating epidemiological study as well as emerging H-types in the environment.
- Isolation of environmental *E. coli* strains from different aquatic systems, identification by biochemical profiling using API 20E and molecular typing of the isolates by 16S rDNA sequencing, *fliC* sequence genotyping, RFLP and Rep-PCR.
- Survival study of the environmental *E. coli* isolates in aquatic microcosm and mesocosm study. Determination the role of fish population in the net *E. coli* load in aquatic system.
- Comparative growth rate and survival study of marine *E. coli* isolates in freshwater and marine water to delineate the adaptive potential as well as tolerance to high salt concentrations.

- Screening for the environmental *E. coli* isolates, cross reactive to antibodies specific for its close relative *Shigella* spp. by slide agglutination test, to be used as vaccine candidate or potential probiotics against Shigellosis.
- Western blot analysis of LPS and protein extracted from the cross-reactive isolates with *Shigella* spp. specific polyvalent and monovalent antibody to confirm the structural similarity between surface antigens of these two genera.
- PCR detection of virulence associated genes in the environmental *E. coli* isolates to assess its potential as probiotics as well as identifying the prevalence of these pathovar specific genes in the environmental isolates.
- PCR detection of adhesion genes in the environmental *E. coli* isolates to correlate the role of adhesions with the association of colonization in fish gut and to assess the potential as probiotic strains or vaccine candidate.
- Plasmid profile analysis of *E. coli* strains.

2. MATERIALS and MATHODS



The methodologies used for the experiments include collection of surface water samples from different natural aquifers to analyze the prevalence and persistence of different *Escherichia coli* pathovars in Bangladesh. The work will suggest some regime of faster and more reliable methods of environmental monitoring of *E. coli* virulotypes and investigating disease outbreaks. The study also includes isolation and identification of environmental *E. coli* and outlines their adaptive features for persistence and extended survival in water. The experiments also approach to screen environmental isolates cross-reacting to anti-*Shigella* spp. antisera and to assess their potential as vaccine candidate. All the media compositions, chemicals, reagents used in this study have been provided in the Appendix sections.

2.1 Sample collection, transport and processing

2.1.1 Sampling sites

Water samples were collected from 46 different natural aquatic locations in Bangladesh between December 2009 and January 2010 (winter season), in April 2010 (summer season) and in July 2010 (rainy season). The sampling points included 35 freshwater and 11 marine/ brackish sites covering a large part of the country. The sampling sites were chosen such that they included major rivers and sea beaches located in regions with higher human activity, economic importance, and tourist interest. The map of Bangladesh marked with the sampling sites (by GPS position coordination) has been shown in Fig. S1 in Appendix III.

2.1.2 Measurement of physicochemical parameters

Physicochemical parameters e.g. temperature, salinity, conductivity and total dissolved solids of the water samples were measured by 'sension5 conductivity Meter' (Hach Company, USA), pH and dissolved O_2 were measured by 'CyberScan pH/DO Meter' (Eutech Instruments, Singapore) and GPS coordinates recorded by 'eTrex Legend HCx personal navigator' (Garmin Corporation, Taiwan). All the parameters were measured on site, enlisted in Table S1 in Appendix III.

2.1.3 Pre-enrichment and enrichment techniques

Sixty mL of each water sample was added to 20 mL of concentrated pre-enrichment broth to achieve the following final concentrations: Lab-Lemco powder (0.5 g/L), yeast extract (1.0 g/L), peptone (2.5 g/L) and of NaCl (2.5 g/L) at pH 7. After 8 h of incubation at ambient temperature, one milliliter of each of pre-enriched cultures was transferred to 50 mL of two different enrichment broths; one supplemented with 0.15% bile salts and another with streptomycin (50µg/mL) in a broth containing peptone (3 g/L), NaCl (1 g/L), K_2HPO_4 (1 g/L) and KH_2PO_4 (1 g/L) at pH 7. The enrichment cultures were incubated for 16 hrs at 37°C with shaking at 120 rpm.

2.2 Tracking of *E. coli* in the aquatic environment

2.2.1 Extraction of DNA

Chromosomal DNA was extracted from pure culture of isolates as well as from pellets collected from direct water filtrate, pre-enrichment and the two enrichment cultures from each sample as described by Faruque *et al.*, (2002). DNA extracted from direct water filtrate was further purified using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Germany).

2.2.1.1 Harvesting of cells

One or two discrete colonies from nutrient agar (NA) plate of pure culture was inoculated into 6 mL Luria Bertani (LB) broth and incubated at 37 °C at 120 rpm for overnight for the extraction of DNA from pure culture. The cells were then harvested by centrifugation (NÜVE, Turkey) of 1.0 mL of culture (pure culture isolate, pre-enrichment and enrichment culture) at 12000 rpm for 10 min. The supernatant was drained off. For harvesting cells directly from water sample, 100 mL of water for

each sample was filtered through 0.45 μm membrane filter (Millipore, USA). The filter was then soaked in 2.0 mL of normal saline (0.85% NaCl) in a sterilized petridish and the pellet was scrubbed out by sterile pipette tip. The suspended pellet was then harvested by centrifugation at 12000 rpm for 10 min.

2.2.1.2 Lyses of the cells

Cell harvest, taken from isolate, water filtrate, pre-enrichment and enrichment cultures, was resuspended in 200 μL DNA extraction solution 1 (20% sucrose and 50mM EDTA in 50mM Tris HCl Buffer) containing lysozyme (20mg/mL) and incubated for 30 min at 37 °C. Then 300 μL of DNA extraction solution 2 (50mM NaCl and 1% SDS) containing 5 μL of proteinase K solution (200 mg/mL) was added to each tube and the sample was incubated at 55 °C for 60 min in a water bath (Memmert, Germany).

2.2.1.3 Phenol extraction of DNA

After the lysis treatment, DNA was extracted using phenol: chloroform: isoamylalcohol (25:24:1). Tubes were mixed well until a milky white appearance was observed. The tubes were then centrifuged for 5-7 min at 12000×g, which produced three phases, the top aqueous phase with DNA, middle solid phase consisted of cellular proteins and debris and the lower phase of phenol. Using pipette, the top phase was recovered carefully and transferred into the fresh microcentrifuge tube.

2.2.1.4 Ethanol precipitation

After phenol extraction, $1/10^{th}$ volume of the 3M sodium acetate (pH 5.2) was added to the DNA solution and mixed well by flicking the tube several times with a finger. Double volume of ice-cold 95% ethanol was added and mixed well by inverting the tubes, and was kept at -30 °C for overnight. DNA precipitate was collected by centrifugation followed by washing with 70% ethanol. Finally the pellet was dried in a concentrator (Eppendorf, Germany) under vacuum. Dried DNA pellet was dissolved in 30 μL of TE buffer and stored in -20 °C.

2.2.1.5 Purification of DNA

DNA extracted from direct water filtrate contained some impurities giving rise to a reddish color of DNA suspension, which could not be removed by the procedure followed. For these samples, a further purification step was followed with the column provided with GenElute bacterial genomic DNA kit (Sigma, USA). The column was prepared with 0.5 mL of column preparation solution. The 50 μL of DNA suspension extracted before was mixed with 200 μL of ethanol and was poured into the column. The column was centrifuged at 12000×g for 1 min followed by a washing step by 0.5 mL washing solution and was eluted with elution buffer provided with the DNA extraction kit.

2.2.1.6 Determination of DNA concentration

DNA concentration was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA) using deionized water or elution buffer (as applicable) as blank.

2.2.2 *fliC* PCR assay for detection of *E. coli*

fliC is the flagellin encoding gene responsible for H-type specific serological heterogeneity for most *E. coli*. There are certain *E. coli* strains, which have flagellin proteins encoded by genes other than *fliC* but they still possess this gene. The primer used to amplify *fliC* gene of *E. coli* co-amplify the gene of other closely related members of Enterobacteriaceae e.g, *Shigella* spp., *Salmonella* spp., *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp.

2.2.2.1 PCR amplification of *fliC*

DNA extracted from direct water filtrate, pre-enrichment and enrichment cultures were used as template for the PCR of *fliC* gene. The amplification of the gene by PCR was done using the primer pair (Machado *et al.*, 2000): 5'-CAAGTCATTAATACMAACAGCC-3' (forward) and 5'-GACATRTTRGAVACTTCSGT-3' (reverse). Amplification was performed in 50 µL reaction volume for each specimen containing 2 µL of template DNA, 0.5 µL of 2 U/µL DyNAzyme™ DNA polymerase, 5 µL of 10× buffer for DyNAzyme, 1.0 µL of a mixture of deoxynucleoside triphosphates (25 mM of each), and 1.0 µL of 25 µM solution of each primer (Sigma-Aldrich, Germany). The thermo-cycler conditions with a PTC 200, Peltier thermal cycler (MJ Research, USA) was as follow: a 3-minute denaturing at 95°C followed by 35 cycles of 3-step panel having "denaturing at 95°C for 30 sec, annealing at 60°C for 60 sec and extension at 72°C for 2 min" with a final extension at 72°C for 7 min.

2.2.2.2 Electrophoresis and observation of PCR product

The PCR products were separated through agarose gel electrophoresis. A horizontal slab of 1.5% agarose was made. Agarose (SeaKem® LE Agarose, Norway) was dissolved in 1× Tris-acatate-EDTA (TAE) buffer. Following solidification of the gel, it was submerged in 1× TAE buffer in an electrophoresis tank. PCR product was mixed with 2 µL of 6× gel loading dye and was loaded into the slots of the gel with the aid of a micropipette. Electrophoresis was continued at constant volts (60-70). Upon EtBr staining, DNA bands were observed in a UV-trans-illuminator (Gel Doc, BioRad, USA). Photographs were taken using prefixed camera in Gel Doc (BioRad, USA) machine attached to a computer and DNA bands were analyzed with 'Quantity One' software (BioRad, USA).

2.2.3 Confirmation of *E. coli*-specific *fliC* gene by Southern blot hybridization

2.2.3.1 Gel pretreatment

Standard protocols for denaturation and neutralization of the gel described by Sambrook and Russel (2001) were followed. The gel was immersed in 100 mL of 0.25N HCl solution in a tray and was shaken gently for 15 min for depurination followed by washing with dH_2O. The gel was then immersed in 100 mL of denaturing solution and was shaken gently for 15 min. Prior to neutralization, the gel was rinsed well for 2 to 3 times in deionized water and then dipped into neutralization solution and was shaken for 15 min again in room temperature.

2.2.3.2 Capillary transfer of DNA onto nitrocellulose membrane

Capillary transfer of DNA was done using 20× SSC with a vacuum blotter (BioRad, USA). Whatman filter paper and nitrocellulose membrane was cut into the shape of the agarose gel but slightly bigger

in size to overhang the gel. The Whatman paper and membrane were soaked in 2× SSC and placed on the porous plate of vacuum blotter and was covered by the precut (to a size slightly smaller than the gel but the window must accommodate the electrophoresis zone of the gel) window gasket. The gasket covered the entire O-ring to ensure proper sealing. The gel was then placed on the window and sealed the entire frame and DNA loading well by melted agarose to prevent breach in the well. The sealing frame was placed and locked onto latch posts. The vacuum pump was switched on and the pressure was adjusted to 5 mm Hg. The chamber was kept flooded with 20× SSC for 90 min with additional gentle pouring of the buffer. The gel was then removed and discarded. The membrane was then placed in a Whatman paper soaked in 10× SSC and the wet membrane placed under UV for cross-linking. The filter was then washed with deionized water briefly and allowed to air-dry.

2.2.3.3 DIG-labeling of probe DNA

2.2.3.3.1 PCR amplification probe DNA

Probe for hybridization was PCR-generated amplicon of oligoncleotide primers 5'-CAAGTCATTAATACMAACAGCC-3' (forward) and 5'-GACATRTTRGAVACTTCSGT-3' (reverse) from *E. coli* O157:H7 clinical strain.

2.2.3.3.2 Purification of probe DNA

PCR products were purified using GenElute PCR clean-up kit (Sigma, USA). Before starting the purification, wash solution was diluted using 48 mL of 100% ethanol. GenElute Miniprep binding columns were inserted into collection tube and 0.5 mL of column preparation solution was added to the column. Upon centrifugation at 12000 ×g for 1 min, elute was discarded. Five volume of binding solution was added to one volume of PCR product and mixed well. The mixture was transferred into the binding column and centrifuged at 12000 ×g for 1 min. Elute was discarded retaining the collection tube. The column was washed with addition of 0.5 mL of wash solution and centrifuges for the second time for 2 min to remove the residual ethanol. The column was then placed into a fresh collection tube. Fifty mL of elution solution was applied to the column and incubated at room temperature for 1 min. The column was then centrifuged at 12000 ×g for 1 min to elute PCR product and stored at -20°C.

2.2.3.3.3 DIG-labeling

DIG DNA labeling and detection kit (Roche, Germany) was used for labeling. Digoxigenin (DIG) - labeled DNA probes are generated according to the method of random primed labeling based on the hybridization of random oligonucleotides to the denatured DNA template. The complementary DNA strand is synthesized by Klenow enzyme using random oligonucleotides as primers and mixture of deoxyribonucleotides containing DIG-11-dUTP, alkali-labile for elongation. Digoxigenin is a steroid hapten used to label probe to ease subsequent detection of hybridization by enzyme immunoassay. The product list provided with the kit is listed in Appendix II. Two to three µg of purified probe DNA were diluted with PCR grade water to make a volume of 15 µL. Subsequently the control DNA 2 (vial 2 of the supplied kit) was also diluted by adding 10 µL of water to 5 µL of DNA. The DNA was denatured by boiling for 10 min and immediately chilled into ice. The tube was then briefly centrifuged and 2.0 µL of hexanucleotide mixture, 2.0 µL of dNTP labeling mix and 1.0 µL of

Klenow enzyme were added followed by gentle mixing and incubation at 37°C for overnight. The reaction was stopped by heating the probe at 65°C for 10 min.

2.2.3.4 Determination of labeling efficiency

Determination of the yield of labeled probe is important to optimize reproducible hybridization. A series of dilutions of labeled probe was prepared and applied as drops on positively charged nitrocellulose membrane. A series of defined dilutions of DIG-labeled control DNA (vial 4) were also applied. The membrane was UV-fixed and subjected to immunological detection with anti-digoxigenin-AP conjugate (vial 8) and freshly prepared color substrate solution. The color intensity of the probe was compared with the control to measure the yield or efficiency of probe.

2.2.3.5 Hybridization and detection

The membrane filter was prehybridized in 20 mL of prehybridization buffer in the rotor bottle for 30 min at hybridization temperature (the hybridization temperature, T_{opt} was determined by the equation $T_{opt} = T_m - 20\text{-}25°C$) in the oven. The prehybridization solution was poured off and 20 mL of pre-warmed hybridization solution was added to the membrane together with 50 µL of denatured DNA probe. The membrane was then incubated in hybridization buffer at hybridization temperature for 16 h with gentle rotation in the hybridization oven. The membrane was then washed twice in washing buffer for 15 min at room temperature followed by stringent wash at 68 °C for 30 min. The membrane was then washed in washing buffer with 0.75% Tween 20 solution for 2 min and then in maleic acid buffer for 30 min. The membrane was then subjected to incubation at room temperature with continuous shaking in maleic acid buffer containing 1/10000 volume of DIG-AP conjugate followed by washing twice in buffer for 15 min each and once in detection buffer for 5 min. The membrane was placed into nitroblue-tetrazoliamchloride/5-bromo-4-chloro-3-indoylphosphate (NBT/BCIP) color substrate containing detection buffer and kept in dark at room temperature until a desire color appeared in the positive colony blot. The color may appear within couple of minutes to several hours.

2.2.4 Multiplex PCR assay for detection of virulence-associated genes

2.2.4.1 Target genes and PCR primers

Detection of virulence marker genes was performed by multiplex PCR (Svenungsson *et al.*, 2000; Nguyen *et al.*, 2005) using 9 primer pairs (Table 2.1) targeting the virulence-related genes e.g. *eaeA* (intimin of EHEC and EPEC strains), *bfpA* (bundle-forming pilus of EPEC strains), *vt1* and/or *vt2* (Shiga toxins 1 and 2 of EHEC strains), *eltB* and/or *estA* (enterotoxins of ETEC strains), *ial* (invasion-associated locus in EIEC and *Shigella*), *ipaH* (in large invasive plasmid and the chromosomes in *Shigella* and EIEC strains) and pCVD (*Eco*RI-*Pst*I DNA fragment of pCVD432 of EAEC). The multiplex PCR reaction was segregated into 3 different sets choosing primer pairs generating PCR products of different sizes which could easily be separated and distinguished by agarose gel electrophoresis.

Table 2.1 List of oligonucleotide primers used in the multiplex PCR assay for the detection of genes associated with diarrheagenic *E. coli* virulotypes.

	Primer	Target gene	Primer sequence	Ref.	Amp. size
Set 1	ST	*EstA*	5'-GCTAAACCAGTA$^{G}_{A}$GGTCTTCAAAA-3' 5'-CCCGGTACA$^{G}_{A}$GCAGGATTACAACA-3'	(Moseley *et al.*, 1983)	147
	SHIG	*Ial*	5'-CTGGTAGGTATGGTGAGG-3' 5'-CCAGGCCAACAATTATTTCC-3'	(Frankel *et al.*, 1990)	320
	bfpA	*BfpA*	5'-TTCTTGGTGCTTGCGTGTCTTTT-3' 5'-TTTTGTTTGTTGTATCTTTGTAA-3'	(Yatsuyanagi *et al.*, 2002)	367
	EA	Pcvd	5'-CTGGCGAAAGACTGTATCAT-3' 5'-CAATGTATAGAAATCCGCTGTT-3'	(Pereira *et al.*, 2007)	630
Set 2	VT1	*vt1*	5'-GAAGAGTCCGTGGGATTACG-3' 5'-AGCGATGCAGCTATTAATAA-3'	(Pollard *et al.*, 1990)	130
	VT2	*vt2*	5'-ACCGTTTTTCAGATTTT$^{G}_{A}$CACATA-3' 5'-TACACAGGAGCAGTTTCAGACAGT-3'	(Lindqvist, 1997)	298
	Eae	*eaeA*	5'-CACACGAATAAACTGACTAAAATG-3' 5'-AAAAACGCTGACCCGCACCTAAAT-3'	(Svenungsson *et al.*, 2000)	376
Set 3	ipaH	*ipaH*	5'-GCTGGAAAAACTCAGTGCCT-3' 5'-CCAGTCCGTAAATTCATTCT-3'	(Tornieporth *et al.*, 1995)	423
	LT	*eltB*	5'-TCTCTATGTGCATACGGAGC-3' 5'-CCATACTGATTGCCGCAAT-3'	(Inoue *et al.*, 1993)	322

2.2.4.2 Composition of PCR reaction mixture and thermocycler conditions

PCR was performed in 20 µL reaction mixture containing 1 µL of template DNA, 0.2 µL of 2 U/µL DyNAzyme^{TM} DNA polymerase, 2 µL of 10× buffer for DyNAzyme, 0.4 µL of a mixture of deoxynucleoside triphosphates (25 mM of each), and 0.5 µL of 25 µM solution of each primer (Sigma-Aldrich, Germany). The thermocycler conditions with a PTC 200, Peltier thermal cycler (MJ Research, USA) were as follows: a regime of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles, with an initial denaturation at 96°C for 4 min and a final 7 min extension at 72°C.

2.2.4.3 Agarose gel electrophoresis

PCR products (10 µL) were evaluated by electrophoresis through a 1.5% (w/v) agarose gel at 70 V for 30 min. Upon EtBr staining, DNA bands were observed, photographs were taken and the bands were analyzed according to the Section 2.2.2.2.

2.2.4.4 Determination of detection limit

To determine the detection limit of the multiplex PCR reaction, a series of mixed cultures were prepared having cell ratios (determined by cfu/mL) of 1:1 to 1:10^{13} (with 10-fold dilution) of an avirulent laboratory control strain (*E. coli* DH5α) and two virulent strains designated as MMLA (possessing *vt1* and *vt2*) and B171 (possessing *bfpA*). DNA was extracted and multiplex PCR was

performed as for the test DNA samples. The sensitivity of the assay was defined as the lowest ratio of diarrheagenic to non-diarrheagenic *E. coli* that yielded positive results. In case of *bfpA* and *vt1*, at least $1:10^3$ was the ratio of diarrheagenic to non-diarrheagenic cells needed for a positive PCR reaction, while for *vt2*, it was $1:10^4$ (Fig. S3 in Appendix III).

2.2.4.5 Data Analysis

Chi-square test was used to assess the significance of the data. Relative standard used in the research is $p > 0.05$.

2.2.5 PCR assay for detection of 'cytolethal distending toxin' (Cdt)-associated genes

2.2.5.1 Target genes and PCR

Seven *cdt* genes (*cdt-IA*, *cdt-IIA*, *cdt-III*, *cdt-IVB*, *cdt-VA*, *cdt-VB* and *cdt-VC*) were targeted to detect five different *cdt* alleles (*cdt-I* to *V*) reported in *E. coli*. The PCR primers and sequences for target genes are listed in Table 2.2. PCR was performed in 20 µL reaction mixture, which was prepared according to Section 2.2.4.2 with *cdt*-specific primers pairs. The PCR reaction was performed with an initial denaturation at 94°C for 5 min followed by 30 cycles of the specific program given in Table 2.3 with a final 7-min extension at 72°C.

Table 2.2 List of oligonucleotide primers used in the PCR assay for the detection CDT-associated genes.

Target gene	Primer sequences	Amplimer size (bp)	References
cdt-IA	5'-TGG TGA GAA TCG GAA CTG-3' 5'-CAT TCC ATC AGG TTT GTC-3'	418	(Bielaszewska *et al.*, 2004)
cdt-IIA	5'-AAT CCC TAT CCC TGA ACC-3' 5'-GTT CTA TTG GCT GTG GTG-3'	542	(Bielaszewska *et al.*, 2004)
cdt-III	5'-AAA CAG GAC GGT AAT AAT GAC TAA TA-3' 5'-GTG ATC TCC TTC CAT GAA AAT ATA GT-3'	2230	(Clark *et al.*, 2002)
cdt-IVB	5'-CCTGATGGTTCAGGAGGCTGGTTC-3' 5'-TTG CTC CAG AAT CTA TAC CT-3'	350	(Toth *et al.*, 2003)
cdt-V	5'-AGC ATT AAA TAA AAG CAC GA-3' 5'-ATG GTC ATG CTT TGT TAT AT-3'	2449	(Janka *et al.*, 2003)
cdt-VA	5'-AGC ATT AAA TAA AAG CAC GA-3' 5'-TAC TTG CTG TGG TCT GCT AT-3'	1329	(Janka *et al.*, 2003)
cdt-VB	5'-AGC ACC CGC AGT ATC TTT GA-3' 5'-AGC CTC TTT TAT CGT CTG GA-3'	1363	(Janka *et al.*, 2003)
cdt-VC	5'-GTC AAC GAA CAT TAG ATT AT-3' 5'-ATG GTC ATG CTT TGT TAT AT-3'	748	(Janka *et al.*, 2003)

Table 2.3 PCR conditions for the detection of different *E. coli*-associated *cdt* genes.

Target	PCR conditions (Temperature and time)		
	Denaturing	**Annealing**	**Extension**
cdt-IA	94°C for 30 sec	51°C for 30 sec	72°C for 60 sec
cdt-IIA	94°C for 30 sec	52°C for 60 sec	72°C for 60 sec
cdt-III	94°C for 30 sec	54°C for 60 sec	72°C for 180 sec
cdt-IVB	94°C for 60 sec	55°C for 60 sec	72°C for 60 sec
cdt-V	94°C for 30 sec	51°C for 60 sec	72°C for 180 sec
cdt-VA	94°C for 30 sec	52°C for 60 sec	72°C for 60 sec
cdt-VB	94°C for 30 sec	52°C for 60 sec	72°C for 60 sec
cdt-VC	94°C for 30 sec	49°C for 60 sec	72°C for 60 sec

2.2.5.3 Agarose gel electrophoresis and data analysis

PCR products (10 µL) were evaluated by agarose gel electrophoresis. Upon EtBr staining, DNA bands were observed, photographs were taken and the bands were analyzed according to the Section 2.2.2.2.

2.2.6 *fliC*-sequence genotyping

2.2.6.1 PCR amplification, purification and cycle sequencing

Amplified PCR products from Section 2.2.2.1, randomly selected for sequence genotyping, were purified by GenElute PCR clean-up kit (Sigma, USA) as described in Section 2.2.3.3.2. Cycle sequencing was performed as described in Section 2.3.3.1.3.

2.2.6.2 Sequence Analysis and Phylogeny

The chromatogram sequencing files were edited using Chromas 2.32 software (Technelysium, Queensland, Australia). The homology of the 16s rRNA gene sequences was compared with the genes of other organisms already submitted to the GenBank database (Benson *et al.*, 2005) using blast (Altschul *et al.*, 1997) to identify its closest relatives. Alignments for phylogenetic analysis were made by using clustalX (Thompson *et al.*, 1997). A phylogenetic reconstruction was produced using phylip software (version 3.63) with the Jukes–Cantor distance matrix (Jukes and Cantor, 1996) and the neighbour-joining algorithm (Saitou and Nei, 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1985).

2.3 Isolation and identification of *E. coli*

2.3.1 Preliminary screening

2.3.1.1 Use of selective and differential media

Culture from pre-enrichment and enrichment broths were streaked onto specific differential and selective media for *E. coli* e.g. onto MacConkey agar No.3 (MAC-3), Eosin Methylene Blue agar

(EMB), Xylose Lysine Deoxycholate agar (XLD), Deoxycholate Citrate Agar (DCA), Sorbitol MacConkey Agar and Hektoen Enteric agar (HE). Colony morphology (size, shape, margin, elevation, pigmentation etc.) were carefully observed after 18 h to 24 h (overnight) of incubation. On the basis of colony morphology and characteristics on several differential and selective media, presumptively identified *E. coli* colonies were purified by repeated subculture. Pure cultures were undergone further microscopic and biochemical profiling.

2.3.1.2 Microscopy

Microscopic studies such as cell size, shape, arrangements and Gram reaction were observed in a bright field microscope (Olympus, Japan) using magnification of 100× under oil immersion lens. Motility test was performed by Hanging Drop method and observed in the bright field microscope.

2.3.1.3 Biochemical screening

Pure discrete colonies from NA plates were subjected to oxidase and catalase test. The oxidase-negative with catalase-positive isolates were streaked on Simmon's Citrate Agar to test for citrate utilization capacity. Citrate non-utilizer isolates were then tested in MIU (Motility-Indole-Urease) soft agar (Himedia, India) for the three tests concurrently. After 24 h of incubation, the motility pattern and urease activity of the isolates were observed and recorded. Indole production was observed upon addition of Covac's reagent. Urease-negative isolates were inoculated in butt and slant of Kligler's iron agar (KIA). The characteristic fermentation pattern in KIA and non-urease activity was considered to screen the *E. coli* isolates. Methyl red and Voges-Proskeur tests were also carried out as supplementary tests for confirmation. The compositions of these media and chemicals used are given in Appendix I and Appendix II respectively.

2.3.2 Biochemical Identification

2.3.2.1 Biochemical profiling

For confirmatory biochemical characterization, API 20E kit (Biomeriaux, France) was used. It is a standardized identification system for Enterobacteriaceae and other non-fastidious, Gram negative rods using 21 miniaturized biochemical tests and standard database. The API 20E strip consists of 20 microtubes containing dehydrated substrates. These microtubes were inoculated with freshly grown respective bacterial suspension. Selected pure colonies are streaked on NA. Upon 24 h of growth, discrete colonies were picked up and a suspension of approximately 1×10^7 cfu/mL was prepared in the inoculation fluid. The strips were then inoculated with the suspension and incubated for 18-24 h at 37°C. During incubation, bacterial metabolism induces colour changes that are either spontaneous or revealed by the addition of reagents. The addition of reagents and the interpretation of reactions were done according to the manufacturer's direction. The observation was recorded according to the recommended reading table (supplied with the kit) and the identification was obtained by referring to the analytical profile index or using the identification software (NB: For some isolates, incubation time may need an extension to 48 h, recommended during observation of result through identification software).

2.3.2.2 Identification using the fluorogen MUG

Chromogens and fluorogens, substrates that produce characteristic color and fluorescence respectively upon cleavage by a specific enzyme, have been used for many years to detect and identify coliform bacteria, including the fecal pollution indicator *E. coli* (Manafi *et al.*, 1991). *E. coli* colonies were detected by the fluorescence of 4-melhyl umbelliferone, produced by the cleavage of 4-methylumbelliferyl-β-D glucuronide (MUG) by β-glucuronidase. After inoculation on EC medium with MUG followed by incubation at 37°C and 44.2°C for 24 h, the plates were placed about 6 inch below the long wave UV-lamp for optimal differentiation of fluorescent colonies.

2.3.3 Molecular identification of *E. coli*

2.3.3.1 16S rRNA gene sequencing

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

2.3.3.1.1 PCR primers and conditions

The 16S rRNA gene sequence was selectively PCR-amplified using universal primer pair 5′-GAG TTT GAT CCT GGC TCA G-3′ and 5′-GAA AGG AGG TGA TCC AGC C-3′ (Loffler *et al.*, 2000). The PCR was performed with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 50°C for 30 s and extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min.

2.3.3.1.2 PCR product purification

PCR products were purified using GenElute PCR clean-up kit (Sigma, USA). Detail of PCR product purification protocol has been described in Section 2.2.3.3.2.

2.3.3.1.3 Cycle sequencing

In cycle sequencing, the ABI-prism BigDye Terminator cycle sequencing ready reaction kit was used in which the following reagents were premixed into a single tube of ready reaction mix of BigDye terminator: deoxynucleotide triphosphates, ampliTaq DNA Polymerase, $MgCl_2$ and Buffer. In this step, only one strand of the desired DNA was amplified and DNA fragments of different length were produced by termination with different dideoxynucleotide triphosohates (ddNTPs). The complete reaction mixture composition for cycle sequencing is shown in Appendix IV. The thermal cycle program initiated with initial denaturation at 96°C for 1 min, followed by 25 cycles of the following steps: denaturation at 96°C for 10 s, annealing at 50°C for 30 s followed by extension at 60°C for 4 min, a single final extension was done at 60°C for 10 min. The products were purified by ethanol precipitation to remove the unincorporated dye. The purification procedure was as follows: 2 µL 3M Na-acetate (pH 4.6) and 50 µL of 96% ethanol was added to each of the tubes containing cycle sequenced product precipitation and centrifugation was done at 13,000 rpm for 20 min. The

supernatant was carefully aspirated to discard and precipitate was washed by addition of 200 µL of 70% ethanol and centrifugation at 14,000 rpm for 10 min. The supernatant was carefully aspirated and discarded again. The tubes were subjected to air-drying and 16 µL of template suppressor reagent (hi-diformamide) was added to each pellet. The whole content was then transferred to a sequencing tube which was placed in a thermal cycler at 95°C for 3 min for denaturation and analyzed by electrophoresis in an ABI-prism 310 genetic analyzer. DNA was separated through the POP6 contained in a capillary tube and detected by laser beam (Manual, ABI Prism USA).

2.3.3.1.4 Sequence Analysis

The chromatogram sequencing files were edited using Chromas 2.32 software (Technelysium, Queensland, Australia). The homology of the 16s rRNA gene sequences was compared with the genes of other organisms already submitted to the GenBank database (Benson *et al.*, 2005) using blast (Altschul *et al.*, 1997) to identify its closest relatives.

2.4. Clonal identification by genomic fingerprinting

2.4.1. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphisms of specified genes or total genomic DNA have long been used to identify the intra- and inter-species clonal relationship and variation.

2.4.1.1. rRNA gene RFLP fingerprinting

The value of rRNA gene RFLP analysis as a tool for species identification was evaluated over the past decades, the establishment of classification scheme based on 16S rRNA restriction fragment length polymorphism (RFLP) patterns has enabled the accurate and reliable identification and classification of a wide range of bacteria (Aakra *et al.*, 1999; Wei *et al.*, 2007).

2.4.1.1.1 PCR amplification of 16S rRNA gene

Extraction and purification of chromosomal DNA of the test isolates was carried out and used as template for the PCR of 16S rRNA gene. The 16S rRNA gene sequence was PCR-amplified. The primer pair used and PCR condition applied have been described in Section 2.3.3.1.1. The concentration of PCR product was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA).

2.4.1.1.2 Restriction digestion

Digestion of the amplified product with restriction endonuclease HhaI and electrophoresis for fragment separation were performed following the protocol of Machado (2000). Reaction mixture was prepared adding 2.5 µL of 10× NE buffer, 2.5 µL of Hha1 enzyme (20,000 U/ml) and 0.25 µL of purified BSA in 20 µL of PCR product (15ng/µL). The mixture was incubated at 37°C for 4 h in an water bath.

2.4.1.1.3 Electrophoresis and band pattern analysis

Digested product was separated by 2% agarose gel electrophoresis. Gels were stained with ethidium bromide, and images of band patterns were electronically captured using a transilluminator. Tagged image file format (TIFF) images were exported to Gel analysis software (developed by Svein

Norman at the Dept. of Biology, University of Bergen, Norway) and cluster analyses were done to make the Dendrogram according to the band pattern.

2.4.1.2. *fliC* RFLP fingerprinting

The *fliC* gene has been successfully used for PCR/RFLP and sequence genotyping of both motile and nonmotile strains might be advantageous over classical H-serotyping and a useful for epidemiological investigations and studies of the emergence.

2.4.1.2.1 PCR amplification of *fliC*

Extraction and purification of chromosomal DNA of the test isolates was carried out and used as template for the PCR of *fliC* gene. The amplification of flagellin genes by PCR was done as described in Section 2.2.2.1 and concentration of PCR product was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA).

2.4.1.2.2 Restriction digestion, electrophoresis and band pattern analysis

Digestion of the amplified product with endonuclease Hhal and electrophoresis for fragment separation were performed as described in Section 2.4.1.2.2. Digested product undergone electrophoretic separation, staining and band pattern analysis as described in Section 2.4.1.2.3.

2.4.2 Rep-PCR genomic fingerprinting for clonal identity

2.4.2.1 Oligonucleotide primers

The pair of 18-mer inosine containing primers was used for Rep-PCR (Versalovic *et al.*, 1991): 5'- I I I-ICG-ICG-ICG-ICA-TCI-GGC-3' and 5'- ICG-ICT-TAT-CIG-GCC-TAC-3'.

2.4.2.2 PCR conditions

Amplification was performed in 30 µL reaction volume for each specimen containing 1 µL of template DNA extracted from the isolates. The PCR condition in the thermocycler was as follow: a 5-min denaturing at 95°C followed by 35 cycles of 4 step pannel having "denaturing at 94°C for 30 s, denaturing at 92°C for 30 s, annealing at 30°C for 1 min and extension at 72°C for 8 min" with a final extension at 72°C for 20 min.

2.4.2.3 Observation

Twenty µL of each PCR product was observed upon electrophoresis in 1% agarose gel followed by Et-Br staining under UV illuminator (Gel Doc, BioRad, USA). Photographs were taken using Gel Doc and bands were analyzed with 'Quantity One' software (BioRad, USA).

2.5 Isolation and characterization of pathogenic *E. coli*

2.5.1 Colony blot and dot blot hybridization with DIG-labeled DNA probe for preliminary screening of diarrheagenic environmental *E. coli* isolates

2.5.1.1 DIG-labeling of probe DNA

Multiplex PCR amplification was performed targeting nine different virulence-associated genes for screening potent virulent isolates in the environment. The PCR primers and conditions have given in Section 2.2.4. The bacterial strains used as templates for the multiplex PCR are enlisted in Appendix IV. PCR products were purified using GenElute PCR clean-up kit (Sigma, USA) as described in Section 2.3.3.1. PCR products were undergone DIG-labeling according to the Section 2.2.3.3 and labeling efficiency was determined.

2.5.1.2 Colony blotting, prehybridization and hybridization

A nitrocellulose membrane was placed carefully on the NA agar plate with colonies to be blotted and removed upon complete soaking. The membrane was then placed on a fresh NA plate and incubated for 3 h at 37°C. The colony of positive control was marked properly on the membrane. Upon incubation, the membrane was removed from the NA plate and placed on a Whatman filter paper soaked with 5 mL of denaturing solution. After 10 min, the filter paper was placed on another Whatman filter saturated with neutralization solution and kept on it for 3 min. The filter was then removed and air-dried. The membrane was then dipped in prehybridization buffer and incubated at hybridization temperature for 1 h. The membrane was then rubbed with non-absorbent cotton bud to remove cell debris with soft hand. The membrane was then placed in a roller bottle containing 6 mL of hybridization buffer containing 6 µL of labeled denatured (by boiling for 5 min and immediate chilling in ice) probe and allowed hybridization overnight at hybridization temperature (T_{opt}). Subsequent washing, developing and detection procedure was followed as described in Section 2.2.3.5.

2.5.2 Identification of virulent genes by multiplex PCR method

Identification of virulent *E. coli* isolates was performed using multiplex PCR assay targeting nine virulence-associated genes. The PCR primers and conditions have been described in Section 2.2.4. One µL of DNA extracted from the isolates was used as template.

2.5.3 Identification of adhesion genes

DNA extracted from the isolates were subjected to PCR assay for the detection of genes for type 1 fimbriae, *pap*, *afa*, *csg*A, *crl* and *sfa* specific for adhesion and virulence of *E. coli*.

2.5.3.1 PCR reaction mixture and primers

Amplification was performed in 30µL reaction volumes for each specimen containing 1 µL of template DNA, 0.3 µL of 2 U/µL DyNAzyme™ DNA polymerase, 3 µL of 10× buffer for DyNAzyme, 0.6 µL of a mixture of deoxynucleoside triphosphates (25 mM of each), and 0.6 µL of 25 µM solution of each primer (Sigma-Aldrich, Germany). Chilling temperature was maintained throughout the preparation procedure. The PCR tubes were then transferred to a DNA thermal cycler (BioRad, Mexico) for amplification. Primers used for each of the genes are given in the Table 2.4.

Table 2.4 List of primers used for detection of adhesion genes

Target genes	Primer Sequences	References
type 1	F: 5'- CGA CGC ATC TTC CTC ATT CTT C-3' R: 5'- ATT GGT TCC GTT ATT CAG GGT T-3'	(Johnson and Stell, 2000)
papC	F: 5'- GAC GGC TGT ACT GCA GGG TGT G-3' R: 5'- ATA TCC TTT CTG CAG GGA TGC A-3'	(Johnson and Stell, 2000)
Afa	F: 5'- GCT GGG CAG CAA ACT GAT AAC T-3' R: 5'- CAT CAA GCT GTT TGT TCG TCC G-3'	(Johnson and Stell, 2000)
*csg*A	F: 5'- ACT CTG ACT TGA CTA TTA CC -3' R: 5'- AGA TGC AGT CTG GTC AAC-3'	(Maurer *et al.*, 1998)
Sfa	F: 5'- CTC CGG AGA ACT GGG TGC ATC T-3' R: 5'- CGG AGG AGT AAT TAC AAA CCT G-3'	(Yamamoto *et al.*, 1995)
Crl	F: 5'- TTT CGA TTG TCT GGC TGT ATG-3' R: 5'- CTT CAG ATT CAG CGT CGT C-3'	(Maurer *et al.*, 1998)

2.5.3.2 PCR conditions

All the PCR tubes were heated at 94 °C for 3 min in the thermal cycler to ensure the denaturation of all DNA templates. The PCR reaction was then continued with the conditions stated in Table 2.5. Thirty cycles of these segments were repeated with a final extension of 10 min at 72 °C.

Table 2.5 PCR conditions for the detection of genes associated with adhesion of *E. coli*

Target gene	PCR conditions (time and temperature)			Amplified product size (bp)
	Denaturation	Anneal	Extension	
papC	94 °C for 1 min	65 °C for 1 min	72 °C for 1 min	328
afa	94 °C for 1 min	65 °C for 1 min	72 °C for 1 min	750
Type 1	94 °C for 2 min	65 °C for 1 min	72 °C for 1min	700-750
*csg*A	94 °C for 30sec	60 °C for 30sec	72 °C for 30 sec	250
sfa	94 °C for 2min	65 °C for 1 min	72 °C for 1min	410
crl	95 °C for 45 sec	60 °C for 45 sec	72 °C for 45 sec	250

2.5.3.3 Electrophoresis and evaluation

PCR products (10 µL) were evaluated by electrophoresis through a 1.5% (w/v) agarose gel at 70 V for 30 min. DNA bands were visualized and photographed under a UV illuminator (Bio-Rad, USA) after the gel was stained with ethidium bromide.

2.6 Plasmid profile analysis

2.6.1 Increasing of plasmid copy number

Increasing the copy number of plasmid per cell was induced by suppressing protein synthesis mechanism by addition of chloramphenicol in fresh culture of *E. coli* isolates. Inhibiting protein

synthesis usually inhibits the chromosomal replication and hence allowing the auto-replicating plasmid to increase their copy per cell. Overnight bacterial cultures grown in LB broth were centrifuged to harvest the cells and re-suspended in fresh LB broth and chloramphenicol solution was added to the final concentration 125 µg/mL. The cells were incubated again at 37°C overnight.

2.6.2 Extraction of Plasmid DNA

Plasmid DNA extraction from the test isolates was carried out according to the procedure described by Kado and Liu (1981). Overnight culture of LB broth with chloramphenicol was centrifuged at 12,000 ×g for 7 min and pellets were resuspended in 100 µL of autoclaved plasmid extraction solution I (composition is given in Appendix-II) and kept at room temperature for 5 min. One hundred and fifty µL of freshly prepared solution II (Appendix-II) was then added, mixed gently and kept in ice for 10 min. This was followed by addition of 150 µL of solution III (Appendix-II), mixed gently and kept in ice for 5 min. The suspension was centrifuged at 12,000 ×g for 5 min and 300 µL of supernatant was collected. Double volume of ice-cold ethanol (95%) was added to the supernatant, mixed well and kept at room temperature for 10 min. Following centrifugation at 12,000 ×g for 5 min, the pellet was taken out and washed with 1 mL of 70% ethanol. Finally, the collected pellet was dried and resuspended in 50 µL TE buffer and kept at 4°C for 24 h to dissolved the pellet and was stored at -20°C.

2.6.3 Observation of Plasmid

Extracted plasmids were observed by electrophoresis through a 0.8% (w/v) agarose gel at 70 V for 60 min. Upon ethidiumbromide staining, DNA bands were observed on UV transilluminator (Gel Doc, BioRad, USA). Photographs were taken using Gel Doc (BioRad, USA) machine attached to a computer and bands were analyzed with 'Quantity One' software (BioRad, USA). The size of the plasmid was calculated observing relative mobility of the plasmid bands in agarose gel compared to supercoiled DNA ladder (Invitrogen).

2.7 Survival study of *E. coli* in laboratory microcosm/mesocosm

2.7.1 Survival study of *E. coli* in sterile natural water microcosm

2.7.1.1 Set up of a microcosm

Both freshwater and seawater microcosm were set up. Freshwater was collected from a pond (Curzon Hall, University of Dhaka) in Dhaka and seawater from the sea beach at Kolatoli point of Cox's Bazar. Water samples were filtered through 0.45 µm membrane filters (Millipore, USA) to remove sand, clay particles and other debris. Fifty mL aliquots of water sample were taken per 100 mL laboratory bottles (Duran, Germany) and autoclaved. The setup was incubated at 25°C with 80 rpm in an orbital shaker. The physicochemical parameters measured in the microcosm water before autoclaving are listed in Table 2.6.

Table 2.6 Physicochemical parameters of water in the microcosm

Microcosm	Total dissolved solids (mg/L)	Conductivity (µS per cm)	Salinity (‰)	pH	Temperature
Seawater	25100	50100	32.8	7.78	28°C
Freshwater	231	462	0.2	8.29	28°C

2.7.1.2 Inoculation

One or two colonies of test *E. coli* isolates from NA plates were inoculated in 10 mL of nutrient broth and were incubated at 37 °C for 24 h. One mL of culture was harvested by centrifugation at 12,000 ×g for 5 min. The pellet was washed twice, once with normal saline and once with respective autoclaved microcosm water, and was resuspended in 100 µL of respective autoclaved microcosm water. Fifty µL of the bacterial suspension was added into 50 mL-microcosm. The inoculum number (cfu/mL) was determined by drop plate method following 10-fold serial dilution of the inoculated microcosm.

2.7.1.3 Assay of survival

Hundred µL of water from inoculated microcosms were sampled from freshwater microcosms and seawater microcosms up to 53 d. Survivability of the isolates was measured by identifying the cfu/mL in the samples by drop plate method following 10-fold serial dilution.

2.7.2 Survival study of gfp-marked *E. coli* in natural water mesocosm

2.7.2.1 Set up of the mesocosm

Untreated freshwater mesocosms were set up using water from a pond in Dhaka. Each of the mesocosms was built in a round glass jar containing 10 L of untreated water, approximately 100 g of sediment (collected from the same pond), a few floating water hyacinths and 6 fishes of *heteropneustes fossilis* species. The setup was kept under shade and temperature recorded during the experiment was between 25 to 31 °C. The physicochemical parameters measured in the mesocosm were: 145.7 mg/L of total dissolved solids, 292 µS per cm of conductivity, 0.01 ‰ of salinity and pH 6.9.

2.7.2.2 Transforming test isolates with pGFP by electroporation

Electroporation provides a rapid and simple method for transforming cells for most bacterial species. Test isolates were transformed with pGFP, which contained genes for green fluorescent protein coding and expression to assist visual identification of the colony under UV and ampicillin resistant marker to assist selection of transformed cells on ampicillin containing media. All the recipe of media and buffer used in this experiment are listed in Appendix I and II respectively.

2.7.2.2.1 Preparation of electrocompetent cells

A pure and discrete colony of the test isolate was inoculated in 5 mL of L-broth and incubated for overnight. The culture was then transferred to 200 mL of fresh L-broth and incubated at 37 °C with constant rotation at 300 rpm in orbital shaker to achieve an OD_{600} of 0.4-0.5 to ensure actively growing cells in log phage. The cells were chilled in ice for 30 min and harvested by centrifugation at 4 °C. The cell pellet was gently resuspended and washed four times by ice-cold 10% glycerol. The competent cells can be stored at -70 °C for further use.

2.7.2.2.2 Electroporation

The cells and the electroporation cuvette (supplied with Gene Pulser Xcell Electroporation system, BioRad, USA) were kept in ice to chill. Forty µL of cell suspension (1-3 $\times 10^{10}$ cells/mL) were mixed

with 2.0 µL of pGFP DNA and transferred into the cuvette. From the home screen on the Gene Pulser, pre-set protocols screen was opened and choose the bacterial protocol option and selected the protocol for the *E. coli* to pulse at 2.5 kV. Cuvette was placed in the pulser slot and pulsed once. As soon as the pulse completed, the cuvette was removed and 1 mL of SOC medium was added to the cuvette and mixed well to suspend the cells. The cell suspension was pipetted out into a collection tube and incubated at 37 °C for 1 h with shaking at 200 rpm. Upon incubation, the cell suspension was spread on LB-agar plates containing 5 µL/mL ampicillin. Successful transformed cells formed colonies, which glowed under UV with bright green fluorescence.

2.7.2.3 Inoculation

One or two colonies of test *E. coli* isolates from NA plates were inoculated in nutrient broth and were incubated at 37 °C for 24 h. One mL of culture was harvested by centrifugation at 12,000 rpm for 5 min. The pellet was washed once with normal saline and resuspended in 1 mL of autoclaved pond water and added into each microcosm. The inoculum number (cfu/mL) was determined by drop plate method following 10 fold serial dilution of the inoculated microcosm on ampicillin containing LB agar and observed under UV.

2.7.2.4 Assay of survival

Hundred µL of water from each inoculated microcosms were sampled each consecutive day for 1 week. Approximately 1 g of sediment, superficial film on water, rhizoid of water hyacinths was also sampled along with water. Fishes of microcosms were sacrificed and samples were collected from skin, gill and intestine of each fish. Survivability of the isolates was measured by identifying the cfu/mL of GFP-marked bacteria in the samples by drop plate method following 10-fold serial dilutions on ampicillin-LB plate and observed under UV.

2.7.2.5 Determination of adhesive property of inoculated *E. coli* cells

2.7.2.5.1 Measurement of hydrophobicity

Hydrophobicity of the bacterial suspension was measured, according to the method described by Klitzke and Lang (2007) with slight modification, as a partitioning coefficient between the aqueous suspension (bacterial suspension in deionized water having conductivity 0.47 µS/cm at 28°C) and a solid hydrophobic phase (silica gel, nontoxic adsorbant). 0.5 g of silica gel was added to 5 mL of each bacterial suspension and shaken in orbital shaker for 1 h at 80 rpm at 28°C to allow the hydrophobic cells to adsorb onto the solids. Afterwards, the suspensions were pipette out of the gel and measured the turbidity (T2) of the suspension.

Hydrophobicity (H) was calculated according to the following formula:

$$H = (T1 - T2)/T1,$$

(T1 and T2 are the turbidity measured before and treatment of bacterial suspension with silica gel).

2.7.2.5.2 Quantitative Biofilm assay

Quantitative biofilm assay was measured with slight modification of the method used by Wakimoto *et. al.* (2004). Overnight bacterial culture in LB broth was centrifuged, washed and resuspended in autoclaved lake water having optical density of approximately 0.6 at 600nm. A 96-well round-

bottom polystyrene microtiter plate was inoculated with 200 µL of the bacterial suspension in each well. The microtiter plate was incubated at 28°C for 30 min, 2 h, 8 h, 24 h, 48 h and 72 h without shaking and then washed with the autoclaved water. The plate was stained with 200 µL of 0.5% crystal violet in each well for 30 min followed by washing. The biofilm was quantified after addition of 200 µL of 95% ethanol in each well by an enzyme-linked immunosorbent assay plate reader at 630 nm.

2.7.3 Determination of growth rate

2.7.3.1 Preparation of growth media

Growth curve experiment was performed to measure and compare the generation time of environmental and clinical *E. coli* isolates in both marine and fresh water with added nutrients. The growth medium was prepared by using the autoclaved marine and fresh water having salinity of 3.28% and 0.02%, respectively. As nutrient supplement, 1% peptone and 0.5% yeast extract was added. In both the media, pH was adjusted to 7.0.

2.7.3.2 Inoculation

One or two colonies of test *E. coli* isolates from NA plates were resuspended in 100 µL of normal saline and added to 100 mL of corresponding growth media. The flasks were incubated at 37°C for 24 h. The bacterial number of inocula and growing cultures (cfu/mL) was determined by drop plate method following 10-fold serial dilution.

2.7.3.4 Estimation of growth rate and doubling time

One mL of each growing culture was sampled in every 2 h interval for 24 h. The number of cells per sample was determined by drop plate method. Numbers of bacterial cells (cfu/ml) at different time interval were plotted. For the constant growth period, in log phage, the plotted dots aligned on a straight line, which slope identified the growth rate. For a constant growth rate of *r%*, the formula for the doubling time T_d was calculated by the equation $T_d = \log(2)/\log(1+r/100) \approx 70/r$

2.8 Immunochemical methods

2.8.1 Slide agglutination

A number of *E. coli* strains have already been reported to cross-react with *Shigella* spp. (Lefebvre *et al.*, 1995). Environmental *E. coli* isolates were screened for their cross-reactivity to *Shigella*-specific antisera (Denka Seiken, Japan) by slide agglutination. The isolates were screened by type-specific polyvalent anti-*Shigella* antisera (A to D) and then grouped by corresponding monovalent antisera. The test was carried out on glass slides: a homogenous suspension of cells in normal saline was dropped at the centre of the slide and then a drop of test antiserum was added and mixed well by tilting back and forth for several times observing the agglutination against a light background. The cells itself clumping in the saline suspension were rejected for the test and termed in practical as auto-agglutinating strains.

2.8.2 Extraction of protein for immunochemical study

Bacterial cells were harvested from overnight grown pure culture and washed in autoclaved PBS by centrifugation at 12000 ×g for 10 min at 4 °C. Cells were resuspended in lysis buffer followed by boiling in water bath for 5 to 10 min. Equal volume of deionized water was added to the suspension and was boiled for an additional 10 min. The lysed sample was centrifuged at 12000 ×g for 10 min. Supernatant containing the soluble whole cell protein extract was pipette out and stored at -30°C until further use.

2.8.3 Extraction of lipopolysaccharide (LPS)

Lipopolysaccharide, traditionally named as somatic antigen or O-antigen, forms an integral part of the outer membrane of Gram-negative organisms. For many-Gram negative bacteria, the structure of lipid A and core LPS are highly conserved. However, considerable heterogeneity can exist in the sugar composition of the subunits that comprise the long chain moiety of LPS, and the number of repeating subunits per chain. This variation in LPS structure forms the basis for the various chemotypes. LPS is usually extracted to define the chemotype of isolates and for detection of immunochemical properties.

2.8.3.1 Chemical lysis method

LPS was extracted following method described by Henrik Chart and Griffiths (1985). Cells were harvested from overnight bacterial culture in Nutrient Broth in a pre-weight microcentrifuge tubes and centrifuged at 12,000 ×g for 5 min. After measuring the weight of the harvested pellet, SDS-PAGE solubilization buffer was added with an amount of 30 µL per mg of culture and dissolved by vortex mixing followed by incubation in boiling water bath for 10 min. Similar amount of the buffer containing proteinase K (100 µg per 30 µL buffer) was added and mixed to lyse the protein and peptides. Upon incubation at 60°C in water bath for 2 h, the LPS solution was stored at -30°C until further use.

2.8.4 SDS-PAGE analysis

PAGE analysis has been used in the study of protein and lipopolysaccharide biochemistry at molecular level. The most popular one is the discontinuous SDS-PAGE, first described by Laemmli (1970). We have used the technique described by Menelaos Costeas (1995), which broadly based on the method of Laemmli but has been significantly modified. To determine the protein and LPS profiles, SDS-PAGE analysis was done followed by comassic blue and silver staining respectively and for detection of immunogenicity, Western blot was performed upon SDS-PAGE. A vertical slab electrophoresis unit (Bio-Rad, USA) was used with a reliable power pack (Bio-Rad, USA) for running the gel.

2.8.4.1 Preparation of polyacrylamide gel

Clean and fresh glass plates were assembled in gel casting chamber to make a cassette. A thin film of soft white paraffin was applied on the spacer to ensure sealing. A 12.5% separating gel mixture was prepared and was poured to a level of three centimeter below the upper edge of glass plates and allowed to solidify. The gel was carefully overlaid by 80% ethanol to avoid gel drying and to ensure a horizontal sharp edge of the separating gel. The polymerization process was faster under UV and

high ambient temperature. After the polymerization of the separating gel, observed by appearance of a sharp line between gel and ethanol phase, a 4.5% stacking gel mixture was freshly prepared. The ethanol was drained out and stacking gel mixture was rapidly poured above the previously constructed separating gel. A 10-toothed comb was then pressed carefully between the two glass plates so that no bubbles can form inside the comb channels. The gel was then allowed to settle undisturbed for 1 h to complete polymerization. The comb was then removed and the glass chamber was fixed in the electrophoresis unit. The whole unit was then placed gently in the electrophoresis tank and flooded by electrophoresis buffer. Twenty µL of each samples was added to wells. Ten µL of protein marker was also loaded in a well to assist the detection of molecular weight of the peptides in the sample. After loading, the electrophoresis unit was connected with the power pack adjusting at 20 mA current. As soon as the tracking dye reached the bottom level of the gel, the power supply was turned off.

2.8.4.2 Coomassic Brilliant blue staining for protein and peptides

Upon SDS-PAGE run, the gel was released from the glass plates and immersed in 0.1% Coomassic Brilliant blue (R 250) staining solution. The gel was kept for approximately 1 h shaken on a rotator shaker (Memmert, USA). The gel was then taken out of the staining solution and flooded with destaining solution (10% Acetic acid), which was placed on a shaker for overnight. When the gel background became transparent, it was taken out of the destaining solution and immersed in distilled water.

2.8.4.3 Silver staining for lipopolysaccharide

The polyacrylamide gel loaded with extracted LPS was run as described in Section 2.8.4.1. Upon electrophoresis, the gel was removed from the cassette and placed in a clean plastic box containing approximately 200 mL fixing solution; the lid was applied and mixed gently on a shaker for at least 2 h. The gel can be left in fixing solution for overnight if necessary. After fixation step, the fixing solution was poured off and, 100 mL of oxidizing solution was added and mixed for 5 min on a rotator. After removing the oxidizing solution, the gel was washed with approximately 200 mL deionized water thrice each for 15 min. During the last washing step, silver staining solution was made as mentioned in the Appendix II and poured into another clean box. The gel was then carefully placed into the staining solution; the lid of the box was put on and mixed for 10 min. After discarding the staining solution, the gel was washed for 3×10 min in deionized water. During the last washing step, developing solution was prepared and 200 mL of the solution was placed into another clean plastic box. The gel was then transferred to developing solution and mixed by gentle shaking. The gel started to get stained after about 5 min. When a desired intensity appeared, the staining reaction was stopped with 10% acetic acid solution and finally was washed with deionized water. The whole procedure was handled wearing gloves and taking precautions to reduce the background staining of the gel.

2.8.5 Western Blot Analysis

Western blot technique (Towbin *et al.*, 1979) was applied to check the immunological response and the cross-reactivity of both the immunogenic proteins and LPS of the sero-cross reactive environmental *E. coli* isolates.

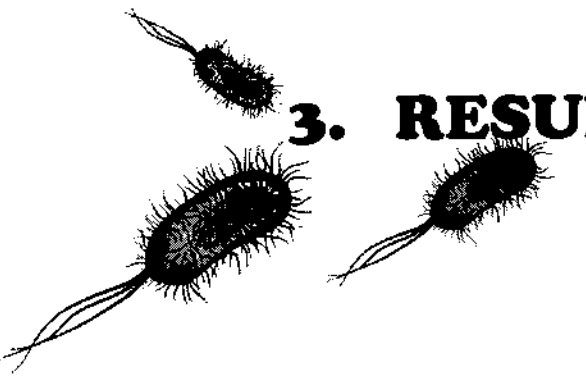
2.8.5.1 Transfer of protein to nitrocellulose membrane:

The polyacrylamide gel was prepared, immunogenic component (protein and LPS) was loaded and undergone electrophoresis according to the procedure described in Section 2.8.4. Upon electrophoresis, the gel was rinsed in transfer buffer for a few min. Each component of transferring unit was soaked in the transfer buffer before assembly. The sandwich components were assembled with the gel following the instruction guide. The cassette was then placed in the proper orientation and the tank was filled with the transfer buffer to a level that covered the electrode panels. The power supply was then connected with the power pack adjusted at a constant volt of 12 and kept for overnight transfer.

2.8.5.2 Detection of immunogenic protein/LPS:

After overnight transfer, the nitrocellulose membrane was removed from the cassette. The whole membrane was then cut into strips through lanes and dipped in blocking solution (2% skim milk) in a clean box. The strips were incubated in the blocking solution for 1.5 h at 37 °C on a rotary shaker. Strip of marker was kept undisturbed for the calibration of molecular weight of immunogenic proteins/LPS. After the blocking, strips were washed with PBS, PBS-Tween and PBS consecutively for 5 min each. The strips were then placed in a new box containing blocking solution (2% skim milk) having primary antibody (polyclonal or monoclonal antibody as required, Denka Seiken, Japan) at a concentration of 1: 200 and incubated at 37 °C for 1.5 h in rotary shaker. The membrane strips were washed again like before and then were transferred to a solution of secondary antibody at a concentration of 1: 2000 in 2% skim milk (Difco, USA) followed by incubation at 37 °C for 1 h in a rotary shaker.

For polyvalent primary antisera, alkaline phosphatase (AP) conjugated secondary antiserum (anti pig IgG developed in rabbit) and for monovalent primary antiserum, horse reddish peroxidase (HRP) conjugated secondary antiserum (anti rabbit IgG, developed in goat, SIGMA, USA) were used. After incubation, the membrane strips were washed once more at the same way it was previously done. Substrate was prepared for AP by dissolving 33 µL of NBT and 16.5 µL of BCIP dye in 5 mL of AP buffer (Appendix II). For HRP, substrate was prepared by dissolving 1 mg of 3,3′-Diaminobenzidine (DAB, Sigma) in 2 mL citrate buffer (pH 5.2) and then H_2O_2 was added to it at concentration of 3 µL/mL. The strips were placed in a clean container and overlaid by respective substrate buffer preparation. When protein/LPS bands appeared in desired intensity, membranes were washed several times in the deionized water to stop the reaction. The membrane strips were then air dried and stored away from light.



3. RESULTS

3.1 Temporal changes in water quality in aquatic environment of Bangladesh

Water samples were collected from 46 different natural aquatic locations, 35 freshwater and 11 marine/brackish sites, in Bangladesh in three successive seasons covering a large part of the country. Sampling sites were chosen to cover major rivers and sea beaches in sites of greater human activity, economic importance and tourist interest. The respective sampling sites are shown in Fig. S3.1. Physico-chemical parameters, e.g. temperature, salinity, conductivity and total dissolved solids of the water samples were measured and GPS coordinates recorded. All the parameters were measured on site, and are listed in Table S3.1 (Appendix III).

Temporal variations in the physicochemical parameters of the water bodies were observed. Water temperature varied between seasons. In winter, it ranged between 16°C and 25°C. It minutely differed between summer and rainy season and ranged between 26°C and 32°C. The salinity of water samples changed unexpectedly with seasons in both fresh and marine sampling sites in Bangladesh. In summer, the marine water samples were most saline (~37 ‰) followed by winter (~34 ‰). In rainy seasons, the salinity decreased due to rain water runoff. Out of 11 marine sites, drastic fall in salinity was observed in six sites in rainy season while the rest five sites were rather stable. Out of 35 freshwater sites, 28 sites were more or less stable in their salinity between seasons. The rest seven sites were found to have significantly higher salinity in the summer. Conductivity and TDS (total dissolved solids) were proportional to the salinity of the water (Table S3.1). The salinity of freshwater samples (rivers) distant from the sea rarely varied with seasons. However, the salinity of rivers closer to the sea increased in the summer. Because of the lack of rain and blockage of the river flow (Farakka dam and Tista barrage) from the source, the water level decreased in rivers during the summer, allowing seawater to enter. The salinity varied with the distance from the sea. In the rainy season, the situation was inversed because of considerable water both from monsoon rainfall and the opening of the river barrages. Conductivity and TDS were found to be proportional to the salinity of the water.

3.2 Detection of *E. coli* in the natural water systems using *fliC*

fliC PCR was performed to trace *E. coli* in all the 46 water samples collected in this study. Four different DNA templates from direct water filtrate (P), pre-enrichment culture (PE), bile salt-enrichment culture (BSE) and streptomycin-enrichment culture (SE), of each water samples were analyzed to detect the presence of *E. coli*-flagellin encoding gene. The primer pair used in the study had successfully amplified *fliC* gene of *E. coli* along with other related bacteria. Most of the water samples gave rise to multiple *fliC*-PCR products (Fig. 3.1). Southern blot hybridization was performed to selectively identify *fliC* belong to *E. coli* in the water samples with DIG-labeled probe synthesized from *fliC*-PCR product amplified from clinical O157:H7 *E. coli* strain (Fig. 3.2).

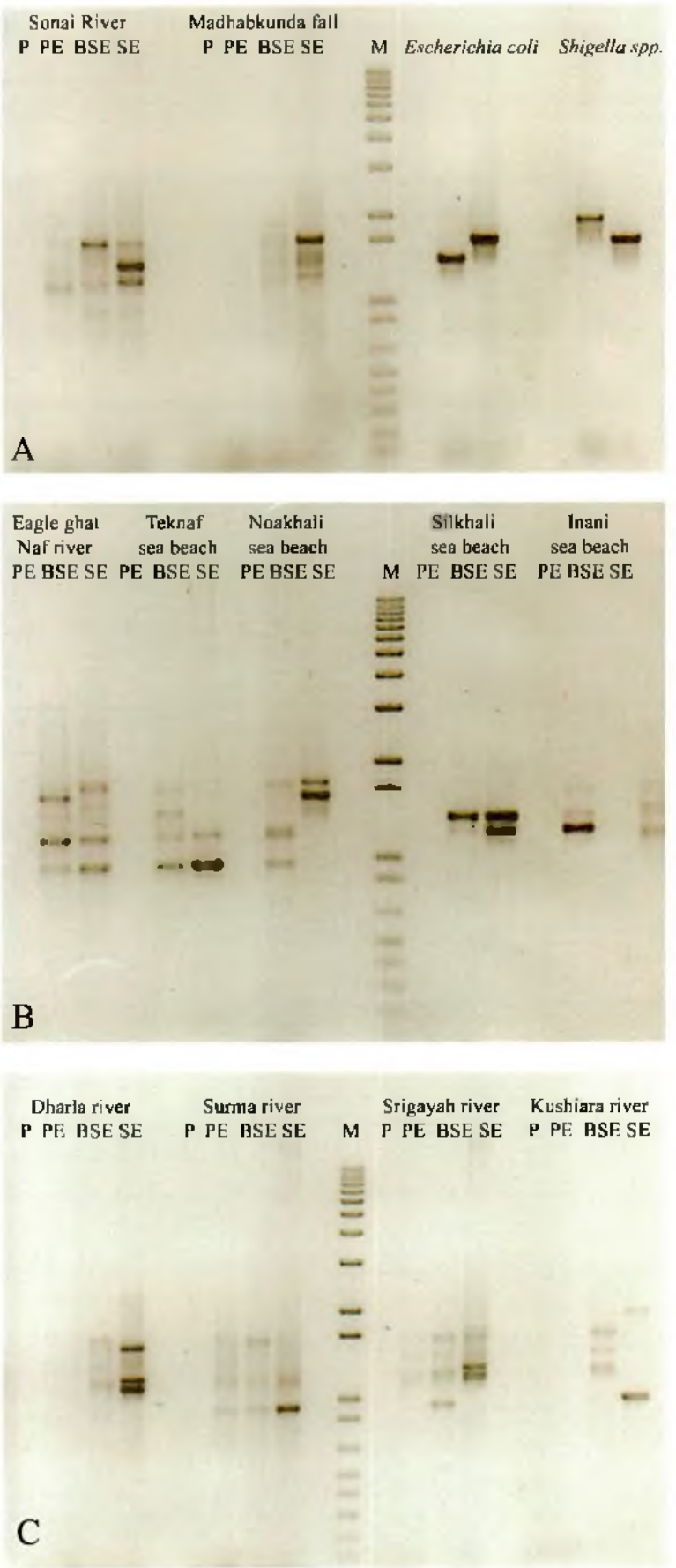


Fig. 3.1 ***fliC*-PCR analysis of DNA extracted from water filtrate (P), pre-enrichment culture (PE), bile salt enrichment culture (BSE) and streptomycin enrichment culture (SE) of each water samples; in winter (A), in summer (B) and in the rainy season (C). Two *E. coli* strains (an environmental isolate, S-13 and a clinical O157:H7 strain) and two *Shigella* spp., i.e. *Shigella dysenteriae* type 4 and *Shigella boydii* 15 (ATCC 12034) were also amplified as positive controls (A).**

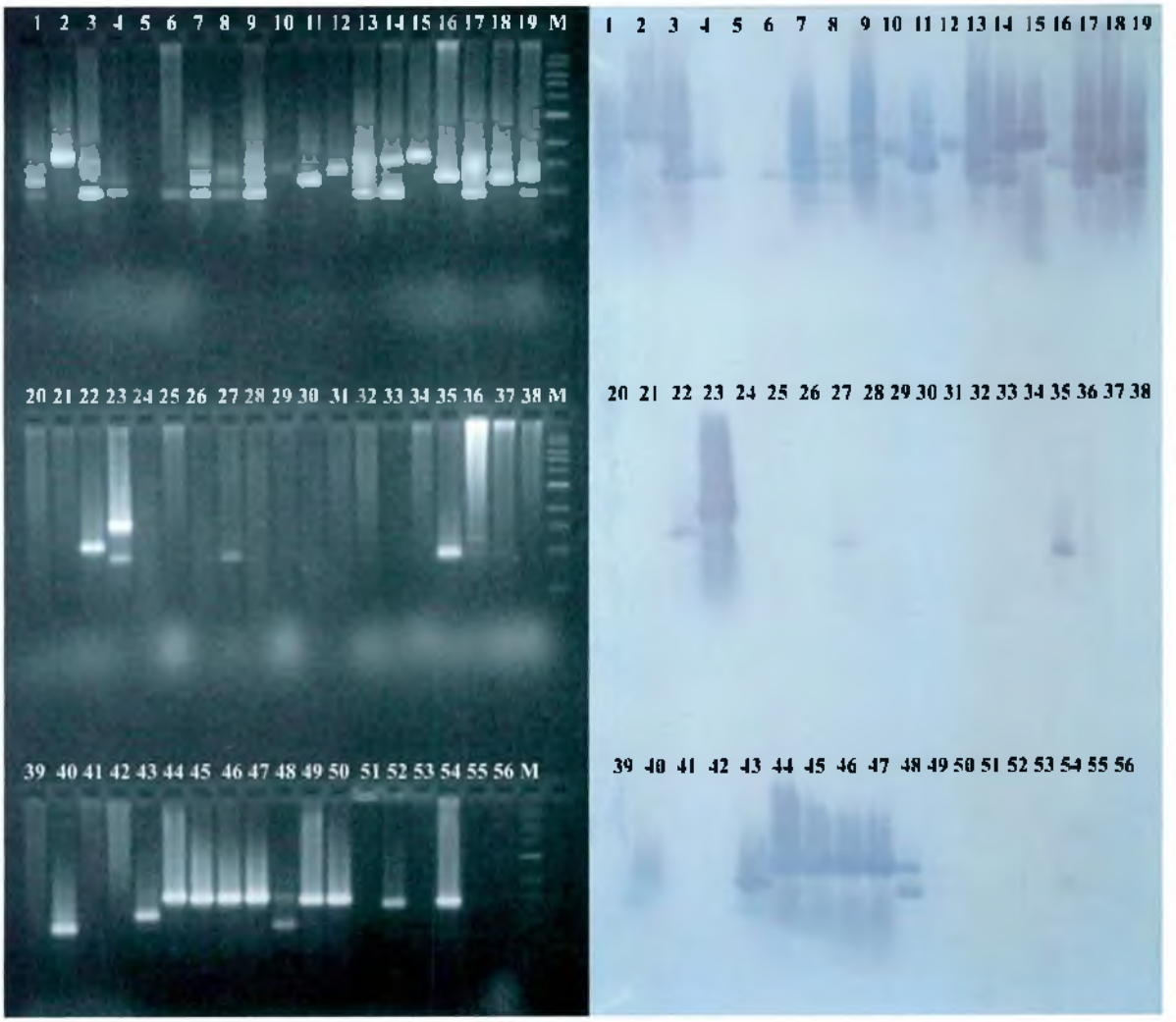


Fig. 3.2 ***fliC*-PCR analysis (left) and corresponding Southern blot hybridization with DIG labelled *E. coli*-specific *fliC* probe (right). Lanes 1 through 42, *fliC*-PCR products amplified in the DNA extracted from pre-enrichment culture (PE), bile salt enrichment culture (BSE) and streptomycin enrichment culture (SE) of different water samples (randomly selected); lane 43, *E. coli* O157:H7 as positive control, lane 44, *Shigella dysenteriae* type 4; lane 45, 46 and 47, *S. flexneri* 2a, 2b and 5a respectively; lane 48, *S. boydii* 15; lane 49 and 50, *S. sonnei* phase I and II respectively; lane 51, *Enterobacter aerogens*; lane 52, *Klebsiella pneumonae*; lane 53, *Citrobacter freundii*; lane 54, *Salmonella enterica*; lane 55 and 56, negative controls.**

3.3 Occurrence and prevalence of *E. coli* virulotypes in aquatic environment of Bangladesh

All the 46 sampling sites of this study were monitored for the presence of *E. coli* virulotypes in three consecutive seasons. DNA templates from P, PE, BSE and SE of each water sample were analyzed to detect the presence of pathogenic genes. Using three sets of multiplex PCR, nine different pathogenic genes possessed by five different *E. coli* virulotypes were assessed and its distribution and seasonal fluctuations were analyzed.

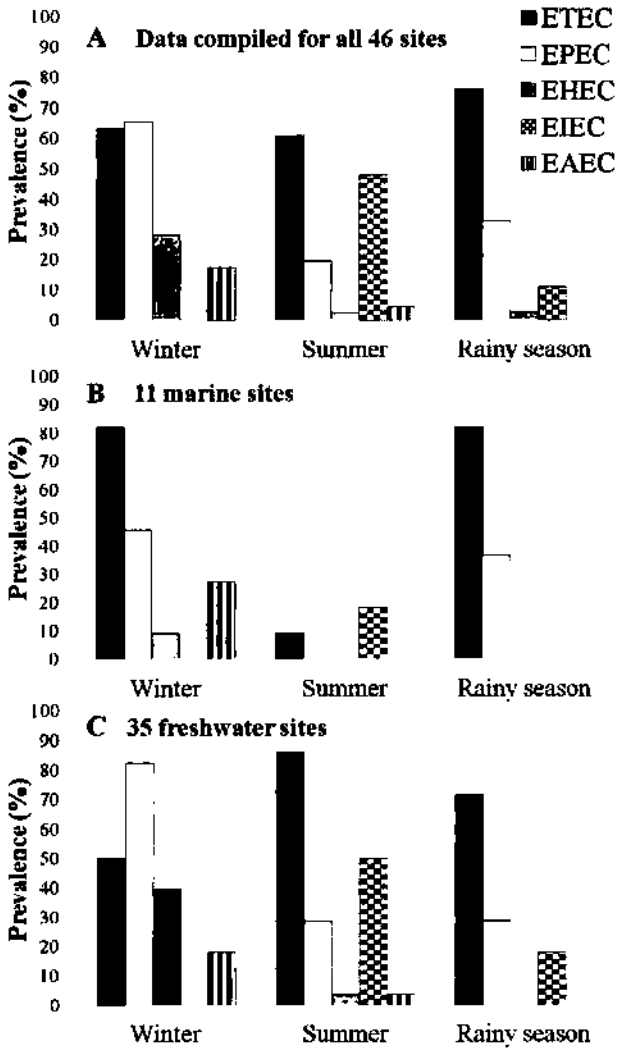
3.3.1 Efficacy of enrichment media on resuscitation of *E. coli* virulotypes

I have analysed the distribution and seasonal fluctuations of *E. coli* virulotypes using three sets of multiplex PCR assays. The detailed results of the analysis are given in Table S3.2 and summarized in Table S3.3. The use of a number of pre-enrichment (PE) and enrichment (BSE and SE) media designed for this experiment enabled enrichment of different virulotypes of *E. coli*. The PE medium was designed to resuscitate stressed or injured environmental bacteria, while the BSE medium was designed to enrich the enteric bacterial population, and streptomycin was added for enriching streptomycin-resistant *E. coli*. A total of 138 DNA samples were evaluated per enrichment. No genes could be amplified from DNA extracted from direct water filtrate. Both *eltB* and *vt1* genes were mostly detected in PE; 72 PE were positive for *eltB*, but only 41 BSE and 26 SE were found to harbour this gene (Table S3.4). Similarly, five PE were positive for the *vt1* gene, but only three BSE and two SE were positive for this gene. The addition of bile salt enabled enrichment of *E. coli* harbouring the *estA*, *vt2*, and *eaeA* genes. The *estA* gene was detected in 18 BSE, 11 PE and 10 SE. The *vt2* gene was detected in nine BSE but only in two PE and one SE. The *eaeA* gene was detected in 45 BSE but only in 20 PE and 11 SE. Streptomycin enrichment medium successfully enriched the EAEC and EIEC virulotypes in the water samples. The pCVD gene was detected in nine SE, one BSE and none in the PE. The *ipaH* gene was detected in 24 SE, eight PE, but none in the BSE, indicating that bile salt suppresses the EAEC and EIEC virulotypes.

3.3.2 Prevalence of diarrheagenic virulotypes in three successive seasons

In this investigation, ETEC was found to be the most prevalent virulotype among the five diarrhoeagenic *E. coli* virulotypes examined (Fig. 3.3A and Table S3) in the sampling sites. The prevalence of the ETEC virulotype in winter, summer, and the rainy season was 63%, 61%, and 76%, respectively. Among the ETEC-containing samples, *eltB* was present in most cases, i.e. in an average 60% of the sampling sites (Table S3.3). *estA* was more prevalent during winter (34%) compared to summer (8.6%) and the rainy season (6.5%). In most cases, *estA* was present along with *eltB*; only 4% of the sampling sites harboured *estA* alone (Table S3.3).

A total of six sites tested positive for *estA* alone, but with seasonal variations; sites 4 and 9 in the winter, sites 7 and 8 in the rainy season and site 10 and 24 in the summer. Except for site 24, all these sites were adjacent to southern sea beaches with marine or brackish salt concentrations. EPEC was the second most prevalent type found followed by ETEC. The prevalence of EPEC was 65% in the winter, which was greater than that of ETEC in that season. In the summer and in the rainy season, only 19% and 32% of the sampling sites, respectively, showed positive results for the EPEC virulotype (Fig. 3.3A and Table S3). In all the EPEC-positive sites, only the *eaeA* (no *bfpA*) gene was detected. The *eaeA* signal could also be due to the presence of EHEC strains.



To aid in interpretation, all *eaeA*-positive samples were considered as EPEC-positive samples, and *vt1*- and/or *vt2*-positive samples were considered as EHEC-positive samples, regardless of the presence of *eaeA*. Like EPEC, the prevalence of EHEC (28%) and EAEC (17%) was also higher in the winter (Fig. 3.2A). In the summer, the prevalence of EHEC and EAEC was 2% and 4%, respectively and in the rainy season, the prevalence of EHEC was also 2% while EAEC was absent. Unlike the other virulotypes, EIEC was relatively more prevalent in the summer (48%). In the rainy season, EIEC was found in 11% of the sampling sites and was absent in the winter. Although the EPEC and ETEC virulotypes were almost evenly distributed throughout the country, regional distribution patterns were observed for certain virulotypes with distinct temporal variations (Fig. 3.4).

Fig. 3.3 The prevalence (in terms of proportion of positive sampling sites) of five different diarrheagenic *E. coli* virulotypes in the aquatic system of Bangladesh assessed in three successive seasons. A, data compiled for all sites; B, in 11 marine sites; and C, in 35 freshwater sites. The Figure has been adopted from Akter *et al.*, 2013.

3.3.3 Effect of salinity on diarrheagenic virulotypes

Significant differences were observed in the virulotype prevalence in freshwater and marine samples ($p > 0.05$). In marine samples (Fig. 3.3B), the ETEC virulotype was found to be the most prevalent virulotype both in winter (82%) and in the rainy (82%) season, but in the summer, its prevalence dropped to 9%. EPEC was the second most prevalent virulotype in the winter (46%) and in the rainy (37%) season, but it could not be detected in the summer. The EHEC and EAEC virulotypes were detected only in the winter, in 9% and 28% of the sampling sites, respectively, whereas EIEC was detected only in the summer in 19% of the sites. In freshwater sites (Fig. 3.3C), the most prevalent was the ETEC virulotype, both in the summer (86%) and in the rainy (72%) season, but in the winter, the EPEC was the most prevalent virulotype (83%). Similar to the results for the marine samples (Fig. 3.3B), EIEC was also prevalent in freshwater samples (Fig. 3.3C) only in summer, but it had higher prevalence in freshwater samples (50%) compared

to marine samples (19%). Neither the EHEC nor EAEC virulotype was detected in the rainy season in the freshwater samples (Fig. 3.3C). In the summer, the prevalence of the EHEC and EAEC virulotypes in freshwater samples was still low (4%), but it increased to 29% and 18%, respectively, in the winter.

3.3.4 Regional distribution pattern of occurrence of *E. coli* virulotypes

It was found that the aquatic systems of the country harbored different virulotypes of diarrheagenic *E. coli* persistently throughout the year and regional distribution patterns for certain virulotypes with distinct temporal variations were observed (Fig. 3.4). Although, EPEC and ETEC were almost evenly distributed throughout the country, these virulotypes maintained regional distribution patterns and had temporal fluctuations.

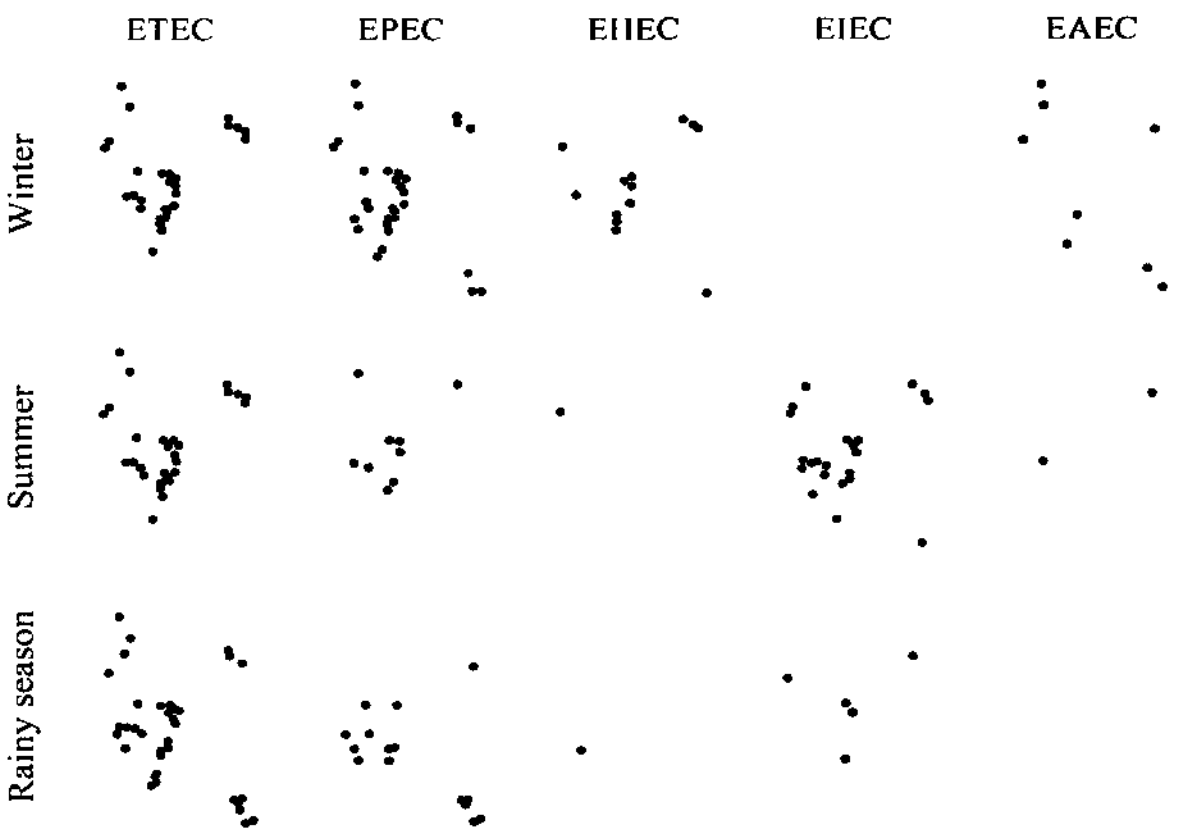


Fig. 3.4 The regional and temporal distribution patterns of five different diarrheagenic *E. coli* strains in three seasons in the aquatic system of Bangladesh. The black circles on the map of Bangladesh indicate the presence of different diarrheagenic *E. coli* virulotypes in the corresponding geographical location. The Figure has been adopted from Akter *et al.*, 2013 and the map has been adopted from the Banglapedia database (Banglapedia, 2006).

In the winter and summer, ETEC was absent in sea beaches but emerged in the rainy season. The decrease in salinity in the water close to seashore or huge runoff from the river in rainy season might have influenced the temporal occurrence of ETEC in that region. In the summer, salinity close to the sea shore was the highest and that could be responsible for the absence of *E. coli* virotypes in the region. In summer, the salinity of the sea beaches was the highest and the least incidence of *E. coli* virotypes were found in sea beaches in that season. The rivers of central part

of the country, around the highly populated capital Dhaka, were the most polluted. All the *E. coli* virotypes were also found to be present in that region.

3.3.5 Effect of seasonal variations on prevalence of virulotype

The marine sites were grouped into two; sites with no salinity fluctuations and sites with lower salinity in rainy season. Significant differences ($p > 0.05$) were observed in virulotype prevalence in two groups of marine sites in the rainy season (Figs. 3.5A and 3.5B). In both the groups, only the ETEC and EPEC virulotypes were detected in the rainy season, but prevalence differed. In the first group, where salinity was rather stable, the prevalence of the ETEC and EPEC virulotypes was 60% and 40%, respectively (Fig. 3.5A).Whereas, the prevalence of ETEC and EPEC was 100% and 26%, respectively (Fig. 3.5B) in the latter group of marine sites where salinity dropped in the rainy season.

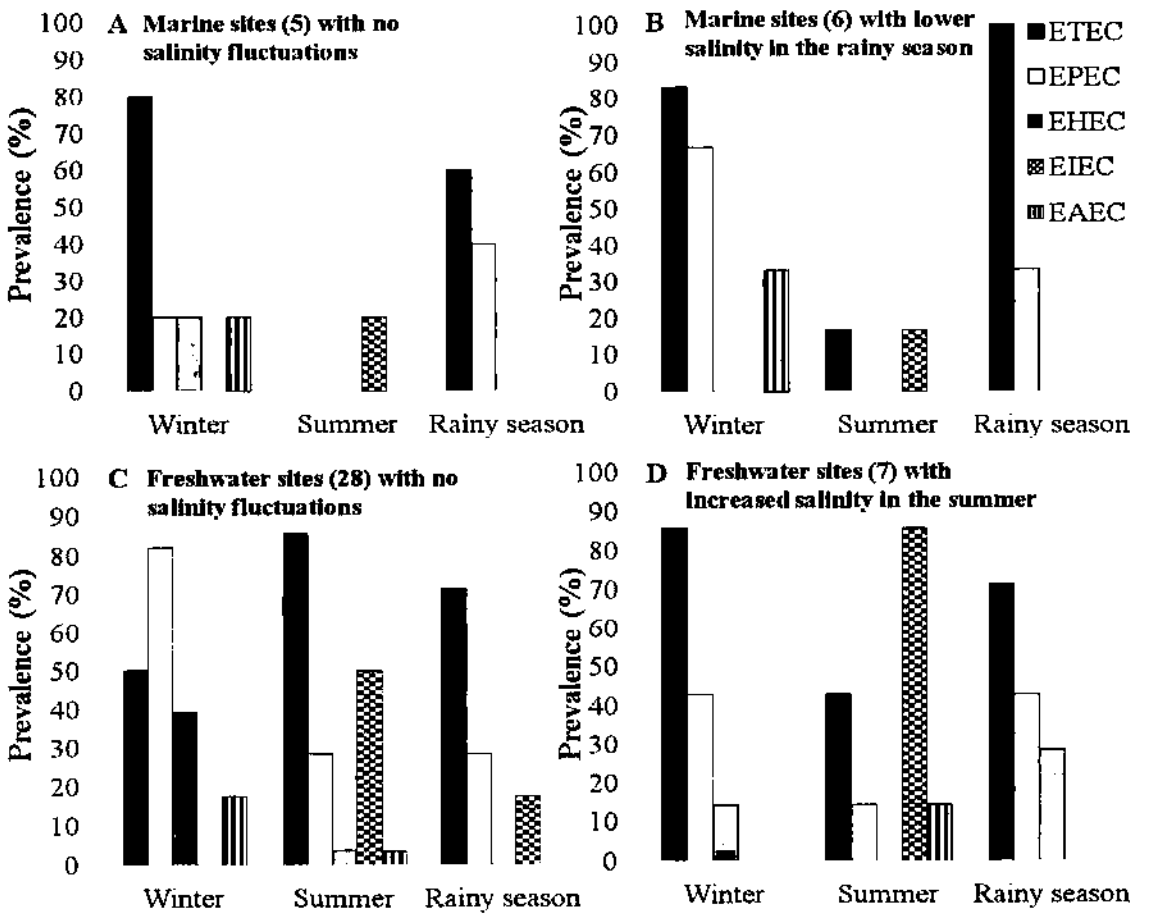


Fig. 3.5 The effect of seasonal fluctuations in salinity on the prevalence of diarrheagenic *E. coli* virulotypes in marine ('A' and 'B') and freshwater ('C' and 'D') sites. A, the prevalence pattern of diarrheagenic *E. coli* in five marine sites where salinity did not fluctuate between seasons; B, the prevalence pattern of diarrheagenic *E. coli* in six marine sites where salinity significantly dropped in the rainy season; C, the prevalence pattern of diarrheagenic *E. coli* in 28 freshwater sites where salinity did not fluctuate between seasons; and D, the prevalence pattern of diarrheagenic *E. coli* in seven freshwater sites where salinity significantly increased in the summer. The Figure has been adopted from Akter *et al.*, 2013.

Out of 35 freshwater sites, the salinity at 28 sites was mostly stable between seasons. The other seven sites had significantly increased salinity in the summer. The pattern of the prevalence of virulotypes differed in these two groups of freshwater sites (Figs. 3.5C and 3.5D). In the winter, the prevalence of the EPEC virulotype was the highest in the salinity-stable freshwater sites (Fig. 3.5C), whereas that of the ETEC virulotype was the highest in the salinity-increased freshwater samples (Fig. 3.5D). The EAEC virulotype was absent in those samples. In the summer and in the rainy season, the virulotype prevalence patterns also differed in these two groups of freshwater sites (Figs. 3.5C and 3.5D).

3.3.6 Prevalence of Cytolethal Distending Toxin (Cdt) producing *E. coli*

In this study, we used PCR to detect all the presently known *E. coli-cdt* alleles to determine the frequency and distribution of *cdt* among the natural water bodies in Bangladesh. Forty six aquatic samples collected in April 2010 were assessed in this experiment. Among the five different *cdt* alleles reported in *E. coli*, encoding Cdt-I, Cdt-II, Cdt-III, Cdt-IV and Cdt-V respectively; *cdt-IA* was the most prevalent type, detected in 34 sites out of 46 aquatic sites around the country followed by *cdt-IIA*, detected in 29 sites (Fig. 3.6). From the rest, *cdt-VA* and *cdt-VC* were detected in 11 sites, *cdt-IVB* in 9 sites, *cdt-III* in 5 sites and *cdt-VB* in only 2 sites. Detailed results of this experiment have been enlisted in Table S3.5.

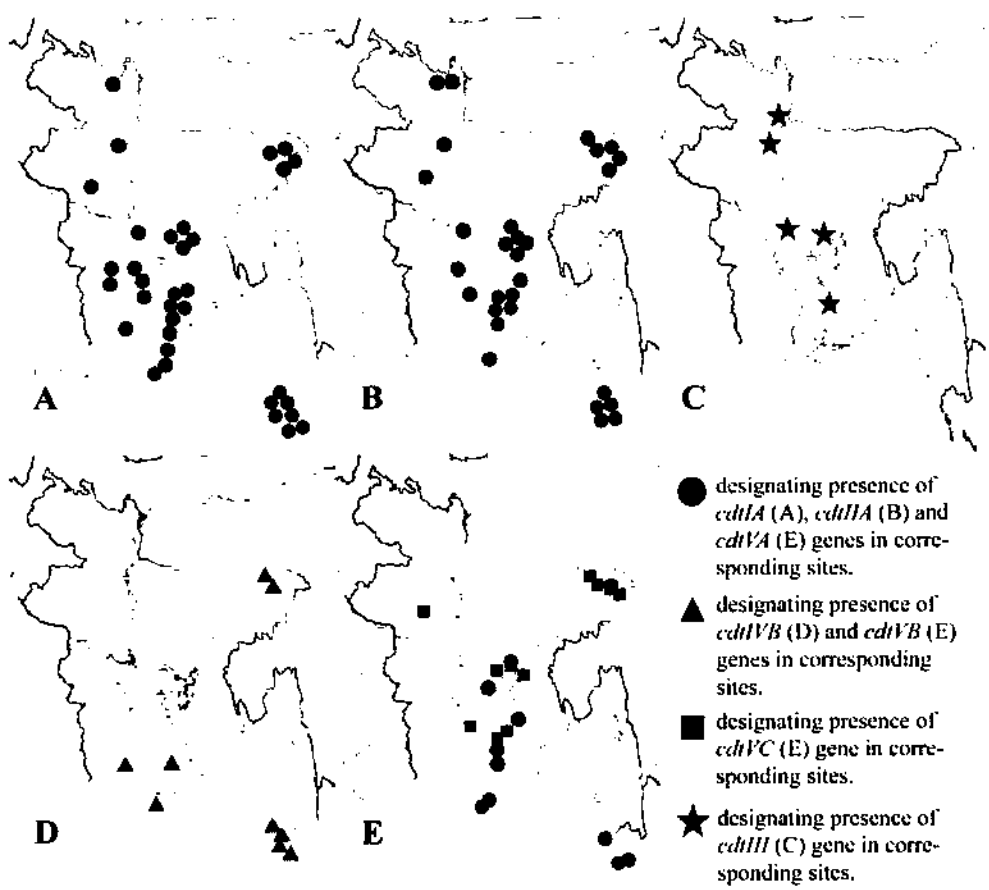


Fig. 3.6 Distribution of different *E. coli* associated *cdt* alleles among the aquatic sites in Bangladesh. The symbols are marked at sampling sites corresponding to the GPS position in respect to Fig. S3.1 (the name of the sampling sites are listed in Table S3.1).

3.3.7 Effect of salinity on Cdt producing *E. coli*

Prevalence pattern of the *cdt*-alleles varied with the salinity of the aquatic environment (Fig. 3.7). In marine sites, prevalence of *cdt-IA* was 80% but *cdt-IIA* dropped to 33%. In freshwater sites, *cdt-III*, *cdt-VB* and *cdt-VC* were detected in 15%, 16.5% and 35.5% sites respectively but couldn't be detected in any of the marine sites. *cdt-IVB* and *cdt-VA* were detected in both the freshwater and marine sites, which also differs in their prevalence percentages (Fig. 3.7). Salinity of the water samples affected prevalence of both the *cdt* alleles and diarrheagenic virulotypes. In linear regression plot (Fig. 3.8), prevalence of *cdt-IA* and *cdt-IVB* were found to be proportional to the salinity of the respective sites but *cdt-IIA*, *cdt-III* and *cdt-VC* were inversely proportional to that. However, prevalence of *cdt-VA* and *cdt-VB* were not affected by the salinity of the sites .

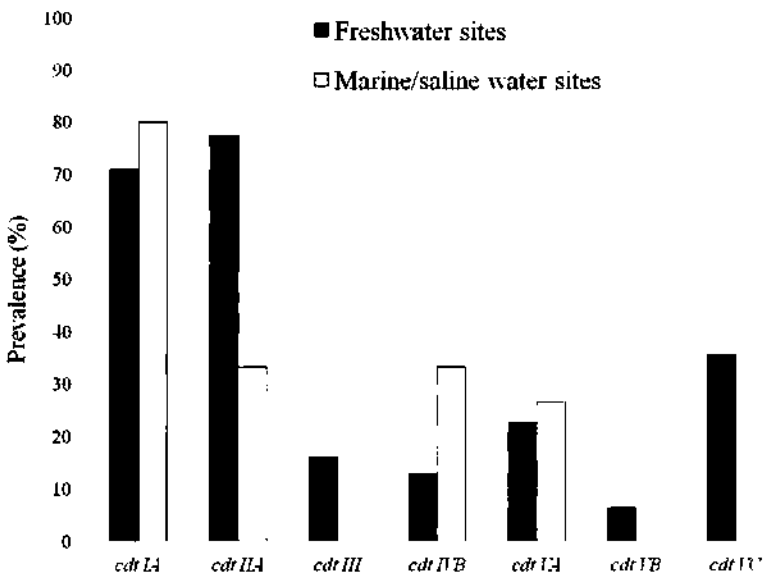


Fig. 3.7 Prevalence of different *E. coli-cdt* alleles in freshwater and marine water sources in Bangladesh.

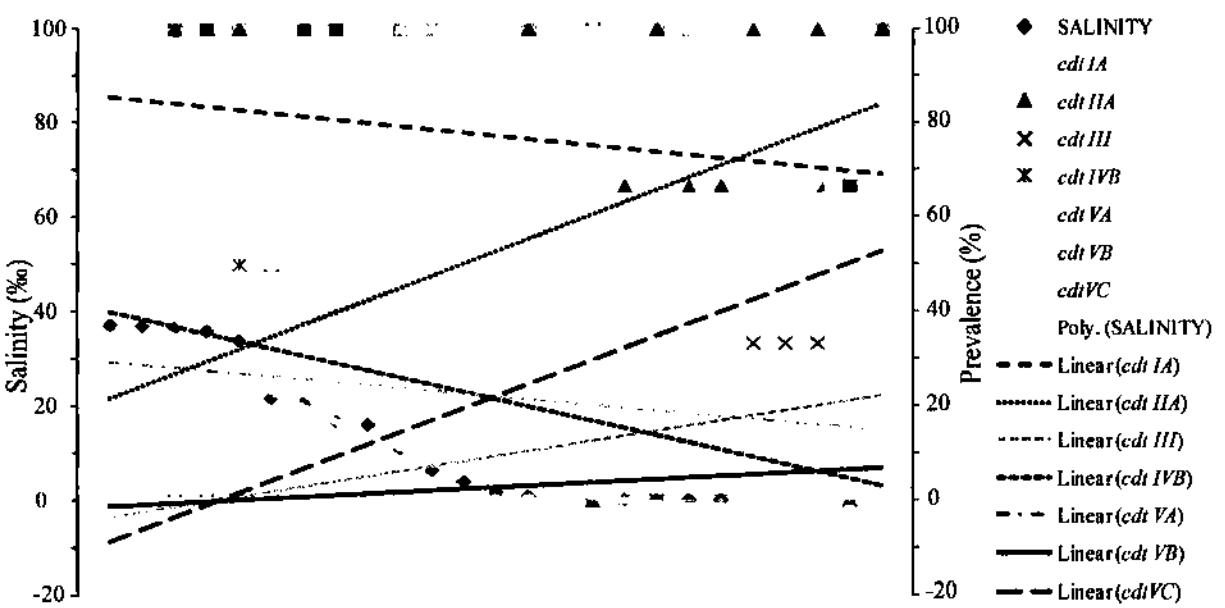


Fig. 3.8 Graphical representation of linear regression analysis, illustrating the effect of salinity on the prevalence of different *cdt*- alleles present in the aquatic systems of Bangladesh.

3.3.8 *fliC*-genotype, as a tool for epidemiological investigation

Sixty six *fliC* PCR products were assessed in this study, in which 39 were found in 37 environmental DNA samples (two of those samples gave rise to double bands, which were excised and re-amplified) randomly selected from pre-enriched, bile salt-enriched and streptomycin-enriched cultures. The rest 27 *fliC* PCR products were from environmental *E. coli* isolates. The isolates were identified by API 20E biochemical profiling and enlisted in Table 3.4. The test sequences were aligned with the reference H type specific sequences (Figs. 3.9 and 3.10) and upon phylogenetic analyses type H4, a recently reported epidemic STEC strain, was found to be the most prevalent in the study (Table 3.1). Eight out of 39 sequences from enriched cultures (Fig. 3.9) and 6 out of 27 sequences from environmental isolates (Fig. 3.10) were typed as H4. The STEC outbreak causing serotype, H25, was found as second prevalent type; 5 out of 39 sequences from enriched cultures and 3 out of 27 sequences from environmental isolates were typed as H25. The most common STEC serotype, H7 was also found in one enriched culture and only in one isolate. Though, these H-types were known to cause severe haemolytic uremic syndrome (HUS) but in the study, we could not identify genes for shiga toxins in any of the H4, H25 or H7 isolates. Among the rest *fliC* PCR products from enriched cultures, 6 products were typed as H10, 2 as H5 and another 2 as H12. *fliC* PCR product for each of the flagellar type H7, H9, H11, H18, H20, H27, H33, H34, H42 and H52 were possessed. Eight *fliC* products left unidentified. Among the isolates, three were typed as H28, two as H16 and one isolate was typed as H2, H5, H7, H19, H29, H30, H32, H37, H38, H45 and H52 for each (Table 3.1). Virulence associated genes were found in different samples but could not be correlated with the H-types identified. The list of virulence associated genes found in the respective sites was given in Table S3.5.

Table 3.1 H-types identified in the environmental *E. coli* isolates and enriched samples by *fliC*-sequencing genotyping.

H-type identified	Number of isolates	Number of samples	H-type identified	Number of isolates	Number of samples
H2	1	0	H27	0	1
H4	6	8	H28	3	0
H5	1	2	H29	1	0
H7	1	1	H30	1	0
H9	0	1	H32	1	0
H10	0	6	H33	0	1
H11	0	1	H34	0	1
H12	0	2	H37	1	0
H16	2	0	H38	1	0
H18	0	1	H42	0	1
H19	1	0	H45	1	0
H20	0	1	H52	1	1
H25	5	3	Unidentified	0	8

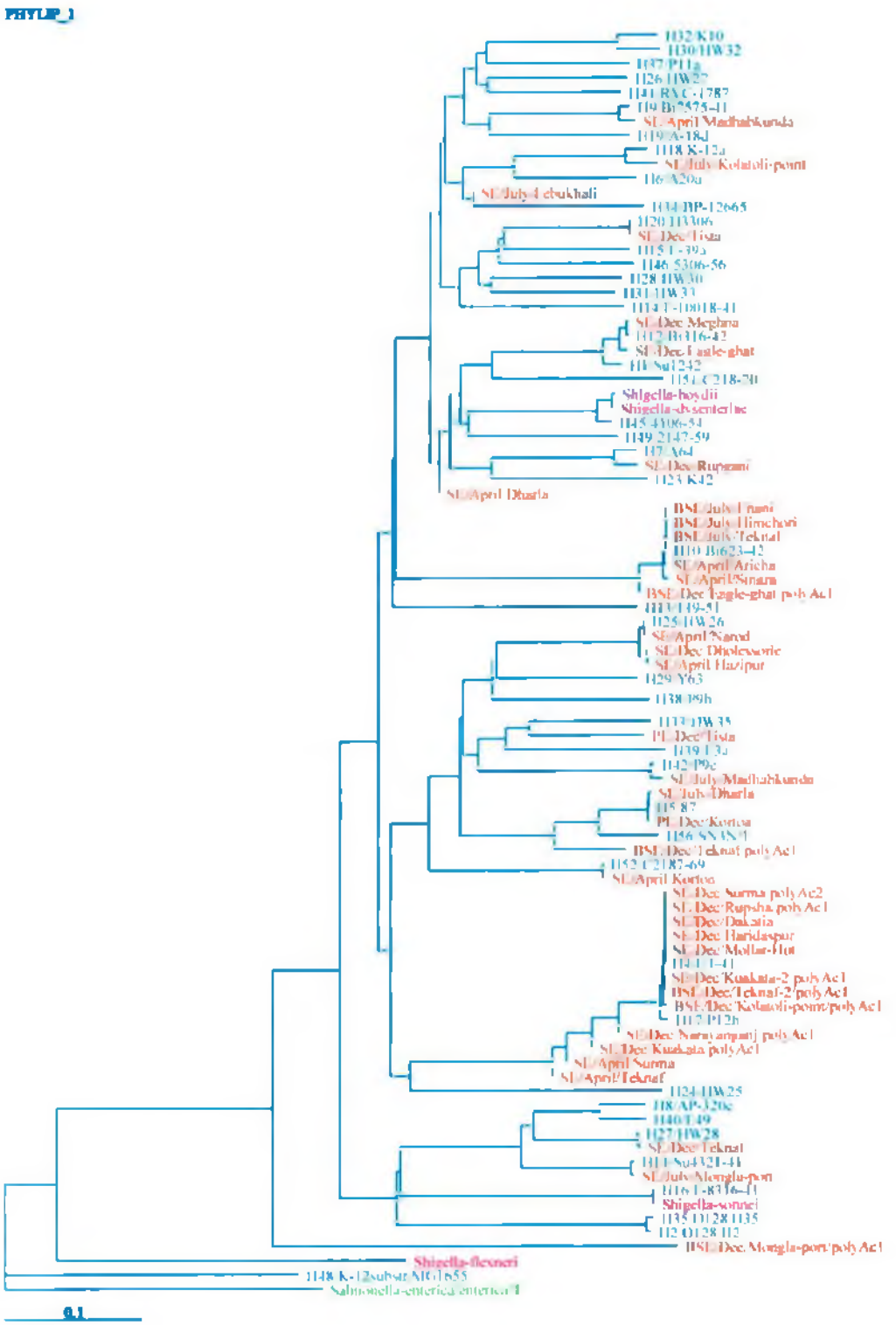


Fig. 3.9 Gene phylogeny of *fliC*-PCR products amplified from environmental DNA. Phylogenetic tree was constructed by the neighbor-joining algorithm using the *fliC* gene sequences of various reference H-type strains (designated as H1 to H53) and the test sequences recovered from environmental enriched samples and rooted by the *fliC* gene of *Salmonella enterica* serover Typhimurium.

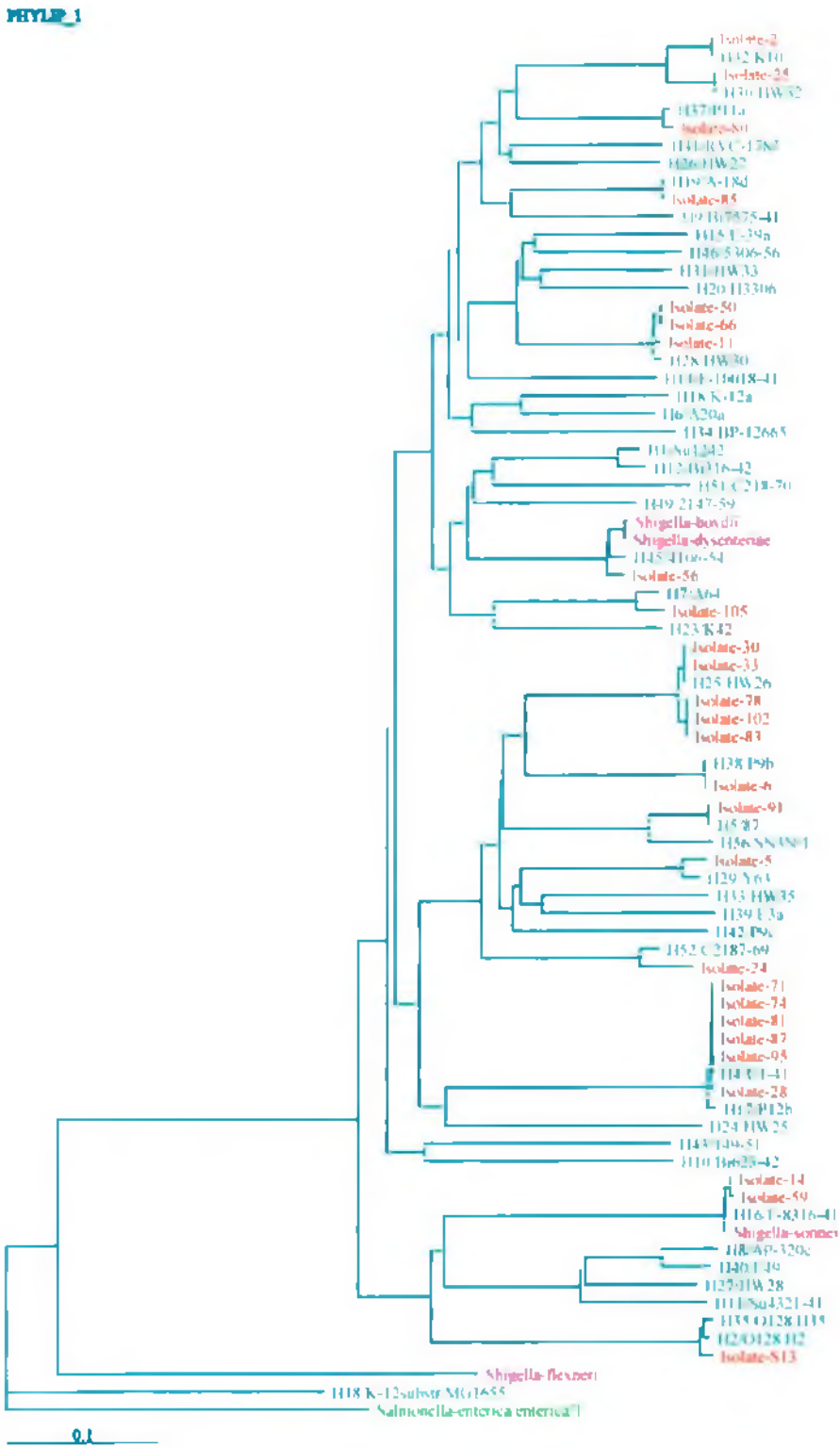
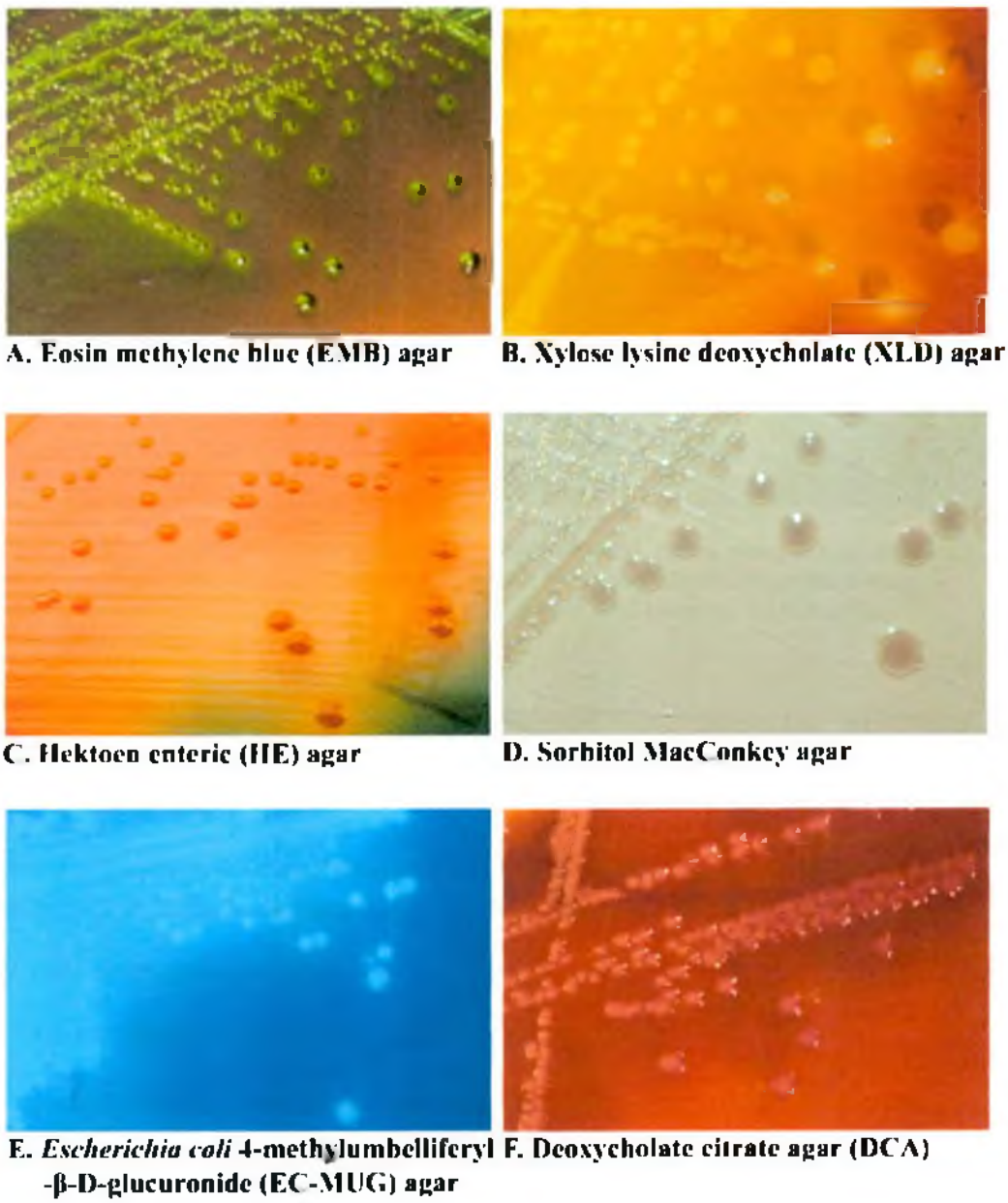


Fig. 3.10 ***fliC*-gene phylogeny of environmental *E. coli* isolates. Phylogenetic tree was constructed by the neighbor-joining algorithm using the *fliC* gene sequences of various reference H-type strains (designated as H1 to H53) and the test sequences amplified from environmental *E. coli* isolates and rooted by the *fliC* gene of *Salmonella enterica* serover Typhimurium.**

3.4 Environmental *E. coli* isolates

3.4.1 Colony morphology

Isolates streaked on eosin methylene blue (EMB) agar showed characteristic green metallic sheen (Fig. 3.11A) whereas, on xylose lysine deoxycholate (XLD) agar, the isolates yielded pale yellow colonies (Fig. 3.11B). The isolates also showed characteristic colony morphology on other selective and differential media e.g., Hektoen enteric agar, sorbitol MacConkey agar and deoxycholate citrate agar (Fig. 3.11C, D and F). On *E. coli*-4-methylumbilliferyl-β-D-glucoronide agar, most of the isolates gave rise to fluorescence under UV (Fig. 3.11E).



A. Eosin methylene blue (EMB) agar
B. Xylose lysine deoxycholate (XLD) agar
C. Hektoen enteric (HE) agar
D. Sorbitol MacConkey agar
E. *Escherichia coli* 4-methylumbelliferyl -β-D-glucuronide (EC-MUG) agar
F. Deoxycholate citrate agar (DCA)

Fig. 3.11 Colony characteristic of environmental *E. coli* isolates streaked on different selective and differential media after 24 h of incubation at 37°C.

S-13 isolates in marine water microcosm, rather than decrease in survival. In freshwater microcosm, all the isolates stably maintained their cfu at that time duration.

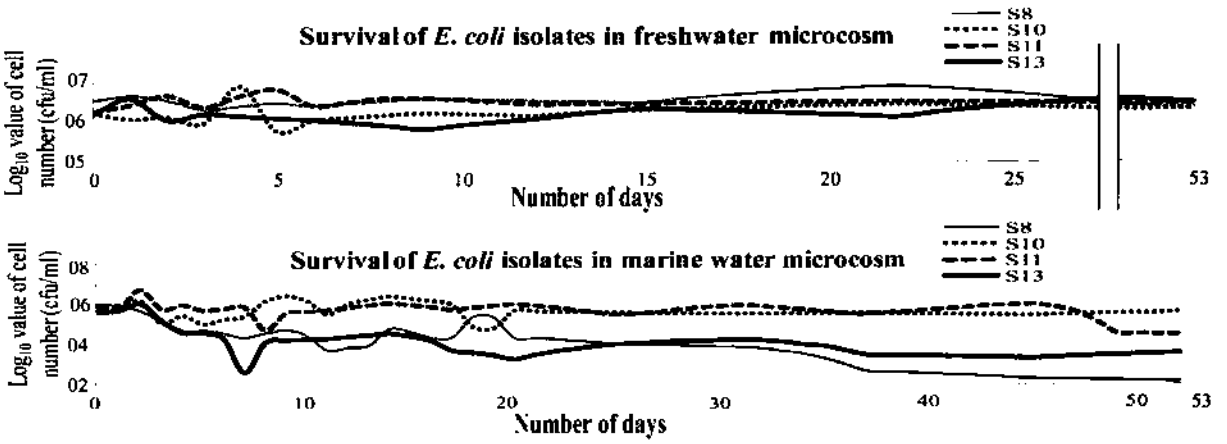


Fig. 3.13 Graphical representation of survival of the salt tolerant *E. coli* isolates in seawater (A) and freshwater (B) microcosm. The X-axis represents the number of days of observation and Y-axis represents the $\log_{10}$ number of cells (cfu/mL).

3.5.2 Growth rate of *E. coli*

From the growth curve experiment, generation time of environmental isolates termed S-8, S-10, S-11, S-13 and a clinical *E. coli* O157:H7 isolate was measured (Table 3.2). The cultures were grown at 25°C with constant shaking at 120 rpm in media containing both the marine water and freshwater respectively. The growth was observed in every 2 h of interval for 26 h in duplicate cultures of each isolate and the mean values were plotted on the graph.

Table 3.2 Generation time of the *E. coli* isolates in media containing both the marine and freshwater measured in growth curve experiment.

***E. coli* test isolates**	**Doubling time of growth at 25°C and 120 rpm**	
	Sea water	Freshwater
S-8	40 min	57.2 min
S-10	60 min	66.67 min
S-11	57.75 min	40 min
S-13	41.1 min	50 min
O157:H7	46.67 min	46.67 min

3.5.3 Adaptation of marine *E. coli* isolates to high salt concentrations

The *E. coli* isolates, during screening for salt tolerance, formed characteristic cell to cell agglutination pattern in media containing higher salt concentrations (Fig 3.14). The culture of isolates growing in 0.85% and 3.0% sea salt media (SSM) did not show any microscopic difference upon 24 h of incubation. All the cells were motile and actively growing. In media containing 5.0% sea salt, the cultures of the isolates formed clumps within 24 h. The microscopic examination was done for each of the culture of different SSM on day-7 upon incubation. All the

isolates showed aggregation and synthesized cell-clumps in 5.0% SSM. Huge numbers of planktonic cells were also observed in the broth. The clumps tended to settle at the bottom of the culture vessels. In the media containing 7.5% sea salt, larger cell clumps were observed with decreased number of planktonic cells. The isolate S-8 and S-11 formed cell clumps with sticky appearance and thread-like structures were viewed under microscope, which disappeared in 10.0% SSM. Isolate S-10 showed similar clumping in 7.5% and even in 10.0% SSM as it showed in culture of 5.0% SSM. The only change found is the decreasing planktonic cells. The isolate S-13 formed brittle film of cells floating at the air-culture interface in 7.5% and 10.0% SSM. The planktonic cells of S-13 were also comparatively lower compared to other isolates in 7.5% SSM and almost nil in 10% SSM. The floating films were larger and intensified in 10% SSM. A clinical O157:H7 strain was kept as control, which showed moderately sticky thread-like appearance in 5.0% SSM, which intensified in 7.5% SSM and surprisingly disappeared in 10.0% SSM. Planktonic cells were found in 10% SSM as well.

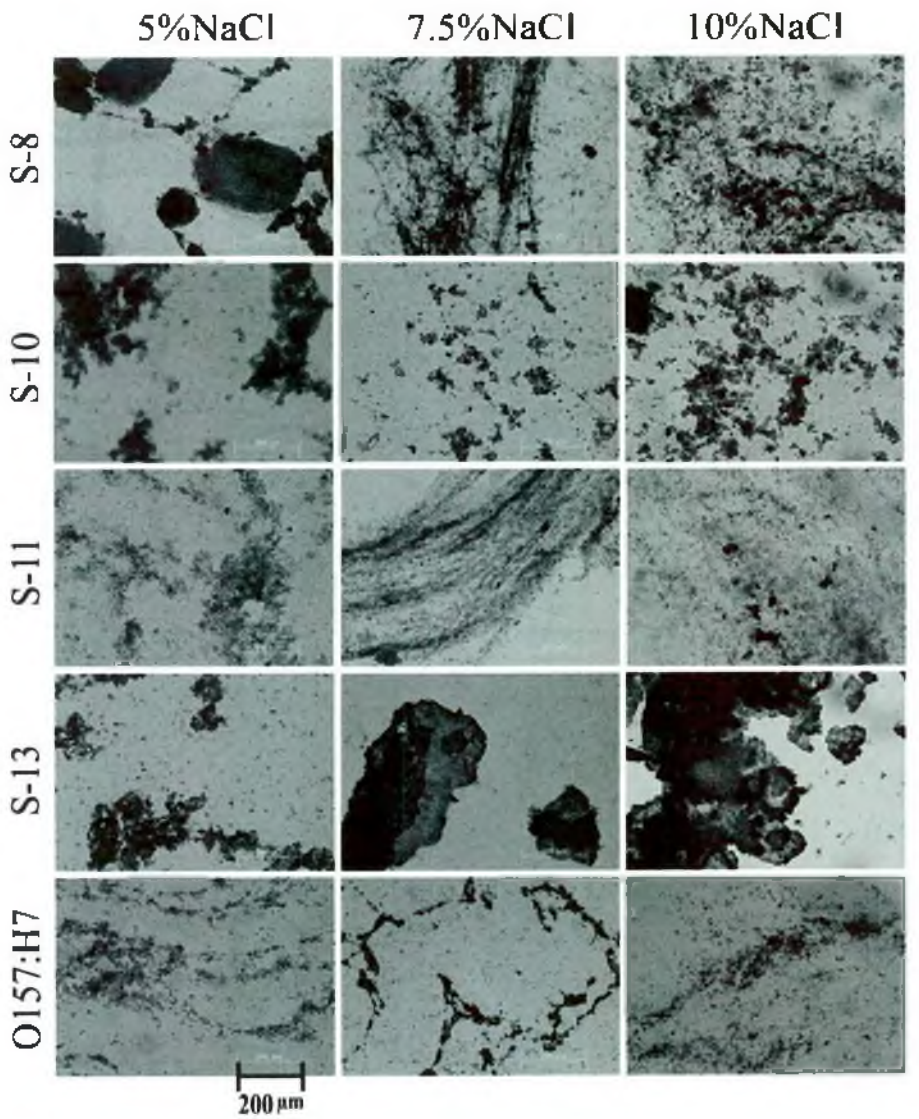


Fig. 3.14 Microscopic observation of salt tolerant *E. coli* isolates in media of different salt concentrations upon 7 d of incubation at room temperature.

3.5.4 Fish as a secondary host in environment

This study was performed with green fluorescent protein (GFP) marked *E. coli* isolates in laboratory mesocosms, which revealed significant bacterial association with fish (*Heteropneustes fossilis*) supplied in the mesocosm. Surface water film, sediments, as well as phytoplankton rhizoids also contained a high proportion of test organisms as compared to bulk water, whereas the bacterial load on fish skin corresponded to that of surrounding water. The bacterial load in the water body was assessed for a week and at the end of the week, most of the bacterial isolates tended to decline in the water body (Fig. 3.15). There was a sharp decline of DUM10 in the water body on fifth day, after the day at which the last fish was taken out of the mesocosm for dissection.

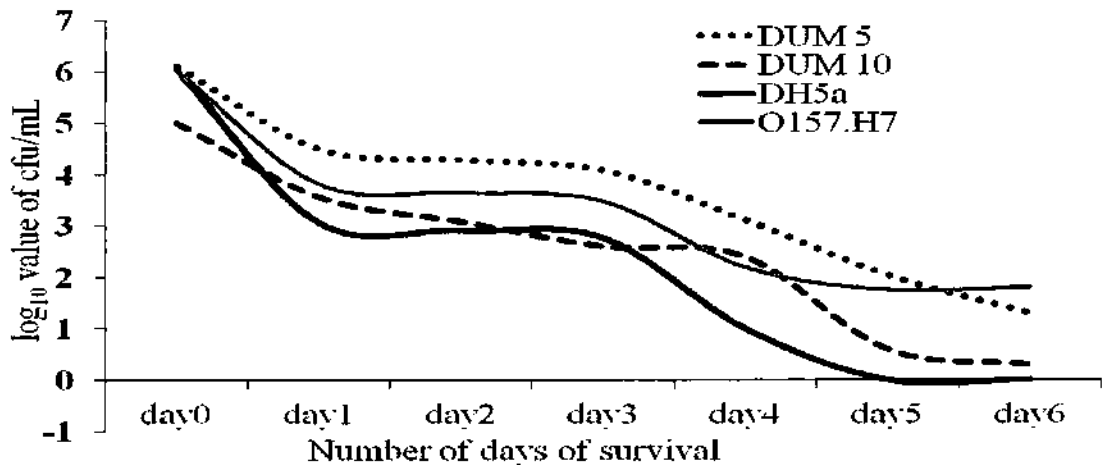


Fig. 3.15 Graphical presentation of survival of *E. coli* strains in microcosm.

Adhesin genes were detected in the test isolates by PCR. The regulatory gene *csg*A was present only in DH5α, whereas the gene for type1 fimbriae was absent only in the DUM5. The afimbrial adhesin gene *afa* was detected in DUM5 and O157:H7 strains. None of the test strains was found to be *sfa* and *pap* positive when screened for adhesion-related genes by PCR. Assessment of hydrophobicity of the four *E. coli* test strains did not show any significant difference as measured in the form of partition coefficient between aqueous suspension and a solid hydrophobic phase. Quantitative biofilm formation assay using all the four isolates reflected a correlation between the presence of *afa* gene and the potential to form biofilm on polystyrene microtiter plates.

No signs of colonization by DUM5, DH5α or O157:H7 strains were detected in the fish gut. However, fish intestinal content of DUM10 containing microcosm had an *E. coli* load of 2.0×10^6 cfu/g, indicating colonization and active growth of the bacterium. This particular isolate did not possess green fluorescence, but Rep-PCR analyses revealed that the gfp-marked DUM10 strain and the bacterium isolated from the respective fish gut belonged to the same clone (Fig. 3.16), indicating loss of the pGLO plasmid while translocating from water to fish gut.

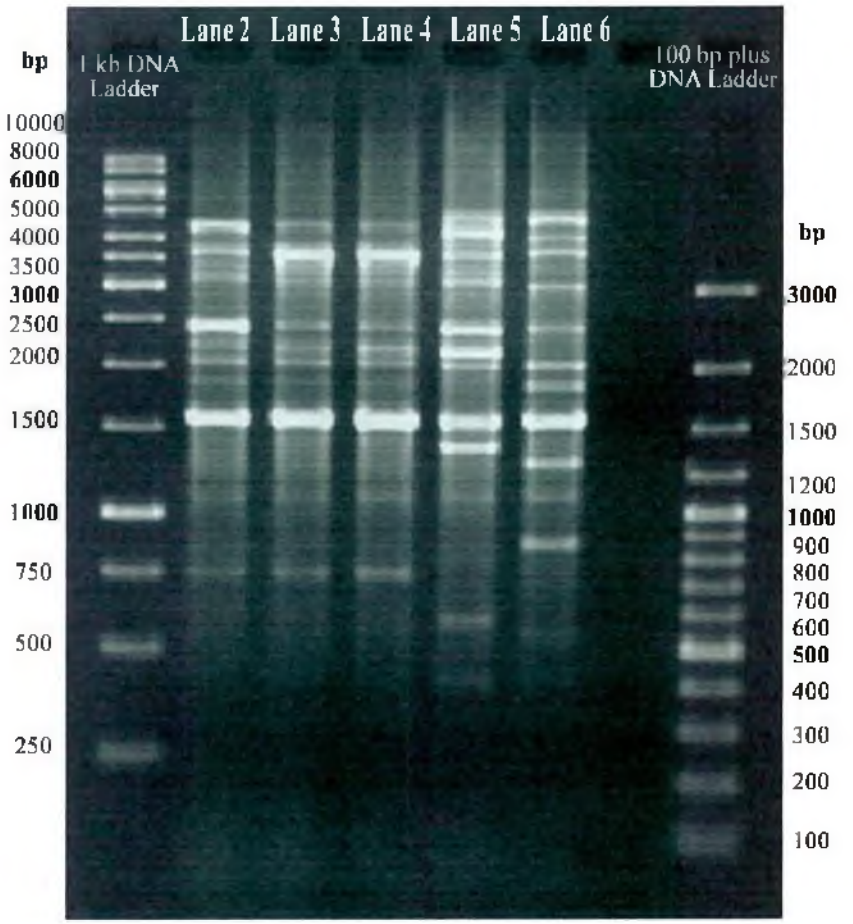


Fig. 3.16 Rep-PCR genomic fingerprint analysis of *E. coli* strains. Lane1 and 7 represent 1 kb DNA ladder and 100bp plus DNA ladder respectively. Lanes 2, 3, 5 and 6 contain rep-PCR bands of DUM5, DUM10, O157:H7 and DH5α correspondingly. Lane 4 contains rep-PCR band of the bacterium isolated from fish gut.

This finding indicates an association between environmental *E. coli* strains and aquatic biota. Fish could be a possible reservoir for the continuous load of enteric bacteria like *E. coli* in water. This could be an alternate explanation for the presence of *E. coli* in water body without recent fecal discharge.

3.6 Environmental *E. coli* isolates cross-reacting with anti-*Shigella* spp. antisera

3.6.1 Screening of serological cross-reactive isolates by slide agglutination test

Environmental isolates, which had been identified as *E. coli* using biochemical profile index, were screened for the detection of cross-reactivity to *Shigella*-specific antisera. Fresh overnight NA cultures were subjected to slide agglutination test with commercially available group and type-specific *Shigella* antisera kit. Out of 140 isolates, 69 showed agglutination with 8 polyvalent antisera belonging to all the four *Shigella* serogroups (Table S3.6). Those were further screened with monovalent antisera for the detection of type specific cross-reaction. Slide agglutination test of the strains varied amongst the isolates. This test revealed that 20 (13%) isolates were serogrouped as *Shigella dysenteriae* and showed monovalent agglutination with different serotypes, 17 (11%) isolates serogrouped as *Shigella flexneri*, 37 (25%) serogrouped as *Shigella*

boydii and 6 (4%) as *Shigella sonnei* (Fig. 3.17). All the isolates showed agglutination with specific monovalent *Shigella* antisera. Thirty isolates were found to agglutinate with polyvalent *Shigella* serogroups but did not react with specific monovalent antisera. Amongst the total 69 isolates, 27 showed cross-reactivity with multiple *Shigella* serogroups (Table 3.3).

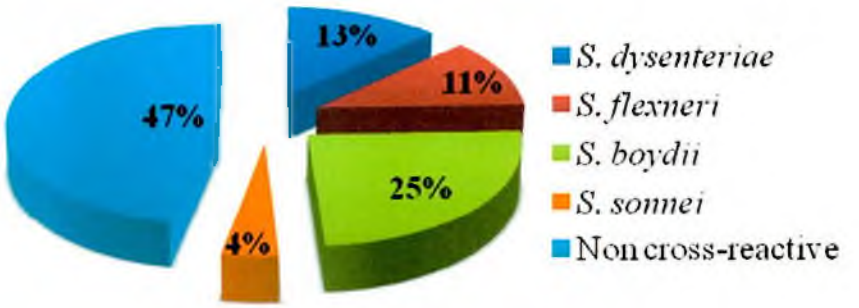


Fig. 3.17 Serological cross-reactivity pattern of the *E. coli* isolates with anti-*Shigella* spp. antisera; 13% of *E. coli* isolates were cross-reactive with *S. dysenteriae*, 11% with *S. flexneri*, 25% with *S. boydii*, 4% with *S. sonnei* and 47% were non cross-reactive.

Table 3.3 *E. coli* isolates from environmental water samples showing multiple sero-cross-reactivity (slide agglutination) with *Shigella* serogroups and serotypes (asterisk '*' designates confirmation by Western blot analysis).

Isolate code	Serogroups	Isolate code	Serogroups
γ 3/8	A1*, B*	γ 2/10	A1*, C
γ 7/12	A1*, B, C2*	γ2/14	B*, C*, C1
θ 2/7	B*, C	β 8/11	A, A1, C
β 4/2	A*, B*, C	s 1/6	A1*, C*, C1*
α 9/1	A1, B*	γ 7/5	A1, C
r 2/10	A*, A1, B*, C*, C2*	α 6/7	A*, C3*
r 5/6	A, C1*, C2*	β 10/7	C3*, D*
α 1/7	A1, B*, C*	α 5/6	C*, D
s 1/5	B*, C*, C2	S-10	A*, C
α 7/6	B, C2	83	B*, C*
α 2/3	B, C3*	2	B*, C
r 4/4	C1*, C2*	85	C*, C1*
α 10/2	C*, D*	β 8/8	A*, C*, C3*
α 6/1	B, D*		

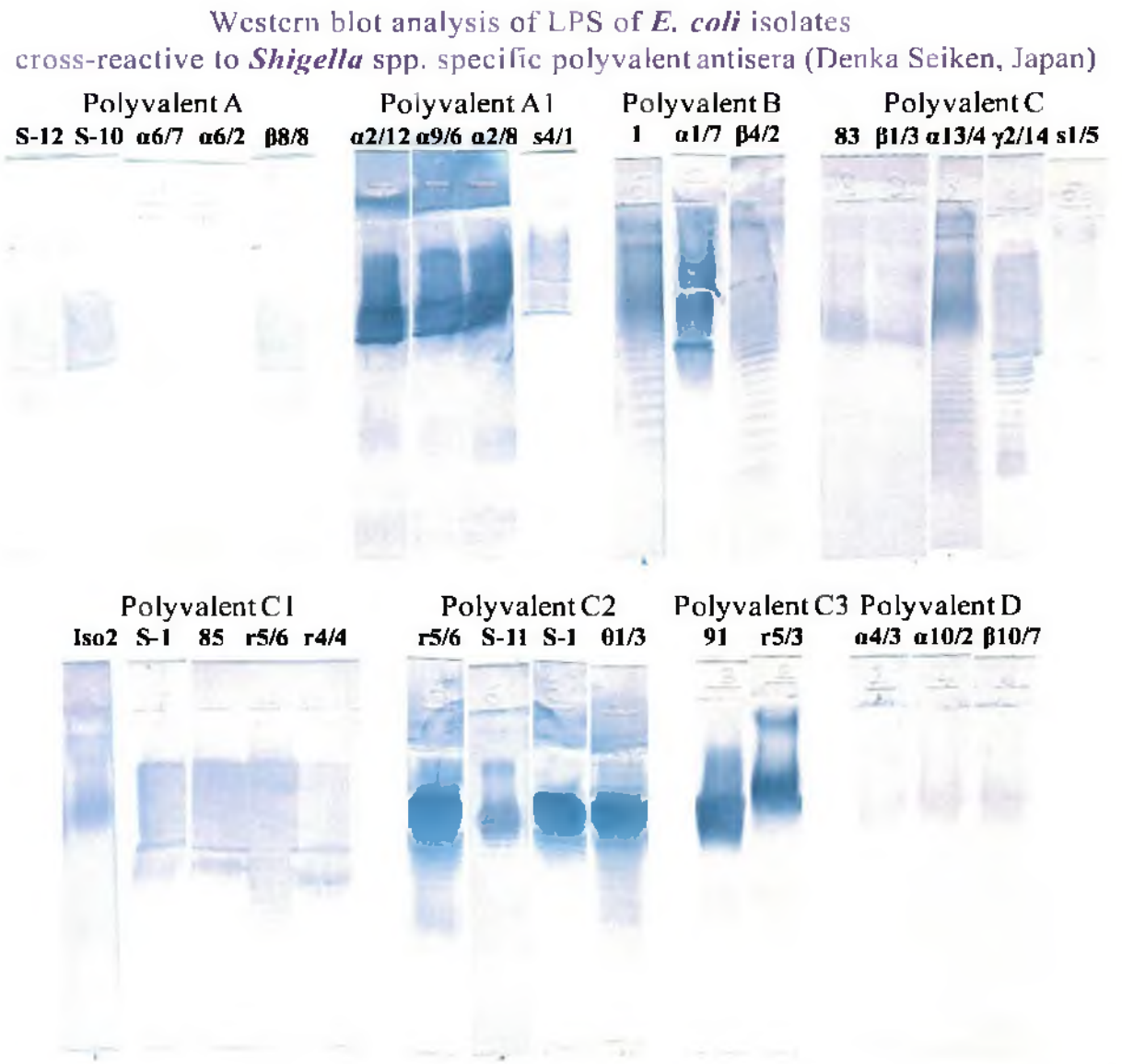


Fig. 3.18 Western blot analysis of LPS extracted from environmental *E. coli* isolates by anti-*Shigella* spp. polyvalent antisera.

Although strains of *Shigella* spp. show certain type of antigen on its surface unique to its serotype, it has been reported that some *E. coli* strains show cross-reactivity with multiple *Shigella* spp.-specific antisera. In this study, a number of *E. coli* isolates were identified to cross-react with anti-*Shigella* spp. antisera, a few of those were not reported earlier. Some of the *E. coli* isolates were concurrently cross-reacting with multiple antisera. List of the isolates showing cross-reactivity to multiple antisera are given in Table 3.3 and the overall serological results are presented in Table S3.6.

The serological cross-reactivity was screened by slide agglutination test and then confirmed by Western blot analysis (Figs. 3.18 and 3.19) using polyvalent and monovalent commercial antisera (Denka-Seiken, Japan) specific to *Shigella* sero-groups and -types respectively.

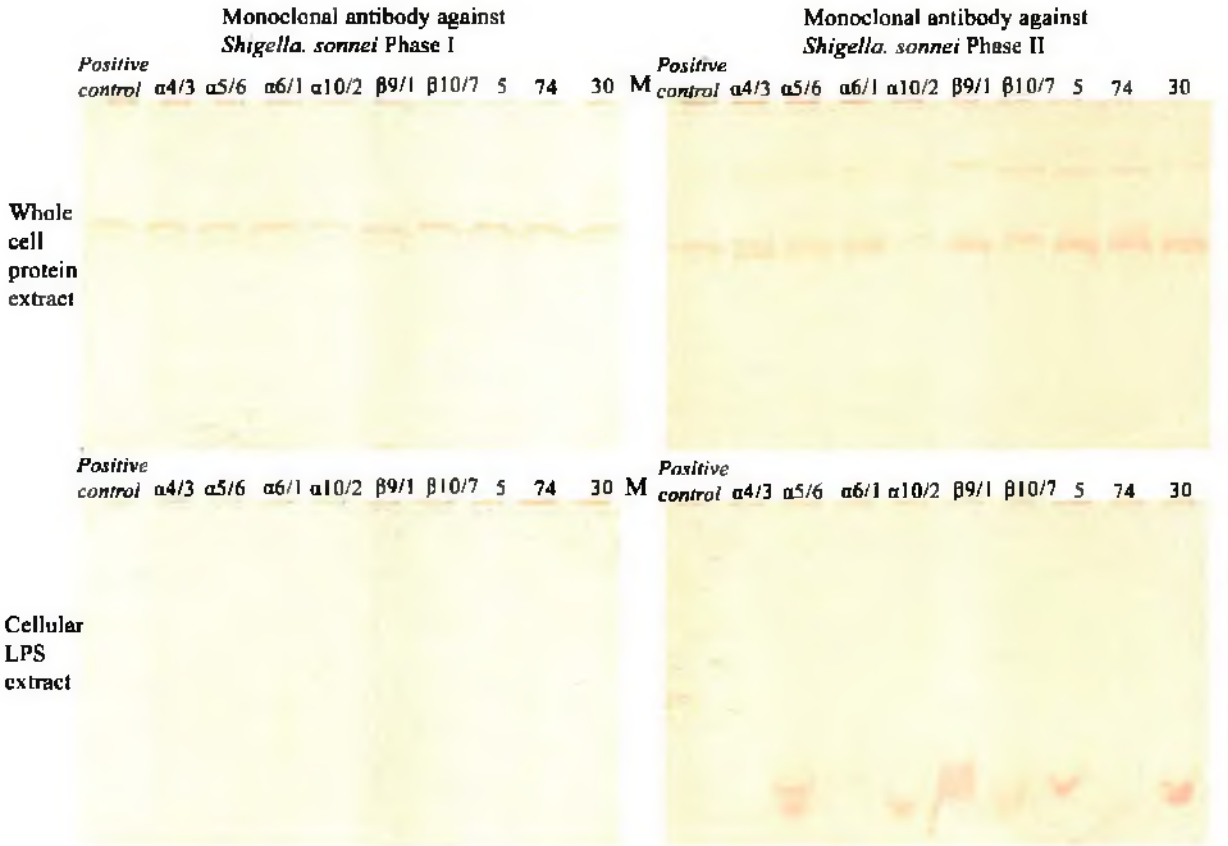


Fig. 3.19 Western blot analysis of total protein and LPS extracted from environmental *E. coli* isolates cross-reacted with anti-*Shigella sonnei* monovalent antisera.

3.6.2 Characteristics of cross-reactive *E. coli* isolates

3.6.2.1 Biochemical characteristics

Altogether 69 different *E. coli* isolates have been tested by using API 20E biochemical strips. Most of the isolates showed more than 90% identity with typical *E. coli*. Detailed sugar fermentation and amino acid decarboxylation pattern of the isolates were tested. Most of the isolates were positive for arabinose, sorbitol and mannitol. They varied in sucrose fermentation and the majority of these isolates could not ferment inositol. Most of the isolates were lysine and ornithine decarboxylase positive and arginine dihydrolase negative. The result has been presented in Table 3.4.

Table 3.4: API 20E biochemical profile index of the *E. coli* isolates.

API 20E code	Isolates	Significant taxa identified	%ID
1144552	α6/11, 1, S-1	*Escherichia coli* 1	99.1%
1044562	B4/2	*Escherichia coli* 1	93.8%
1044552	α14/9, 11, Q1/3,5	*Escherichia coli* 1	96.3%
5144572	S-10, α6/7, S1/6, α9/6, α13/4, γ4/9, α10/2, 78, 85, B10/7, B9/1	*Escherichia coli* 1	99.5%
5144152	S-12, γ7/5, α 4/3	*Escherichia coli* 1	97.7%
5044552	S4/9, B8/8, 66, α 3/5	*Escherichia coli* 1	99.8%
1144550	R2/10	*Escherichia coli* 1	97.7%
5144512	α6/2, B8/11, α14/19, γ7/12, α5/6,74	*Escherichia coli* 1	98.1%
5144722	α 2/12, B3/8	*Escherichia coli* 1	97.9%
5144552	6, S-5, γ3/8, α2/8, γ2/14, α6/1, 83, B1/3, 30	*Escherichia coli* 1	99.9%
5044553	α1/7, α7/6, Q2/7, 2, iso-2, R4/4, S1/1, R5/6	*Escherichia coli* 1	94.7%
5044542	γ2/10	*Escherichia coli* 1	98%
5044562	S4/9, α 2/3, S-8, S4/11	*Escherichia coli* 1	99.8%
1144552	α 10/4, 81	*Escherichia coli* 1	99.1%
5044472	α 5/4, S-3, S1/5	*Escherichia coli* 1	99.9%
5044120	γ6/4	*Escherichia coli* 2	92.3%
3044552	B10/13	*Escherichia coli* 1	96%
1144152	R5/3	*Escherichia coli* 1	91%
1144152	91	*Escherichia coli* 1	91.7%
7044500	α9/1	*Escherichia coli* 2	89%

3.6.2.2 Observation of virulence-associated properties of the cross-reactive isolates

The cross-reactive isolates possess a potential for use as probiotics for protection against shigellosis. So, the virulent properties are important character to assess their potentiality, viability and acceptability of the isolates as protective vaccine agent. Multiplex PCR assay has been used to detect nine different pathogenic genes in the isolates. All the isolates were PCR-negative for any of the nine genes assessed.

3.6.2.3 Plasmid profile analysis

The plasmid profile of the cross-reactive *E. coli* isolates was determined. Heterogeneous plasmid pattern were observed in most of the isolates. Out of sixty nine isolates, thirty seven contained plasmid. The size of the plasmid bands ranged between 250 bp and 43.6 kbp. Twelve isolates harboured multiple plasmids. A representative plasmid profile of agarose gel has been given in Fig. 3.20.

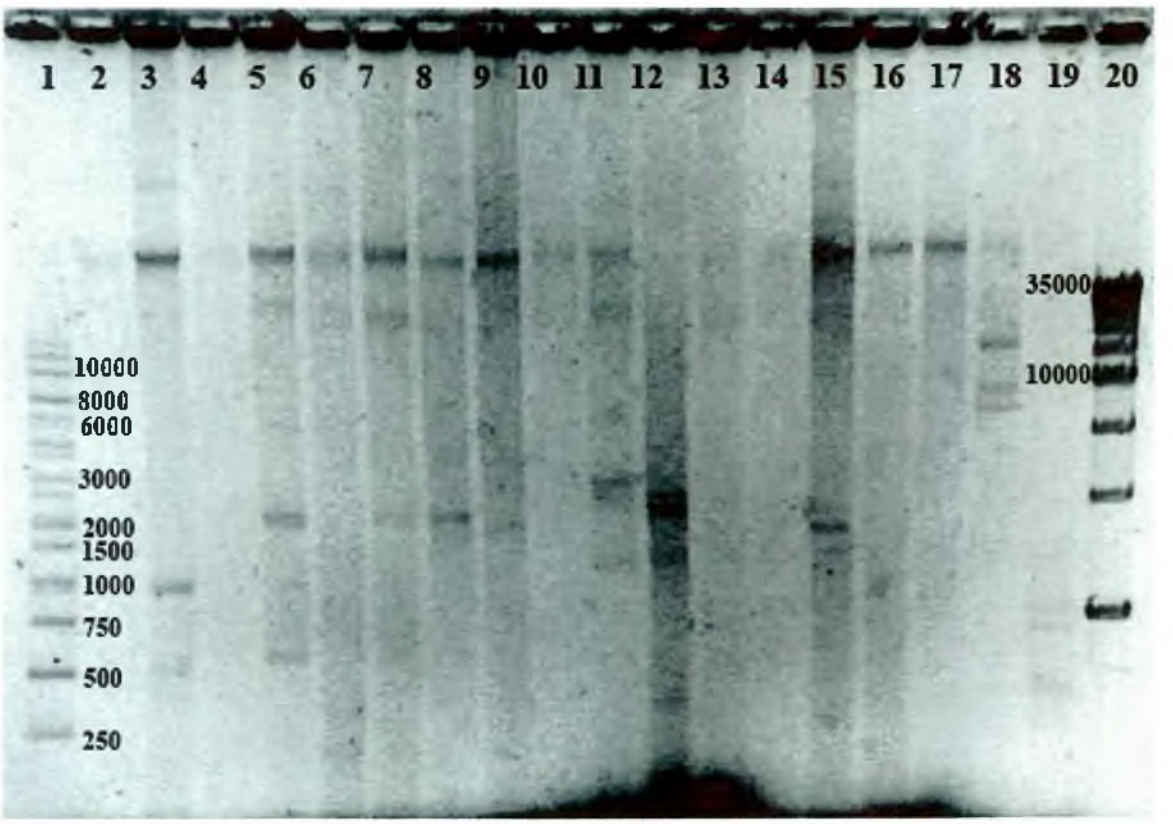


Fig. 3.20 Representative plasmid profile of the cross-reactive *E. coli* isolates (Lane-1: supercoiled DNA ladder, Lane-2-19: Plasmid profile of different *E. coli* isolates and Lane- 20: 35 Kb DNA ladder).

3.6.2.4 Observation of adhesin genes of the cross-reactive isolates

Adhesive properties are important factors contributing to initial attachment of the bacteria to epithelium, either intestinal or extraintestinal surfaces, followed by colonization. The isolates were screened for the presence of adhesin genes. Detection of pancreatitis associated protein (*pap)*, afimbrial adhesin (*afa*) and S-fimbrial adhesin (*sfa*) gene was performed by multiplex PCR. Only the isolates α6/7 and S-8 gave a band which is approximately 328 bp which indicated the presence of *pap* gene. No band appeared for two adhesin (*afa* and *sfa*) genes of pathogenic *E. coli* for any of the isolates.

Curli is a unique group of thin, coiled and fibrous structures found on bacterial surface which is necessary for adhesion of many members of Enterobactericeae. Almost 95% of the isolates under study were found to contain a gene specific for the structure (*csgA*) and for one of the regulator genes (*crl*) of curli expression. Three isolates; s1/6, s4/1 and 1 gave negative results for both the *crl* and *csgA* genes. Isolate 1 and S1/5 were *crl* gene positive but *csgA* negative, whereas S-3 possessed *csgA* without *crl*. The rest of the isolates were both *csgA* and *crl* positive. Representative figures for the agarose gel electrophoresis of *crl* and *csgA* genes are presented in Fig. 3.21 and Fig. 3.22 respectively.

Fig. 3.21 Representative agarose gel of *crl* gene PCR product of *E. coli* isolates (Lane-7: 1 kb plus DNA ladder, Lane-1 and 2: positive control, Lane-3: negative control and lane-4 through 15: *crl* PCR product of different *E. coli* isolates).

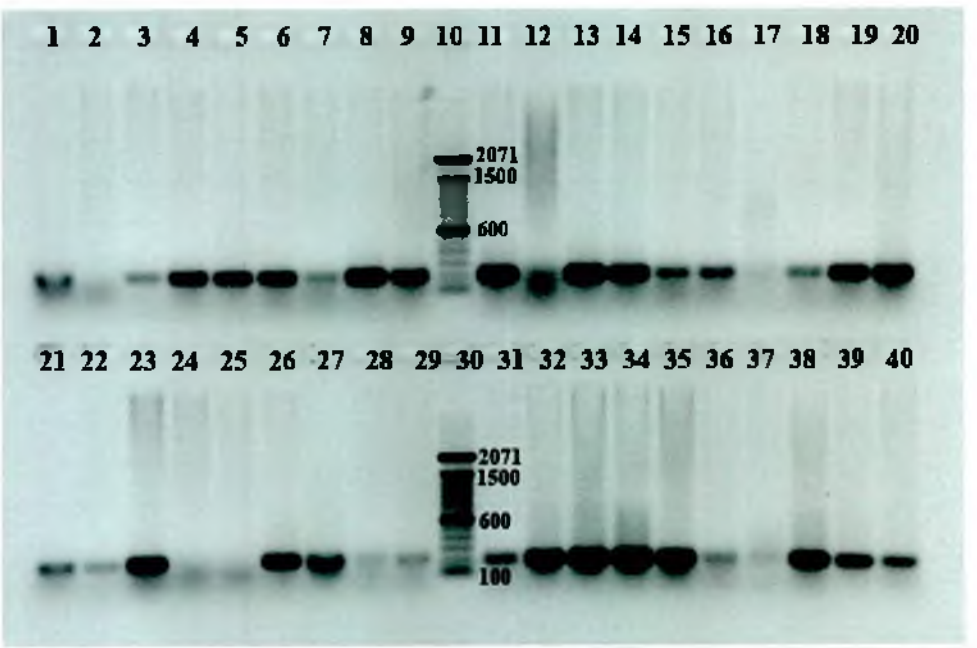
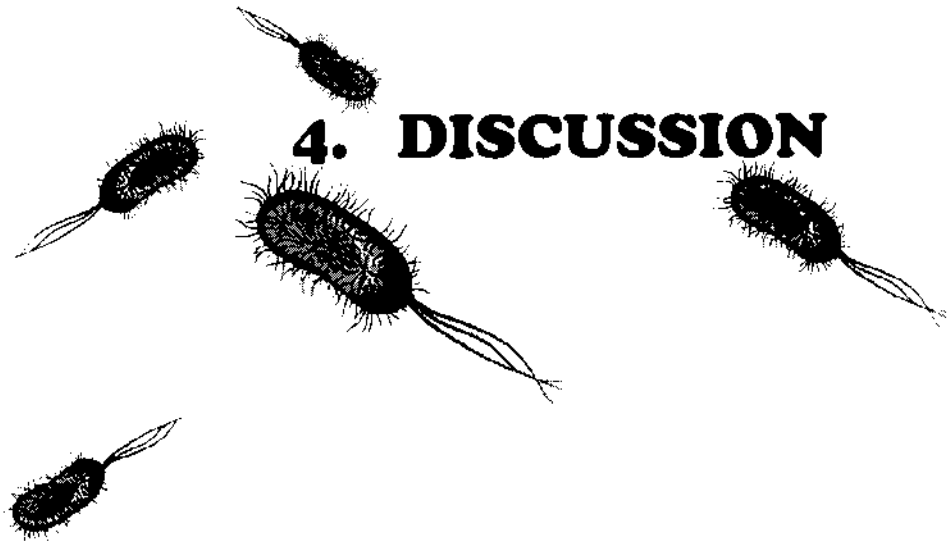


Fig. 3.22 Representative agarose gel of *csgA* gene PCR product of *E. coli* isolates (Lane-10 and 30: 1 kb DNA ladder, Lane-1: positive control, Lane-2: negative control and lane-3 through 40: *csgA* PCR product of different *E. coli* isolates).



4. DISCUSSION

Escherichia coli is one of the most important opportunistic pathogens in developing countries like Bangladesh. Yet its detection and source tracking methods are not well developed. Constant monitoring of the occurrence and survivability of the bacterium in the natural aquatic environments may predict such adaptive changes and assess vulnerability to *E. coli*-associated epidemics and outbreaks. Monitoring of the aquatic samples for *E. coli* pathovars through molecular tracking will help in seasonal risk assessment along with detecting regional distribution pattern and temporal fluctuations of the virulotypes. *E. coli* has long been used as an indicator, but reports on its adaptation and survival in environment introduces skepticism for further use as indicators. Well planned experiments and observations may provide an opportunity to explain some of the mysteries concerning the occurrence, adaptation and extended survival of *E. coli* in aquatic environment and the debate over the credibility of *E. coli* as an indicator of fecal pollution particularly in tropical countries.

4.1 Occurrence and prevalence of *E. coli* in aquatic systems

Global estimates indicate that each year, more than 120 million cases of gastrointestinal diseases are caused by swimming and bathing (Shuval, 2003). In recent years, there has been an increasing concern about the sanitary quality of beach sand and its potential influences on water quality of intertidal zone and on public health (Whitman and Nevers, 2003; Yamahara *et al.*, 2007; Heaney *et al.*, 2009; Whitman *et al.*, 2009). On the other hand, nearly 1.1 billion people in the world are lacking access to safe drinking water, and most of them are living in developing countries (WHO, 2009). In these areas, unsafe drinking water, mostly the surface water sources contaminated by waterborne pathogens, poses disastrous risk to public health. Millions of people die annually from epidemiological diseases due to unsafe drinking water (Boschi-Pinto *et al.*, 2008). Therefore, monitoring of water quality is an urgent issue for improving global access to clean water, and thereby protecting public health and saving lives. *Escherichia coli* is commonly found as normal microflora in the human gastrointestinal tract (Gill *et al.*, 2006) and is intricately involved in the lives of humans. *E. coli* isolates causing serious illness in humans are associated with at least six distinct disease presentations (Kaper *et al.*, 2004; Smith *et al.*, 2007). The first task of this study was to track *E. coli* and monitor potential pathogenic types of *E. coli* in tropical aquatic systems covering a nationwide area and to use different tracking approaches.

4.1.1 Molecular tracking of *E. coli* in aquatic envionment

In this study, *fliC*-PCR was performed for two different purposes. The first one is a generalized tracking method, which involved *fliC* gene amplification in DNA from water filtrate, pre-enriched and enriched cultures. Multiple bands appeared indicating presence of different *E. coli* clones (Fig. 3.1). The primers used in this study also amplified *fliC* gene of *Shigella* spp., *Klebsiella pneumonae* and *Salmonella enterica* (Fig. 3.2A) and thus Southern blot hybridization was performed (Fig. 3.2B) to track *E. coli*-specific *fliC* bands. The specificity of the DIG-labeled *fliC*-probe was checked and found to hybridize *E. coli* and most *Shigella* spp. (excluding *S. sonnei*). Most of the *fliC* genes amplified in environmental samples was also hybridized by the probe indicating presence of only *E. coli-Shigella* group in the aquatic systems.

4.1.2 Prevalence of *E. coli* diarrheagenic virulotypes

Multiplex PCR assays for detecting diarrheagenic *E. coli* have been used earlier for clinical specimens (Nguyen *et al.*, 2005) and environmental isolates. I have used multiplex PCR for the environmental monitoring of *E. coli* virulotypes that evades the traditional isolation procedure and offers a prompt, easy, and reliable method to estimate water-related health risks. DNA extracted from direct water filtrates (P) were assessed for the presence of nine virulence-associated genes of *E. coli,* but none of the samples were found to be positive. The concentration of pathogenic *E. coli* strains in the water samples might be below the detection limit of the PCR method; therefore, three different enrichment procedures were used to enrich *E. coli* virulotypes in the water samples. Pre-

enrichment (PE) culture medium that did not contain any selective agent was more effective for detection of *E. coli* harbouring the *eltB* and *vt1* virulence genes, while bile salt and streptomycin suppressed those virulotypes. *E. coli* harbouring the *estA*, *vt2*, and *eaeA* virulence genes was selectively enriched in bile salt-enrichment (BSE) medium. The reduction in the number of positive samples for these three genes in PE cultures could be explained by overgrowth of non-enteric bacteria present in water samples, which reduced the proportion of *E. coli* in the culture beyond the PCR detection limit. In this study, the detection limit of the multiplex PCR assay was checked and it was found that the ratio of virulent to avirulent *E. coli* strains must be at least $1:10^4$ to obtain positive PCR reaction (Fig. S3.3). The pCVD and *ipaH* genes were mostly detected in the streptomycin-enrichment cultures (SE). It can be assumed that the EAEC and EIEC virulotypes (or *Shigella* spp.) are mostly streptomycin resistant and thus selectively enriched in the SE. These *E. coli* strains might grow slowly and be outcompeted in PE and BSE media.

E. coli is the most common pathogen among travellers with diarrhea (Taylor and Echeverria, 1986). Among the *E. coli* virulotypes, the EPEC and ETEC virulotypes are the most important in terms of the total diarrheal episodes globally (Schmidt *et al.*, 1997). The ETEC virulotype is an important cause of diarrhea in the developing world, where there is inadequate supply of clean water and poor sanitation (Qadri *et al.*, 2005). The ETEC virulotype has already been reported to cause endemic diarrheal disease in Bangladesh (Albert *et al.*, 1999; Qadri *et al.*, 2000). Surface water sources in Bangladesh, in both rural and urban areas, are highly contaminated with the ETEC virulotype. In another study, ETEC strains were isolated from clinical samples as well as from 32% of water samples obtained from the ponds, rivers, and lakes around around the city of Dhaka and from the Matlab rural field area in Bangladesh, where cholera was endemic (Begum *et al.*, 2005). In this study, more than 60% of the surface water samples were found to be contaminated with the ETEC virulotype. The differences might simply be due to increased contamination in recent years or because rivers and sea beaches are more vulnerable to contamination compared to isolated ponds and lakes. The enrichment protocols used in the study probably increased the target organisms to above the detectable limit. In this study, *estA*-harbouring EPEC strains were mostly found in cities and industrial areas. All the five industrial sites harboured both the *estA* and *eltB* gene of ETEC. Out of nine sea beaches, four sites harboured *estA* alone and two sites harboured both *estA* and *eltB*. *estA* harboring ETEC were also prevalent in highly populated areas as eight of 11 sites harboured the *estA* and *eltB* gene pair. EAEC is reported to cause sporadic diarrhea in Bangladesh, particularly in children (Qadri *et al.*, 2005), as well as in Mexico, Chile, and Iran (Nataro *et al.*, 1987; Cravioto *et al.*, 1991; Bouzari *et al.*, 1994; Heuvelink *et al.*, 1995). However, this virulotype was the least prevalent type found in this experiment, although an increase in the prevalence of EAEC was observed in the winter. EAEC was found both in highly populated commercial and tourist sites as well as in rivers in remote villages. EHEC strains are not common pathogens in Bangladesh among hospitalized patients with diarrhea. These strains were found in only 9 out of 410 children in a hospital study in Bangladesh (Islam *et al.*, 2007). Some research findings are available on diarrheal epidemics in the country, but most of those are from regional and

1996; da Silva and da Silva Leite, 2002; Marques *et al.*, 2003) or cytotoxic necrotizing factor type 2 (Peres *et al.*, 1997). In this study, PCR was used to detect all the presently known *E. coli cdt*-alleles to determine the frequency and distribution of *cdt* among the natural water bodies in Bangladesh. Most of the PCR-primers worked perfectly with the environmental DNA samples to amplify *cdt* genes. A few incorrectly sized products were also produced in the *cdt-IA* PCR. These PCR products might have evolved from *cdt* gene-clusters through accumulating deletions or mutations and from nonspecific amplification of similar genes in other bacterial species present in the environment. Both the deletions/mutations in *cdt* gene cluster (Bang *et al.*, 2003) and interspecies sequence identity in CdtA and CdtB has been reported (Heywood *et al.*, 2005). In the present investigation, a range of virulence genes including toxin genes of diarrheagenic *E. coli* were detected, but coexistence with *cdt*-alleles could not be demonstrated.

4.1.4 *fliC*-genotyping, a tool for epidemiological investigation

To monitor the presence of *E. coli* outbreak strains as well as emergence of new *E. coli* H-types, the present study with *fliC*-sequence genotyping could serve as a routine protocol in epidemiological surveys. In this study, H4 was found to be the most prevalent H-type. Though a newly described epidemic outbreak strain was found to carry the H4 allele (a German outbreak in 2010 by the STEC O104:H4 strain), none of our environmental isolates possessed any of the Shiga toxin genes. Another STEC outbreak serotype, H25 was found as the second most prevalent type in this study. An H7 isolate was also found, which is usually known as the most common STEC serotype. These H-types were known to cause severe hemolytic uremic syndrome (HUS), but in this study, I could not identify genes for shiga toxins in any of the H4, H25 or H7 isolates. In case of enrichment cultures, I found virulence-associated genes in different samples but this could not be correlated with the *fliC* H-types. This approach can also be adopted with other environmental samples, food or specimens for rapid source tracking and monitoring distribution of outbreaks and identifying emerging and evolving H-serotypes.

4.2 Adaptation, survival and growth potential of *E. coli* in aquatic systems

Several studies have aimed at understanding of the growth potential of *E. coli* in environments through simulated field (e.g., mesocosm) or direct in-situ experiments (Byappanahalli and Fujioka, 2004; Byappanahalli *et al.*, 2006; Ishii *et al.*, 2006; Whitman *et al.*, 2006). Recent reports indicate that indigenous *E. coli* strains grow better than non-indigenous or transient strains, emphasizing that the indigenous strains are better adapted to the environmental conditions (Ishii *et al.*, 2006).

4.2.1 Salt-tolerance of *E. coli*

One part of this study aimed to isolate salt-tolerant *E. coli* from marine water samples and to assess their survival potential in marine water. The study was initiated by recovery of *E. coli* isolates from two different marine sources of the Bay of Bengal. Most of the isolates were identified with more than 99% similarity with the taxon *E. coli* 1 (typical *E. coli*), though these isolates have shown some minor discrepancies in reaction patterns. The isolate S-10 showed mannose negative and S-8 showed rhamnose negative reactions,

which were in contrast of typical *E. coli* reaction patterns. The isolate S-13 showed 62.1% and 35.6% similarity with the taxa *E. coli* 1 and *E. coli* 2 (atypical) respectively. The per cent ID less than 80% is usually not considered as satisfactory identification, but the API 20E profile did not suggest any other taxon for the isolate. For the confirmatory identification of S-13, 16S rRNA gene sequencing was done, which showed ≥98% sequence similarity with *E. coli* strains, and hence confirmed it as *E. coli*. For the survivability study of these isolates, natural freshwater (lake Dhanmondi) and marine water (Kolatoli point at Cox's Bazar) were used without any nutrient supplements. It has been reported (Gonzalez *et al.*, 1992) that the predation of enteric bacteria is the dominating factor in their disappearance from marine and freshwater aquatic ecosystem. Moreover, a number of studies showed that *E. coli* could survive longer in the absence of protozoa in estuarine and marine habitats (McCambridge and McMeekin, 1981; Sørensen *et al.*, 1999) because it is not an effective competitor in sea water. In this study, we have employed filtration followed by autoclaving to sterilize the natural water. The beneficial effect of autoclaving the water resulted in inactivation or destruction of bacteriophages and predators. Thus the antagonistic and competitive relationship as well as commensal interaction with other bacterial populations was absent in the experimental microcosm. Generally, under optimal conditions, the generation time of *E. coli* is 18-20 minutes at 37°C. To determine the generation time and to analyze the growth rate at high salinity, a growth curve experiment was performed for the isolates along with a clinical control isolate, *E. coli* O157:H7. The comparison of generation time in natural freshwater and marine water at 25°C might help us to predict the adaptive potential of marine *E. coli* isolates in their aquatic environment. The isolates, S-8, S-10 and S-13 showed shorter doubling time in media containing marine water (salinity 3.4%) than that of freshwater, which indicates that these isolates might have adapted to marine water. There is already a considerable number of reports for *E. coli* O157:H7 surviving in marine water and with capability to multiply in sterilized marine water (Miyagi *et al.*, 2001). In a water microcosm study, *E. coli* O157:H7 survived for more than 12 weeks at 8°C and for 7 to 12 weeks at 25°C (Wang and Doyle, 1998). The growth rate of *E. coli* O157:H7 was unaffected by the salinity, while another marine isolate, S-11, showed longer doubling time in media consisting of marine water than freshwater, which can be explained as a partial adaptation (or tolerance) to salinity. This isolate might have been disposed recently to the marine environment compared to the other three isolates. *E. coli* has never been considered as halophilic organism, but the higher growth rate of *E. coli* isolates in marine water suggests not only their tolerance or adaptation but a possible adaptive dependence of salinity. Microbes develop a variety of traits for survival, and growth in natural environments. Some of these traits include spatial separation or isolation, selection of habitat-specific strains, and the ability to utilize diverse organic substrates and competing under extremes of environmental factors. The findings of this study reveal that *E. coli* strains have already been adapted to the saline condition of the marine environment; though their emerging adaptive mechanisms are yet to be explored.

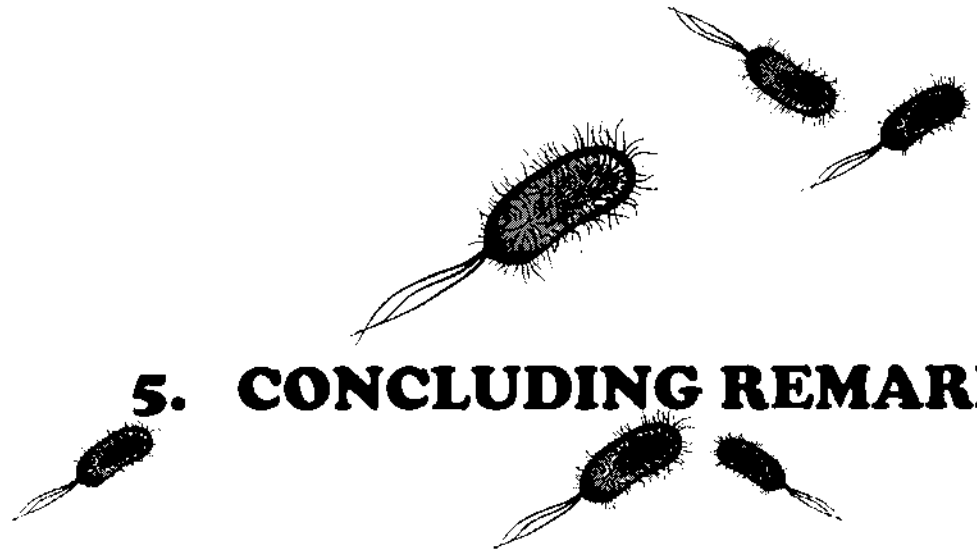
4.2.2 Fish as a secondary host of *E. coli*

Gordon and Cowling (2003) included some key phenomena about *E. coli* reservoir, it stated that the species was more commonly detected in mammals, less frequently in birds, and infrequently in fish, frogs, and reptiles. Aquarium experiments with fish immersed in water containing varying densities of *E. coli* inoculums revealed that higher levels of water contamination resulted in higher levels of *E. coli* in gut contents (Hansen *et al.*, 2008) suggesting that fish can be considered as a vector for *E. coli* rather than a reservoir. A microcosm study was performed in this study with gfp-marked strains which revealed significant bacterial association with fish (*Heteropneustes fossilis*) gut. Surface water film, sediments, as well as phytoplanktonic rhizoids also contained a high proportion of test organisms as compared to bulk water. The bacterial load on the fish skin corresponded to that of surrounding water. No sign of colonization by the test isolates DUM5, DH5α or O157:H7 could be detected in the fish gut. However, in DUM10 containing microcosm, fish intestinal content harboured an *E. coli* load of 2.0×10^6 cfu/g, indicating colonization and active growth of the bacterium in the fish gut. This particular isolate did not possess green fluorescence, but Rep-PCR and RAPD analyses revealed that the gfp-marked DUM10 strain and the bacterium isolated from the respective fish gut belonged to the same clone (Fig. 3.16), indicating loss of the pGLO plasmid while translocating from water to fish gut. This finding indicates a possible adhesion and association between environmental *E. coli* strains and aquatic biota. Fish might be a probable reservoir for the constant load of *E. coli* in water. This could be an alternate explanation for the presence of *E. coli* in water body without recent fecal discharge. I aimed to correlate this association with the physicochemical adhesion property as well as, presence of different adhesin genes in the test *E. coli* isolates. The hydrophobicity of the four *E. coli* test strains was measured, in the form of partition coefficient between aqueous suspension and a solid hydrophobic phase, no significant difference between isolates could be found. Quantitative biofilm formation assay reflected a correlation between the presence of *afa* gene and the potential to form biofilm on abiotic (polystyrene) surfaces. The afimbrial adhesin gene *afa* was detected in DUM5 and O157:H7 strain. None of the test strains was found to be *sfa* and *pap* positive when screened for adhesion-related genes by PCR. The regulatory gene *csg*A was present only in DH5α, whereas the gene for type-1 fimbriae was absent only in DUM5. The interesting finding of DUM10 should be further investigated in relation to colonization factors and multiplication in the fish gut.

4.3 Environmental *E. coli* isolates with potential as probiotics strains or vaccine candidates against shigellosis

Diarrheal disease continues to be a leading cause of morbidity and mortality particularly in the tropical countries, and is ranked as the fourth most common cause of death (Murray and Lopez, 1997). Shigellosis is an important cause amongst the diarrheal deaths; causing an estimated 163 million cases of dysentery annually and 1 million deaths worldwide, predominantly in children in developing countries (Kotloff *et al.*, 1999). In contrary of other diarrheagenic diseases, shigellosis cannot be treated by simple rehydration. An

antibiotic generally cures the disease but this treatment is often impeded by the emergence of resistant strains and by the excessive cost in concerned countries. Probiotic products seem to be promising in this respect (de Vrese and Marteau, 2007). With the progress of isolation, a number of suspected *E. coli* isolates were obtained that showed serological cross-reactivity with *Shigella*-specific highly sensitive monovalent and polyvalent antisera. Biochemical profiling using API 20E identification kit along with genotypic characteristics of the isolates was investigated. These isolates might possess traits to be used as probiotics, additionally having vaccine potential against *Shigella* infection. Out of 140 environmental *E. coli* isolates, 69 showed positive slide agglutination with *Shigella*-specific polyvalent and monovalent antisera (Figs. 3.18 and 3.19). Twenty seven isolates were found cross-reactive with multiple antisera concurrently (Table 3.3). Serologically cross-reactive environmental *E. coli* isolates were subjected to molecular analyses to assess their potential as probiotics. Multiplex PCRs were performed to facilitate detection of *E. coli* and *Shigella* spp. specific virulence genes. None of the isolates were found to contain any of the genes associated with virulence properties. PCR analysis was also conducted for the detection of some adhesin genes commonly found in *E. coli*, which are required for primary adhesion, adsorption, receptor mediated attachment and colonization on abiotic or biotic surfaces. Curli are the major proteinaceous component of a complex extracellular matrix produced by many members of Enterobacteriaceae, to be the envelope structure of major importance for surface colonization (Vidal *et al.*, 1998). Almost 95% of the isolates of this study were found to contain curli adhesin (*crl* and *csgA*) genes and hence were able to express curli fimbriae. Phenotypic expression of curli was also observed on congo red medium. Multiplex PCR was also performed for the detection of *pap*, *afa* and *sfa* adhesin genes normally expressed by uropathogenic and diarrhea-associated *E. coli* strains. Only 2 isolates, α6/7 and S-8 gave a positive reaction for the *pap* gene. No isolates were found to contain *afa* or *sfa* adhesion genes. Through the observation of phenotypic and serological characteristics, it can be concluded that the environmental isolates were identified as *E. coli*, which were serologically related to four *Shigella* serogroups. The lack of virulence genes in the environmental isolates and serological cross-reactivity could make them potential candidates for development of probiotic strains or vaccine candidates against shigellosis. Experiments regarding the safety of the isolates should also be performed. Their preventive effect of cross-protection should also be examined in animal models to assess their potential as safe and effective probiotics and vaccine candidates.



5. CONCLUDING REMARKS

5 CONCLUDING REMARKS

Three to five billion cases of diarrhea were reported in 1992 to occur annually, with approximately 2.6 million diarrhea-related deaths worldwide (Lima and Guerrant, 1992). But a recent review (Fischer Walker *et al.*, 2012) revealed that about 5 billion episodes of diarrhea occur in children alone annually, with 3.2 billion cases in Southeast Asia. Although diarrheagenic agents are known to be food- and waterborne, information about the influence of climatic factors or the occurrence and prevalence of the disease agents in surface waters is limited (Rowland, 1986). The findings of this study might contribute to the data vacuum that presently exists.

5.1 Tracking *E. coli* in aquatic system

- In this study, natural aquatic systems were monitored to detect the presence of *E. coli*. Forty six sites were selected, including both freshwater and marine origins, covering rivers and sea beaches at highly populated urban and rural areas, industrial regions, tourism sites as well as remote villages. Molecular tracking through *fliC*-PCR followed by Southern blot analysis successfully tracked the bacterium in water, assisted by the use of newly designed novel pre-enrichment and enrichment media to resuscitate those to a detectable limit.

- Occurrence and prevalence of diarrheagenic *E. coli* virulotypes were assessed in 46 different sites covering a large part of Bangladesh in three successive seasons. All the five diarrheagenic virulotypes were detected by multiplex PCR but with varying degrees of prevalence. EPEC and ETEC were found as the predominant virulotypes. Temporal fluctuations and regional distribution patterns for each of the virulotypes were identified, and differences in virulotype distribution between freshwater and marine water were recorded. Because of the lack of proper health care facilities in remote areas and the occurrence of many unreported home-treated cases where hospitalization is not available, a comparative analysis with clinical cases could not be performed in the relevant regions. However, our observations on the distribution and prevalence of *E. coli* virulotypes may assist in assessing seasonal risk and identifying vulnerable areas of the country prone to *E. coli*-associated outbreaks.

- Prevalence of cytolethal distending toxin (Cdt)-producing *E. coli* was also studied in the similar sampling sites by PCR targeting five different *E. coli-cdt* alleles (*cdt I* to *V*). This virulotype was previously reported to be associated with diarrhea in Bangladesh. In this study, *cdt-IA* was the most prevalent gene detected followed by *cdt-IIA* in both freshwater and marine sites. *cdt-IVB* and *cdt-VA* were also detected in certain freshwater and marine sites. *cdt-III*, *cdt-VB* and *cdt-VC* were detected in freshwater sites only. The prevalence of these genes was found to be influenced by the salinity of the water samples. No correlation between diarrheagenic *E. coli* virulotypes and *cdt* alleles was observed.

- Outbreaks with *E. coli* are always associated with certain O:H types. *fliC* genotyping could replace traditional serotyping and additionally cover typing of nonmotile, spontaneously agglutinating and newly evolving strains. This study encompasses adoption of *fliC*-genotyping to investigate water samples for outbreak strains bypassing bacterial isolation. H4 was the most prevalent H-type found in randomly selected samples followed by H10. A number of environmental *E. coli* isolates were also included in this study and they depicted similar prevalence pattern. The method can be adapted for epidemiological investigations especially during emergency outbreaks and to track mutated and emerging H-types.

5.2 Coining adaptation and survival of *E. coli* in aquatic systems

- The study also attempted to isolate, identify and characterize *E. coli* isolates from natural aquatic systems. A group of marine *E. coli* isolates were examined to determine the survivability and growth rate in natural waters. *E. coli* is generally not considered as halophilic but the higher growth rate of certain 'marine' *E. coli* isolates in marine water suggests a possible adaptation to salinity. These isolates also showed extreme salt tolerance. These findings reveal that *E. coli* strains have already been adapted to the saline condition of the marine environment; although their adaptive mechanisms are yet to be explored.

- Another microcosm study was performed with *gfp*-marked *E. coli* strains which revealed significant bacterial association with fish (*Heteropneustes fossilis*) gut. The test isolate, DUM10 could colonize and actively growth in the fish gut. This finding coined a possible adhesion and association between environmental *E. coli* and aquatic biota. Fish might be a possible reservoir for constant loading of *E. coli* in water.

- With the progress of isolation techniques, a number of suspected *E. coli* isolates were obtained that showed serological cross-reactivity with *Shigella*-specific highly sensitive monovalent and polyvalent antisera. The *E. coli* isolates have structural O-antigenic similarity with that of *Shigella* spp., as confirmed by Western blot analysis. These isolates possess a potential as live shigellosis vaccine candidates. Experiments on the safety and cross-protection capability are yet to be performed to allow safe probiotic administration or preventive vaccine agents.

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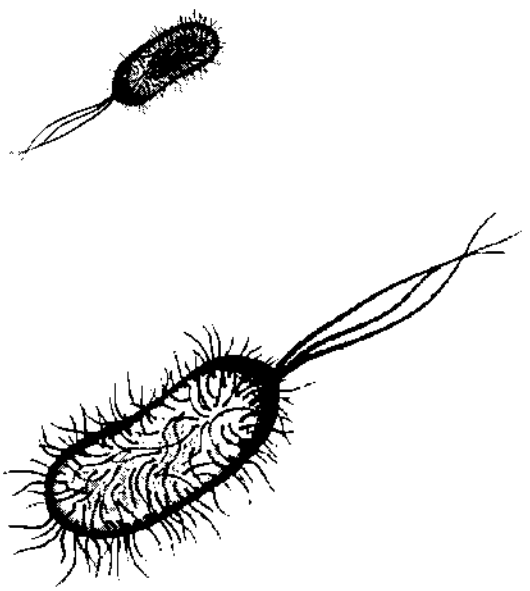

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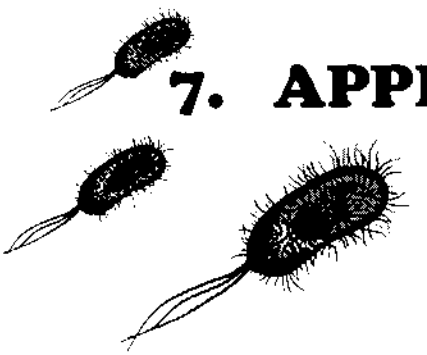


7. APPENDICES

This section deals with the supplementary information to the thesis. Appendix I and II contain the media composition, formulation of reagents and reaction mixers. Appendix III and IV solely entertained the supplementary information to the results section. The section is also available online:
http://vedlegg.uib.no/?id=2d2d43faed979b0fe24b311102f8285b.

APPENDIX I:
RECIPE OF MEDIA USED IN THE STUDY

Preparation of stock reagents and solutions has been given in this chapter. Preparations of working reagents or dilutions

Amino acid decarboxylation test: Amino acid 5g/L, Yeast extracts 3g/L, Glucose 1.0 g/L, Bromophenol purple 0.02 g/L and pH adjusted to 6.1.

Arginine Dehydrolase Medium: Peptone 1.0 g/L, NaCl 5.0 g/L, K_2HPO_4 0.30 g/L, Arginine HCl 10.0 g/L, Phenol red 0.01 g/L, Agar 3.0 g/L and pH adjusted to 7.2.

Brain heart infusion broth: Calf brain infusion solids 12.5 g/L, beef heart infusion solids 5.0 g/L, peptone 10.0 g/L, glucose 2.0 g/L, sodium chloride 5.0 g/L, di-sodium phosphate 2.5 g/L.

Carbohydrate fermentation medium: Trypton 10g/l, NaCl 5 g/l, Sugar 5 g/l, Phenol red 0.02 g/l and pH adjusted to 7.3.

Deoxycholate citrate agar (DCA): Lab-lemco powder 5.0 g/L, peptone 5.0 g/L, lactose 10.0 g/L, sodium citrate 5.0 g/L, sodium thiosulfate 5.0 g/L, ferric citrate 1.0 g/L, sodium deoxycholate 2.5 g/L, neutral red 0.025 g/L and agar 15 g/L.

Hectoen enteric agar (HE): Protease peptone 12.0 g/L, yeast extract 3.0 g/L, Lactose 12.0 g/L, Sucrose 12.0 g/L, salicin 2.0 g/L, Bile salt (no. 3) 9.0 g/L, sodium chloride 5.0 g/L, sodium thiosulfate 5.0 g/L, ammonium ferric citrate 1.5 g/L, acid fuchsin 0.1 g/L, bromophenol blue 0.65 g/L, agar 15.0 g/L.

Lysine Decarboxylase Broth: Yeast extract 3.0 g/L, Glucose 1.0 g/L, Lysine 5.0 g/L, Dye solution 2 mL

Luria Birtani Broth (LB): Bacto tryptone 10.0 g/L, Yeast extract 5.0 g/L, NaCl 10.0 g/L, NaOH (1N) 1.0 mL g/L and pH adjusted to 7.0 ± 0.2.

Kligler's Iron Agar (KIA): Beef extract 3.0 g/L, Yeast extract 3.0 g/L, Peptone 15.0 g/L, Protease peptone 5.0 g/L, Lactose 10.0 g/L, Dextrose 1.0 g/L, Ferrous Sulfate 0.2 g/L, Sodium Chloride 5.0 g/L, Sodium Thiosulfate 0.3 g/L, Phenol Red 0.024 g/L, Agar 15 g/L, Distilled water 1 liter with adjusted final pH7.4 ± 0.2.

MacConkey Agar No. 3 (dehydrated premixed media from Oxoid, UK)**:** peptone 20.0 g/L, lactose 10.0 g/L, bile salts 1.5 (No. 3) g/L, NaCl 5.0 g/L, neutral red 0.3 g/L, crystal violet 0.001 g/L, and agar 12.0 g/L.

MR-VP broth: Peptone 7.0 g/L, Dextrose 5.0 g/L, K_2HPO_4 5.0 g/L and pH adjusted to 6.9.

Muller-Hinton agar: Beef extract 2.0 g/L, bacto casamino acid 17.5 g/L, starch 1.5 g/L, agar 17.5 g/L.

Motility Indole Urea (MIU) Medium: Peptone 30.0 g/L, $KH_2PO_4$2.0 g/L, Sodium chloride 5.0 g/L, Phenol red 0.005 g/L, Urea 20.0 g/L, Agar 4.0 g/L, Distilled water 1 L with adjusted final pH7.2 ± 0.2.

Nutrient Agar (NA): peptone 5.0 g/L, NaCl 5.0 g/L, beef extracts 3.0 g/L, agar 15.0 g/L and pH adjusted to 7.0.

Simmon's Citrate Agar: NaCl 5.0 g/L, $MgSO_4$0.2 g/L, NH_4PO_4 1.0 g/L, K_2HPO_4 1.0 g/L, Sodium citrate 2.0 g/L, Agar 15.0 g/L, Distilled water 1 L with adjusted final pH 6.8 ± 0.2

T1N1 Soft Agar: Trypticase 10.0 g/L, NaCl 10.0 g/L, Agar 2.0 g/L and pH adjusted to 7.5.

Xylose Lysine deoxycholate (XLD) agar: Yeast extract 3.0 g/L, L-lysine 6.0 g/L, Xylose 3.75 g/L, Lactose 7.5 g/L, Saccharose 7.5 g/L, sodium deoxycholate 2.5 g/L, ferric ammonium citrate 0.8 g/L, sodium chloride 5.0 g/L, phenol red 0.8 g/L, agar 15.0 g/L.

APPENDIX II:
REAGENTS AND CHEMICALS USED IN THE STUDY

Preparation of stock reagents and solutions has been given in this chapter. Preparations of working reagents or dilutions were made from these stocks.

A. REAGENTS USED IN GENERAL

1. Normal saline

Prepared by dissolving 0.85 g NaCl in 100 mL of distilled water and sterilized by autoclaving upon adjusting pH at 7.5.

2. 3M NaCl

175.3 g of NaCl was dissolved in distilled water to a final volume of 1 L and autoclaved.

3. 10M NaOH

40 g of NaOH beads was dissolved in distilled water to a final volume of 100 mL and stored in airlock bottle.

4. Phosphate buffered saline (PBS)

8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4 and 0.24 g of KH2PO4 were dissolved in 800 mL of distilled water, pH was adjusted to 7.4 with HCl and the final volume was adjusted to 1 L and sterilized by autoclaving.

5. 1 M tris-Cl

121.1 g of Tris base was dissolved in 800 mL of distilled water, pH was adjusted to desired value as required for different purposes with concentrated HCl and the final volume was adjusted to 1 L and sterilized by autoclaving.

6. 0.5 M EDTA

186.1 g of Na2EDTA.2H2O and 20 g of NaOH beads were dissolved in 800 mL of distilled water, pH was adjusted to 8.0 with few drops of 10 M NaOH and the final volume was adjusted to 1 L and sterilized by autoclaving.

7. 3 M Na-acetate

40.81 g of Na2(CH3COOH).H2O was dissolved in 80 mL of distilled water, pH was adjusted to 5.2 with glacial acetic acid and the final volume was adjusted to 100 mL. The solution was sterilized by autoclaving and stored at 4°C.

8. 10% SDS

10.0 g of SDS (sodium dodecyl sulfate) was dissolved in 80 mL of double distilled water and final volume was adjusted to 100 mL. the solution was autoclaved sterilized and stored at 4°C.

9. TE buffer (pH 8.0)

TE buffer contained 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA, which was prepared by diluting and mixing the stocks of 1 M Tris-Cl and 0.5 M EDTA in double distilled water.

10. TAE buffer (50x)

242 g of Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0) were dissolved in water to a volume of 1 L. Working TAE buffer was made by adding 10 mL of this stock to 490 mL of distilled water.

11. DNA staining solution

10.0 µL of ethidium bromide solution was added to 100 mL of TAE buffer and stored in dark.

12. Gel loading buffer

Six times concentrated loading buffer consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll, 0.5 µg/mL Rnase in PCR grade water.

13. McFarland standard (0.5)

0.5 mL of 1.175% (w/v) barium chloride dehydrate (BaCL2.2H2O) solution was added to 99.5 mL of 1% sulfuric acid.

14. Bradford reagent

A. Stock solution: 300 mg of Sorva blue G (Comassie G-20) was dissolved in 300 mL methanol. 600 mL of 85% phosphoric acid was then added and stirred well.

B. Test solution: 150 mL of the test solution was dissolved in 850 mL of distilles water. The solution was then filtered with Whattman paper in dark onto a brown bottle.

15. Methyl red solution

0.1 g methyl red was mixed with 300 mL of 95% ethyl alcohol and 200 mL distilled water.

16. Barritt's reagent

Solution A was prepared by adding 5.0 g alpha napthol with 95.0 mL of absolute ethanol with constant stirring. Solution B was prepared by 40.0 g potassium hydroxide, 0.30 g creatine and 100 mL distilled water. KOH was dissolved in 75 mL od distilled water. The solution became warm and it was allowed to cool. Then creatin was added and stirred to dissolve. The solution was stored at 4 °C.

17. Kovac's reagent

p-dimethylaminobenzaldehyde was added with 75.0 mL of amyl alcohol and 25.0 mL of concentrated HCl.

B. REAGENTS USED FOR CHROMOSOMAL DNA EXTRACTION

Solution 1

50 mM tris-HCl buffer (pH-7.5) was added with 20% (w/v) sucrose, 50 mM EDTA and 20 mg/mL lysozyme.

Solution 2

50 mM NaCl, 1% (w/v) SDS and 200 µg of proteinase-k (5.0 µL from the 20 mg/mL stock solution).

Phenol- chloroform solution

Phenol-chloroform and isoamyl-alcohol solution mixed in ratios of 25:24:1 respectively saturated by TAE buffer.

C. REAGENTS USED FOR PLASMID DNA EXTRACTION

Solution 1

25 mM Tris-Cl, 10 mM EDTA and 50 mM glucose in deionized water at pH 8.0.

Solution 2

Freshly prepared solution with 1% SDS and 0.2 N NaOH

Solution 3

60 mL of 5M Potassium acetate, 11.5 mL of glacial acetic acid were mixed in 28.5 mL water and pH was adjusted to 4.8.

D. REAGENTS USED FOR SDS-PAGE

1. 30% acrylamide-bisacrylamide solution

Acrylamide	29.0 g
Bis-acrylamide	1.0 g
Distilled water	100 mL

2. Separating gel buffer (pH 8.8)

Tris base	18.17 g
SDS	0.4 g
HCl	To adjust pH at 8.8
Distilled water	Adjusted up to 100 mL

3. Stacking gel buffer (pH 6.8)

Tris base	6.1 g
SDS	0.4 g
HCl	To adjust pH at 6.8
Distilled water	Adjusted up to 100 mL

4. 10% Ammonium peroxodisulphate solution dissolved in deionized water (freshly prepared or aliquote and freeze at -30°C)

5. 10% SDS dissolved in distilled water and sterilized by autoclaving and stored at 4°C.

6. Sample loading buffer

0.5M Tris chloride (stacking gel buffer)	10.0 mL
SDS	10.0 mL
2-mercaptoethanol	1 mL
Glycerol	10 mL
Distilled water	19.0 mL

7. Tracking dye

Bromophenol blue	0.1 g
Distilled water	100.0 mL

8. Electrophoresis buffer (pH 8.3)

Tris base	3.028 g
10% SDS	10.0 mL
Glycine	14.413 g
Distilled water	Adjusted up to 1000 mL

9. Staining Solution (protein)

Coomassie Brilliant blue G250	0.2 g
Glacial acetic acid	10.0 mL
Methanol	25.0 mL
Distilled water	65.0 mL

10. Destaining Solution

Glacial acetic acid	10.0 mL
Methanol	25.0 mL
Distilled water	65.0 mL

11. Protein Extraction (sample lysis) buffer by 'Costas'

0.5M Tris chloride (stacking gel buffer)	70.0 mL
SDS	4.0 g
2-mercaptoethanol	2 mL
Glycerol	20 mL
Distilled water	Adjusted up to 100 mL

12. LPS (crude) exraction (solubilization buffer)

Stacking gel buffer	12.5 mL
10% SDS	30.0 g
2-mercaptoethanol	5 mL
Glycerol	10 mL
1% Bromophenol blue	1.0 mL
Distilled water	Adjusted up to 100 mL

13. Solubilization buffer-Proteinase K

Solubilization buffer (12)	30.0 µl
Proteinase K (200mg/mL)	5.0 µl

14. Composition of SDS-PAGE gel

Seperating gel (12.5%)	***10.05 mL***	***Stacking gel (4.5%)***	***2.552 mL***
Deionized water	3.2 mL	Deionized water	1.5 mL
Separating gel buffer	2.5 mL	Stacking gel buffer	0.625 mL
30% acrylamide bisacrylamide solution	4.2 mL	30% acrylamide bisacrylamide solution	0.375 mL
10% SDS	0.1 mL	10% SDS	0.05 mL
10% APS	40.0 µl	10% APS	10.0 µl
TEMED	10.0 µl	TEMED	2.5 µl

E. REAGENTS USED FOR SILVER STAINING

1. Silver staining Fixing solution

Deionized water	55.0 mL
Methanol	40.0 mL
Glacial acetic acid	5.0 mL

2. Oxidizing solution

Glacial acetic acid	10.0 mL
Methanol	25.0 mL
Distilled water	65.0 mL
Periodic acid	0.7 g

3. Staining solution (freshly prepared and use within 5 min of preparation)

0.1 M NaOH	28.0 mL
Ammonium hydroxide	2.0 mL
20% silver nitrate solution	Drop wise to achieve just saturation
Deionized water	Adjusted to 150.0 mL

4. Developer solution

Citric acid	50.0 mg

Dissolved in Deionized water	1.0 L
Formalin	0.5 mL

5. Color stopper solution

10% acetic acid	50.0 mL
Or Deionized water	50.0 mL

F. REAGENTS USED FOR WESTERN BLOT

1. Amido Black straining solution

Amido black	0.1g
1% acetic acid	100 mL

2. Blocking solution

Skim milk	2.0 g
PBS	100 mL

3. Phosphate buffered saline (PBS) pH 7.4 without adjustment

NaCl	8.0 g
KCl	0.2 g
Na2HPO4	1.44 g
KH2PO4	0.24 g
Distilled water	Adjusted up to 1.0 L

4. PBS Tween 20

PBS	100 mL
Tween 20	100 µl

5. 10% Sodium azide solution (preservative of antibody solutions)

Na azide	1.0 g
Distilled water	10.0 mL

6. Transfer /Blot buffer

Methanol	250.0 mL
Tris base	3.6 g
Glycine	18.0 g
Deionized water	1.0 L

7. 100mM Citrate buffer (pH 5.2)

Sodium citrate	2.94 g
Deionized water	100 mL
Glacial acetic acid	To adjust pH to 5.2

8. Substrate for HRP conjugate

DAB	5 mg tablet
100mM Citrate buffer	10.0 mL
Hydrogen peroxide	30 µl

9. 50mM Tris-HCl (pH 9.14)

Tris base	605 mg
Distilled water	20 mL
HCl	To adjust pH at 9.4

10. Substrate for AP conjugate (Fast red)

AS-MX Naphthol Phosphate	10.0 mg
Fast red TR	20.0 mg
50mM Tris-HCl (pH 9.14)	10.0 mL

11. Alkaline phosphate buffer (pH 9.0)

Tris base	1.2114 g
NaCl	0.8766 g
0.05M MgCl2	2.0 mL
HCl	To adjust pH at 9.0
Distilled water	Adjusted up to 100 mL

12. Substrate for AP conjugate (NBT/BCIP)

Alkaline phosphate buffer (pH 9.0)	10.0 mL
NBT	66.0 µl
BCIP	33.0 µl

G. REAGENTS FOR SOUTHERN BLOT HYBRIDIZATION

1. Washing buffer (400 mL)

Maleic acid	4.64 g
NaCl	3.5 g
Tween 20	1.2 mL
Distilled water	Adjusted up to 400 mL
pH	Adjusted to 7.5

2. Maleic acid buffer (400 mL)

Maleic acid	4.64 g
NaCl	3.5 g
Distilled water	Adjusted up to 400 mL
pH	Adjusted to 7.5

3. Detection buffer (400 mL)

Tris base	4.8456 g
NaCl	2.3376 g
Distilled water	Adjusted up to 400 mL
pH	Adjusted to 9.5

4. Depurination solution (1 L)

11 mL of HCl was added in 989 mL distilled water.

5. Denaturation buffer (400 mL)

NaCl	35.064 g
NaOH	8.0 g
Distilled water	Adjusted up to 400 mL

6. Neutralization buffer (400 mL)

Tris base	24.2 g
NaCl	35.064 g
Distilled water	Adjusted up to 400 mL
pH	Adjusted to 7.5

7. DNA transfer buffer, 20x SSC (1 L) pH 7.0

Tri-sodium citrate	88.23 g
NaCl	175.32 g
Distilled water	Adjusted up to 1 L

8. DNA hybridization buffer (DIG DNA hyb; Roche, Germany) for 100 mL

Working solution	Stock	amount
5x SSC	20x SSC	25 mL
50 % Deionized formamide	Deionized formamide	50 mL
0.1% N-Lauroylsarcosine	1% N-Lauroylsarcosine	10 mL
0.2 % SDS	10% SDS	200 µl
2 % Blocking reagent	Blocking reagent	2 mL
Deionized water	Deionized water	13 mL

9. Reagents supplied with the DIG DNA labeling kit

Vial 1: Unlabeled control DNA 1
Vial 2: Unlabeled control DNA 2
Vial 3: DNA dilution buffer
Vial 4: Labeled control DNA
Vial 5: Hexanucleotide mix
Vial 6: dNTP labeling mixture
Vial 7: Klenow enzyme, labeling grade
Vial 8: Anti-Digoxigenin-AP conjugate
Vial 9: NBT/BCIP
Bottle 10: Blocking reagent

10. Blocking stock solution (10x)
10 g of blocking reagent was dissolved in maleic acid buffer under constant stirring on a heated block at 65 °C to a volume of 100 mL. The solution was sterilized by autoclaving and stored at 4°C. Working blocking solution was freshly prepared by dissolving the stock in 1:10 dilution in maleic acid buffer.

11. Antibody solution
Anti-Digoxigenin-AP conjugate (vial 8) was centrifuged for 5 min at 10000 rpm in the original vial and pipet the necessary amount to dilute at 1:5000 (150 mU/mL) in blocking solution.

12. Color substrate solution
40 µl of NBT/BCIP (vial 9) was added to 2 mL of detection buffer freshly prior to work and stored protected from light

APPENDIX III
(SUPPLEMENTARY INFORMATION TO RESULT)

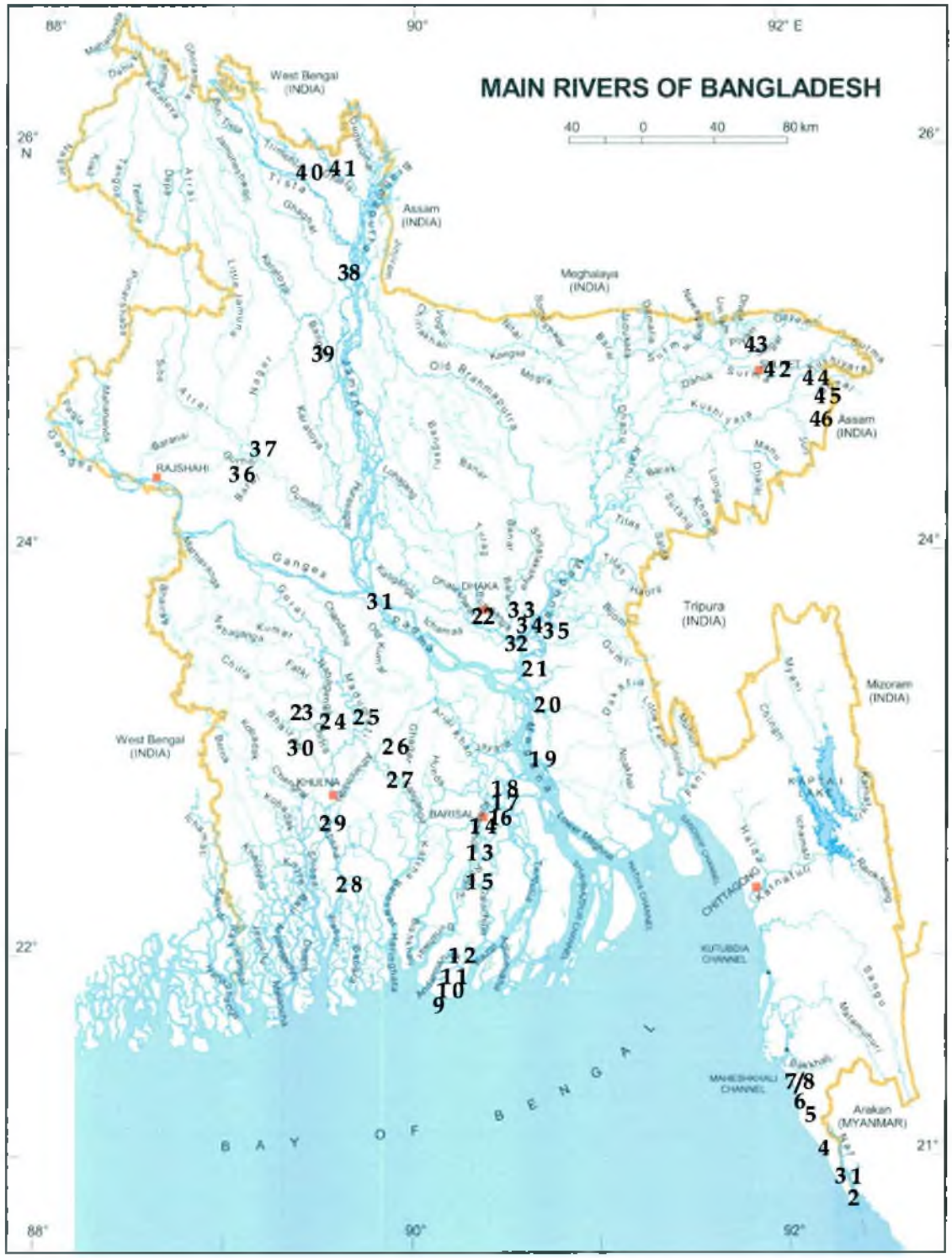


Fig. S3.1 Map illustrating 46 different sampling sites in Bangladesh. The positions on the map were determined by Global Positioning System (GPS) and numbered, which corresponded to the respective sampling sites summarized in Supplementary Table S1. The map has been adopted from banglapedia database [34].

Table S3.1 List of the sampling sites with coordinate GPS positions and physicochemical parameters measured in water samples.

Site	Site type	Season	GPS	Temp. (°C)	pH	Sal (‰)	Cond. (µS/cm)	TDS (ppm)	DO %	DO (mg/ml)
1.Eagle ghat	TRM	W	20°55'23.22"N	24.2	6.52	27.1	42300	21200	51	4.40
		S	92°16'9.72"E	31.2	7.53	37.2	55900	27900	73.4	5.42
		R		28.6	8.11	1.5	28600	1439	76.0	5.89
2. Teknaf	TRM	W	20°50'48.44"N	23.6	6.68	34.1	52000	25900	55	4.82
		S	92°16'17.64"E	33.6	8.28	37.2	56000	27900	85.4	6.02
		R		29.0	8.20	25.2	39300	19720	84.0	6.60
3. Noakhali	TRM	W	20°54'46.50"N	24.7	6.72	33.9	51500	25800	59	4.83
		S	92°13'33.72"E	34.4	8.60	37.1	55100	27800	139.7	9.81
		R		28.5	8.16	0.2	346	171	89.0	6.90
4. Shilkhali	TRM	W	21° 3'19.08"N	26.1	7.36	22.4	35700	17800	114	9.15
		S	92° 9'6.24"E	32.7	8.54	36.5	55000	27300	88.7	6.34
		R		29.1	8.27	27.2	42200	21400	85.2	6.61
5. Enani	TRM	W	21°14'0.75"N	25.5	7.58	34.7	52400	26300	70.9	5.80
		S	92° 2'40.92"E	34.5	8.48	35.9	54200	27000	88.3	6.18
		R		29.4	8.42	27.9	43300	21100	88.2	6.75
6. Himchori	TRF	W	21°21'18.12"N	24.8	8.96	0.1	206	103.4	65.5	5.45
		S	92°1'33.00"E	32.1	9.11	0.1	230	116	79.3	5.9
		R		28.3	8.25	0.1	192.2	96.9	88.4	6.93
7. Laboni point	TRM	W	21°25'32.22"N	25.6	7.85	33.7	51300	25600	62.9	5.03
		S	91°58'12.06"E	31.9	8.57	33.9	51700	25700	86	6.22
		R		28.0	8.51	29.2	45100	29200	64.0	5.1
8. Kolatoli point	TRM	W	21°24'12.69"N	25.3	7.35	33.7	51400	25700	60	4.8
		S	91°08'28.01"E	32.9	8.62	33.9	51200	25600	85.6	6.17
		R		29.1	7.54	28.7	44500	22200	78	6.9
9. Kuakata	TRM	W	21°48'46.26"N	24.7	6.73	12.2	20700	10250	81.4	6.6
		S	90° 7'20.16"E	29.3	8.11	20.5	33000	16440	86.3	6.5
		R		30.8	7.69	0.2	373	186.4	68.2	5.09
10. Mohipur	HP	W	21°51'21.48"N	23.9	6.94	12.2	20500	10240	79	6.4
		S	90° 7'29.04"E	29.3	7.93	21.7	34600	17290	75.7	5.66
		R		30.3	7.59	0.4	895	441	58.1	4.34
11. Hazipur	HP	W	21°53'42.42"N	23.6	7.43	12.2	20300	10310	80	6.6
		S	90° 8'22.26"E	29.5	7.44	21.8	34700	17330	75	5.73
		R		30.1	7.64	0.4	827	415	43.0	3.25
12. Khepupara	HP	W	21°59'0.72"N	23.6	7.7	8.7	14940	7460	73	6
		S	90°13'3.84"E	30.9	8.33	20.7	33100	16550	70	5.10
		R		30.5	7.43	0.6	1150	581	33.8	2.55
13. Lebukhali	HP	W	22°27'49.86"N	22.7	7.6	0.1	232	116.1	74.5	6.33
		S	90°20'28.74"E	31.1	8.68	0.1	171.7	85.6	71.8	5
		R		29.6	7.19	0.1	160.9	80.4	30.0	2.25
14. Dobdobia	HP	W	22°38'40.14"N	22.3	7.58	0.1	248	123.8	77	6.6
		S	90°20'35.88"E	31.4	7.63	0.1	164.7	82.9	75	5.42
		R		30.2	6.76	0.1	163.4	81.7	29.0	2.18

Table S3.1 *(Continued)* List of the sampling sites with GPS positions and physicochemical parameters of water sample

Site	Site type	Season	GPS	Temp. (°C)	pH	Sal (‰)	Cond. (µS/cm)	TDS (ppm)	DO %	DO (mg/ml)
15. Patuakhali ghat	HP	W	22°21'40.68"N	23.1	7.7	0.1	255	127	73	6.12
		S	90°21'0.12"E	31.4	8.88	0.1	282	141.2	73.3	5.34
		R		30.3	7.28	0.1	165.4.	83.0	58	4.3
16. kirtonkhola	HP	W	22°41'56.28"N	22.2	8.18	0.1	251	125.3	67	5.7
		S	90°22'32.52"E	29.8	8.52	0.1	155.9	77.9	87.3	6.59
		R		29.5	7.24	0.1	170.0	85.1	48.9	3.71
17. Aarial kha	RV	W	22°45'52.56"N	20.9	7.7	0.1	253	126.4	75.6	6.77
		S	90°26'38.82"E	28.2	8.30	0.1	155.7	77.9	75.6	5.83
		R		29.4	7.41	0.1	150.9	75.6	54.4	4.14
18. Kalabadar	RV	W	22°48'19.80"N	21.4	7.47	0.1	251	125.7	72	6.3
		S	90°27'13.50"E	27.1	8.16	0.1	148.1	74.1	81.3	6.43
		R		29.4	7.27	0.1	149.3	74.6	57.9	4.42
19. Meghna	RV	W	22°56'26.28"N	21.3	7.56	0.1	241	120.7	73	6.40
		S	90°38'21.00"E	27.6	8.16	0.1	141.1	70.5	76.5	5.93
		R		29.6	7.83	0.1	129.9	65.0	56.3	4.27
20. Chandpur / Dakatia	HP	W	23°15'20.82"N	21.7	7.38	0.1	215	107.4	60	5.27
		S	90°38'38.82"E	26.9	7.50	0.1	128.2	64.1	72.4	5.74
		R		29.6	7.24	0.0	109.5	54.8	54.3	4.13
21. Dholeeshori	RV	W	23°27'37.20"N	22.6	7.39	0.1	188.6	94.4	42.3	3.6
		S	90°35'6.30"E	27.8	6.89	0.0	86.9	43.3	71	5.52
		R		30.2	7.97	0.0	56.3	28.2	56.3	4.18
22. Buriganga	IC	W	23°42'21.24"N	22.1	7.36	0.4	761	739	0.5	0.03
		S	90°24'27.06"E	26.1	6.78	0.3	635	317	27	2.08
		R		29.8	7.69	0.1	156.8	78.4	50.2	3.80
23. Afra	RV	W	23°10'1.80"N	19.4	6.17	0.3	662	331	81.2	7.60
		S	89°26'31.20"E	31	7.27	6.4	11230	5610	4	3.16
		R		31.3	7.67	0.3	536	268	50.1	3.66
24. Rupganj	RV	W	23° 9'44.58"N	20.3	6.21	0.2	461	231	86.3	8.1
		S	89°30'7.44"E	30.9	7.57	4.0	7350	3670	60.7	4.5
		R		31.7	7.27	0.2	454	226	51.0	3.78
25. Kalna	HP	W	23°11'38.94"N	20.0	7.8	0.2	359	179.8	97	8.84
		S	89°41'21.96"E	30.3	8.01	0.9	1729	865	50.5	3.78
		R		32.8	8.43	0.1	189.2	94.3	67.2	4.75
26. Horidashpur	RV	W	23° 1'51.12"N	20.2	6.95	0.2	440	245	77	6.9
		S	89°49'3.48"E	31.8	7.80	0.2	429	215	49.1	3.58
		R		31.1	7.7	0.1	181.7	90.8	60.7	4.40
27. Mollar hat	RV	W	22°55'53.10"N	20.7	6.87	0.2	429	215	89	7.87
		S	89°48'27.36"E	31.4	7.92	1.8	3530	1767	60.3	4.41
		R		30.7	7.47	0.1	190.9	95.4	66.5	4.95
28. Mongla Port	TRM	W	22°28'20.10"N	20.8	7.41	1.8	3440	1722	86.3	7.78
		S	89°35'48.90"E	31.5	8.04	16.8	27400	13680	53.8	3.95
		R		29.9	7.86	2.9	5380	2690	66.0	4.97

Table S3.1 *(Continued)* List of the sampling sites with GPS positions and physicochemical parameters of water sample.

Site	Site type	Season	GPS	Temp. (°C)	pH	Sal (‰)	Cond. (µS/cm)	TDS (ppm)	DO %	DO (mg/ml)
29. Rupsha	IC	W	22°46'38.22"N	21.4	7.6	0.3	668	334	76.4	6.84
		S	89°35'10.38"E	33.8	8.3	16	26200	1310	53.1	3.75
		R		31.4	8.70	0.2	351	175.2	67.9	5.06
30. Nowapara	HP	W	23° 2'11.64"N	20.2	7.68	0.5	1113	555	83	7.63
		S	89°23'40.92"E	32.5	8.38	11.0	18630	9.31	45.1	3.25
		R		31.6	7.95	0.4	777	388	57.2	4.17
31. Aricha	HP	W	23°46'14.64"N	16.8	7.97	0.2	371	185.7	95	8.86
		S	89°47'13.68"E	30.5	9.46	0.1	292	145.7	67.2	5
		R		28.7	6.90	0.3	688	344	30.7	2.32
32. Narayanganj	IC	W	23°36'54.78"N	21.7	6.65	0.4	737	369	3.5	0.29
		S	90°30'20.64"E	32.8	7.97	0.2	509	254	33	2.47
		R		31.1	6.10	0.1	161.5	80.6	43.2	3.18
33. Kanchpur	IC	W	23°42'10.86"N	21.7	6.60	0.3	718	355	12.1	1.01
		S	90°30'59.88"E	30.8	6.9	0.2	428	214	35.2	2.57
		R		32.0	8.50	0.1	193.4	96.6	30.3	2.19
34. Boidderbazar	RV	W	23°38'58.02"N	19.5	6.9	0.1	130.2	65.2	89	7.84
		S	90°37'30.72"E	31	7.36	0.0	79.4	39.3	59.7	4.47
		R		30.1	6.54	0.0	56.8	28.9	54.5	4.09
35. Nangolbondh	RV	W	23°39'30.78"N	20.2	6.6	0.3	664	332	60.6	5.25
		S	90°34'12.00"E	31	6.55	0.1	174.6	87.2	55.2	4.11
		R		30.7	6.31	0.0	85.7	42.8	32.9	2.44
36. Narod	RV	W	24°23'59.10"N	24.7	6.3	0.4	759	373	18.5	1.48
		S	89° 0'32.10"E	34.4	8.1	0.6	1226	620	82	5.7
		R		29.8	7.61	0.8	1577	788	40.4	3.15
37. Singra	RV	W	24°29'59.64"N	23.4	6.95	0.1	150.5	75.3	84.5	6.84
		S	89° 8'22.62"E	33.9	8.9	0.1	147.6	73.8	93.7	6.64
		R		30.6	7.33	0.0	72.3	36.1	46.9	3.50
38. Jamuna	RV	W	25°18'52.68"N	23.5	6.8	0.1	276	137.8	88.8	7.25
		S	89°36'59.16"E	29.8	8.02	0.1	127.8	64	70.3	5.32
		R		28.8	7.22	0.0	75.4	37.7	41.4	3.16
39. Kortoa	RV	W	24°57'45.12"N	23.9	6.77	0.1	229	114.3	83.5	6.81
		S	89°20'48.60"E	34.3	6.53	0.1	145.3	72.6	67.7	4.70
		R		31.3	7.2	0.0	73.1	36.5	43.6	3.19
40. Tista	RV	W	25°47'21.72"N	23.8	7.1	0.1	130.4	65.2	97.2	7.9
		S	89°26'11.88"E	28.7	8.13	0.0	80.2	40.1	68.8	5.31
		R		27.1	8.49	0.0	63.3	31.4	43.4	3.43
41. Dharala	RV	W	25°49'17.28"N	22.5	7.6	0.1	273	135.5	94	8.1
		S	89°39'53.52"E	28.5	8.0	0.1	213	106.3	72	5.56
		R		27.7	6.86	0.0	99.6	50.0	45.7	3.58
42. Shurma	IC	W	24°53'16.38"N	21.7	7.94	0.1	297	152.7	51.9	4.51
		S	91°51'59.94"E	27.7	8.50	0.0	63.7	32.6	58	4.51
		R		27.0	7.76	0.0	60.7	30.3	53.0	4.12

Table S3.1 ***(Continued)*** **List of the sampling sites with GPS positions and physicochemical parameters of water sample**

Site	Site type	Season	GPS	Temp. (°C)	pH	Sal. (‰)	Cond. (µS/cm)	TDS (ppm)	DO %	DO (mg/ml)
43. Srigayan	RV	W	24°59'29.52"N	21.1	7.6	0.0	108.8	54.4	84	7.51
		S	91°51'7.74"E	28.1	7.23	0.0	58.2	29.1	69	5.36
		R		28.1	6.73	0.0	44.0	21.8	54.0	4.21
44. Kushiara	RV	W	24°50'15.42"N	22.8	7.1	0.1	213	106.7	87	7.33
		S	92° 4'54.78"E	27.4	7.5	0.0	75.7	37.7	63	4.95
		R		26.5	6.36	0.0	62.0	31.0	49.5	4.10
45. Sonai	RV	W	24°46'13.62"N	24.1	7.45	0.1	168.6	64.1	89	7.28
		S	92° 9'25.62"E	29.8	6.67	0.0	56.6	28.4	64.8	4.88
		R		29.6	6.4	0.0	56.9	28.5	50.2	3.8
46. Madhab-kunda	TRM	W	24°38'17.58"N	16.3	8.1	0.1	262	132.2	72.3	6.65
		S	92°13'28.80"E	27.2	7.56	0.0	107.8	53.9	69	5.42
		R		28.3	7.02	0.0	89.3	44.5	71.5	5.39

*GPS, Global Positioning System; Temp., temperature; Sal., salinity; Cond., conductivity; TDS, total dissolved solids; DO, dissolved oxygen; W, Winter; S, Summer; R, Rainy season; TRM, tourism site (marine); TRF, tourism site (freshwater); HP, highly populated area; IC, industrial site in a city; and RV, remote village site of big rivers.

Table S3.2 Detailed list of virulence genes identified in pre-enrichment and enrichment cultures by multiplex PCR in water samples from 46 different sites in three successive seasons.

Site	Season	DS	PR	BSE	SE	Type of virulotypes present
1. Eagle ghat	W	-	*eltB*	*eltB estA eaeA*	pCVD	ETEC EPEC EAEC
	S	-	-	-	-	-
	R	-	-	*eltB eaeA*	-	ETEC EPEC
2. Teknaf	W	-	*eltB*	*vt1*	-	ETEC EHEC
	S	-	-	-	-	-
	R	-	-	-	-	-
3. Noakhali	W	-	-	*eaeA*	-	EPEC
	S	-	-	-	-	-
	R	-	*eltB*	*eaeA*	-	ETEC EPEC
4. Shilkhali	W	-	-	*estA*	-	ETEC
	S	-	-	-	-	-
	R	-	-	-	-	-
5. Enani	W	-	*eltB*	-	-	ETEC
	S	-	-	-	*ipaH*	EIEC
	R	-	-	-	*eltB*	ETEC

Table S3.2 Detailed list of virulence genes identified in pre-enrichment and enrichment cultures by multiplex PCR in water samples from 46 different sites in three successive seasons (*Continued*)

Site	Season	DS	PE	BSE	SE	Type of virulotypes present
6. Himchori	W	-	*eaeA*	*eaeA*	*eaeA*	EPEC
	S	-	-	-	-	-
	R	-	*eltB*	*eaeA*	-	ETEC EPEC
7. Laboni point	W	-	*eltB*	pCVD	-	ETEC EAEC
	S	-	-	-	-	-
	R	-	-	*estA eaeA*	-	ETEC EPEC
8. Kolatoli point	W	-	-	*eaeA*	*eaeA*	EPEC
	S	-	-	-	-	-
	R	-	-	*estA eaeA*	-	ETEC EPEC
9. Kuakata	W	-	-	*estA eaeA*	-	ETEC EPEC
	S	-	-	-	-	-
	R	-	-	-	*eltB*	ETEC
10. Mohipur	W	-	*eltB eaeA*	-	-	ETEC EPEC
	S	-	-	-	*estA ipaH*	ETEC EIEC
	R	-	*eltB*	*eltB*	-	ETEC
11. Hazipur	W	-	*eltB*	-	-	ETEC
	S	-	-	-	-	-
	R	-	*eltB*	*eltB*	-	ETEC
12. Khepupara	W	-	*eltB estA*	*estA*	pCVD	ETEC EAEC
	S	-	-	-	-	-
	R	-	*eltB*	*eltB*	*eltB*	ETEC
13. Lebukhali	W	-	*eltB estA vt1 eaeA*	*eltB estA*	-	ETEC EPEC EHEC
	S	-	*eltB*	*eltB eaeA*	*eltB*	ETEC EPEC
	R	-	*eltB*	*eltB*	*eltB*	ETEC
14. Dobdobia	W	-	*eltB estA vt1 eaeA*	*eltB estA eaeA*	-	EPEC ETEC EHEC
	S	-	*eltB*	*eltB*	*eltB estA ipaH*	ETEC EIEC
	R	-	*eltB*	*eaeA*	-	ETEC EPEC
15. Patuakhali ghat	W	-	*eltB estA vt2 eaeA*	*eltB estA vt2 eaeA*	*eaeA*	ETEC EPEC EHEC
	S	-	-	*eltB*	*eltB*	ETEC
	R	-	-	*eaeA*	*ipaH*	EPEC EIEC

Table S3.2 Detailed list of virulence genes identified in pre-enrichment and enrichment cultures by multiplex PCR in water samples from 46 different sites in three successive seasons (*Continued*)

Site	Season	DS	PE	BSE	SE	Type of virulotypes present
16. Barishal ghat/ kirtonkhola	W	-	*eltB estA eaeA*	*eltB estA eaeA*	*eltB estA* pCVD	ETEC EPEC EAEC
	S	-	-	*eltB eaeA*	-	ETEC EPEC
	R	-	*eltB*	*eltB eaeA*	-	ETEC EPEC
17. Aarial kha	W	-	*eaeA*	*eaeA*	-	EPEC
	S	-	*eltB*	-	*ipaH*	ETEC EIEC
	R	-	-	-	-	-
18. kalabadar	W	-	*eaeA*	*eaeA*	-	EPEC
	S	-	*eltB*	*eltB*	*ipaH*	ETEC EIEC
	R	-	-	-	-	-
19. Meghna	W	-	*vt1 eaeA*	*eaeA*	-	EPEC EHEC
	S	-	*eltB*	*eltB*	-	ETEC
	R	-	-	-	-	-
20. Chandpur/ Dakatia	W	-	*eaeA*	-	-	EPEC
	S	-	*eltB*	-	-	ETEC
	R	-	*eltB*	-	-	ETEC
21. Dholeeshori	W	-	*vt1 eaeA*	*eaeA*	-	EPEC EHEC
	S	-	*eltB eaeA*	-	*ipaH*	ETEC EPEC EIEC
	R	-	*eltB*	-	-	ETEC
22. Buriganga	W	-	*eltB estA eaeA*	*eltB estA eaeA*	*eltB estA eaeA*	ETEC EPEC
	S	-	*eltB eaeA ipaH*	*eltB eaeA*	*eltB ipaH*	ETEC EPEC EIEC
	R	-	*eltB ipaH*	*eltB estA*	*eltB ipaH*	ETEC EIEC
23. Afra	W	-	*eltB vt1*	*vt2*	-	ETEC EHEC
	S	-	-	-	*ipaH*	EIEC
	R	-	*eltB*	-	*eltB*	ETEC
24. Rupganj	W	-	-	-	-	-
	S	-	-	-	*estA eaeA ipaH*	ETEC EPEC EIEC
	R	-	*eltB*	*eltB*	*eltB*	ETEC
25. Kalna	W	-	*eltB*	-	-	ETEC
	S	-	*eltB*	*eltB*	*eltB* pCVD *ipaH*	ETEC EAEC EIEC
	R	-	*eltB*	-	-	ETEC

Table S3.2 Detailed list of virulence genes identified in pre-enrichment and enrichment cultures by multiplex PCR in water samples from 46 different sites in three successive seasons (*Continued*)

Site	Season	DS	PE	BSE	SE	Type of virulotypes present
26. Horidashpur	W	-	*eaeA*	*eaeA*	-	EPEC
	S	-	*eltB*	*eltB eaeA*	*ipaH*	ETEC EPEC EIEC
	R	-	-	*eltB eaeA*	-	ETEC EPEC
27. Mollar hat	W	-	*eltB*	*eaeA*	-	ETEC EPEC
	S	-	*eltB*	-	*eltB ipaH*	ETEC EIEC
	R	-	-	-	-	-
28. Mongla Port	W	-	*eltB*	*estA eaeA*	-	ETEC EPEC
	S	-	-	-	*ipaH*	EIEC
	R	-	-	*eaeA*	-	EPEC
29. Rupsha	W	-	*eltB*	*eaeA*	*estA*	ETEC EPEC
	S	-	-	-	-	-
	R	-	-	*eltB vt2 eaeA*	-	ETEC EPEC EHEC
30. Nowapara	W	-	*eltB estA*	*estA*	-	ETEC
	S	-	-	-	*ipaH*	EIEC
	R	-	-	*eltB vt2 eaeA*	-	ETEC EPEC EHEC
31. Aricha	W	-	*eltB estA eaeA*	*eltB estA eaeA*	-	ETEC EPEC
	S	-	-	*eltB*	*eltB*	ETEC
	R	-	*eltB*	*eaeA*	-	ETEC EPEC
32. Narayanganj	W	-	*eltB estA eaeA*	*eltB estA*	*estA vt1*	ETEC EPEC EHEC
	S	-	*eltB ipaH*	*eltB*	*eltB estA*	ETEC EIEC
	R	-	*eltB ipaH*	-	*eltB ipaH*	ETEC EIEC
33. Kanchpur	W	-	*eltB estA eaeA*	*estA eaeA*	*estA*	ETEC EPEC
	S	-	*eltB*	*eltB eaeA*	*eltB*	ETEC EPEC
	R	-	*eltB*	*eltB eaeA*	*eltB*	ETEC EPEC
34. Boidderbazar	W	-	*eltB*	*eaeA*	*vt2*	ETEC EPEC EHEC
	S	-	*eltB*	*eltB*	-	ETEC
	R	-	*eltB*	-	-	ETEC
35. Nangolbondh	W	-	*eltB*	-	-	ETEC
	S	-	-	-	*ipaH*	EIEC
	R	-	*eltB*	-	-	ETEC
36. Narod	W	-	-	*vt1 vt2 eaeA*	*vt1eaeA*	EPEC EHEC
	S	-	-	*eltB vt2*	*eltB ipaH*	ETEC EHEC EIEC
	R	-	*ipaH*	-	*ipaH*	EIEC

Table S3.2 Detailed list of virulence genes identified in pre-enrichment and enrichment cultures by multiplex PCR in water samples from 46 different sites in three successive seasons (*Continued*)

Site	Season	DS	PE	BSE	SE	Type of virulotypes present
37. Singra	W	-	-	*eaeA*	*eaeA* pCVD	EPEC EAEC
	S	-	*eltB*	-	*ipaH*	ETEC EIEC
	R	-	*eltB*	-	-	ETEC
38. Jamuna	W	-	*eltB*	*eaeA*	*eaeA* pCVD	ETEC EPEC EAEC
	S	-	*eltB eaeA*	-	-	ETEC EPEC
	R	-	*eltB*	-	-	ETEC
39. Kortoa	W	-	-	-	-	-
	S	-	*ipaH*	-	*ipaH*	EIEC
	R	-	*eltB*	-	-	ETEC
40. Tista	W	-	-	-	-	-
	S	-	*eltB*	*eltB*	-	ETEC
	R	-	*eltB*	-	-	ETEC
41. Dharala	W	-	*eltB*	*eaeA*	*eltB* pCVD	ETEC EPEC EAEC
	S	-	-	-	-	-
	R	-	-	-	-	-
42. Shurma	W	-	*eltB estA*	*eltB estA eaeA*	*estA eaeA*	ETEC EPEC
	S	-	-	*eltB*	-	ETEC
	R	-	*eltB ipaH*	-	-	ETEC EIEC
43. Srigayan	W	-	*eaeA*	*vt2*	-	EPEC EHEC
	S	-	*eltB ipaH*	*eltB*	*eltB eaeA*	ETEC EPEC EIEC
	R	-	*eltB*	-	-	ETEC
44. Kushiara	W	-	-	*vt2*	-	EHEC
	S	-	*eltB*	*eltB*	pCVD	ETEC EAEC
	R	-	-	-	-	-
45. Sonai	W	-	*vt2*	*vt1 vt2*	*eltB eaeA* pCVD	ETEC EPEC EHEC EAEC
	S	-	*eltB*	*eltB*	*eltB ipaH*	ETEC EIEC
	R	-	*eltB*	-	*eltB*	ETEC
46. Madhabkunda	W	-	-	-	-	-
	S	-	-	*eltB*	*ipaH*	ETEC EIEC
	R	-	-	*eaeA*	-	EPEC

*DS, direct water samples; PE, pre-enriched culture; BSE, Bile salt enriched culture; SE, streptomycin enriched culture; W, Winter; S, Summer; and R, Rainy season.

Table S3.3 Summary of the results of multiplex PCR virulotyping of 46 different water samples collected in three successive seasons.

Categories of *E. coli* virulotypes and associated gene		Number of sites positive for different virulotypes of *E. coli.*		
		winter	**summer**	**Rainy season**
ETEC	*eltB*	13	24	32
	estA	2	2	2
	eltB+estA	14	2	1
	total	**29**	**28**	**35**
	proportion	**63.04%**	**60.86%**	**76.08%**
EPEC	*eaeA*	30	9	15
	eaeA+bfp	0	0	0
	total	**30**	**9**	**15**
	proportion	**65.21%**	**19.56%**	**32.60%**
EHEC	*vt1*	1	0	0
	vt2	1	1	0
	vt1+eaeA	5	0	0
	vt2+eaeA	3	0	1
	vt1+vt2	1	0	0
	vt1+vt2+eaeA	2	0	0
	total	**13**	**1**	**1**
	proportion	**28.26%**	**2.17%**	**2.17%**
EAEC	pCVD	**8**	**2**	**0**
	proportion	**17.39%**	**4.34%**	**0**
EIEC	*ial*	0	0	0
	ipaH	0	22	5
	ial+ipaH	0	0	0
	total	**0**	**22**	**5**
	proportion	**0**	**47.82%**	**10.86%**
No virulotype		**4**	**12**	**7**

Table S3.4 Influence of enrichment conditions on the detection of genes by multiplex PCR analysis of 46 different water samples collected in three successive seasons.

Genes detected	Direct pellet	Pre-enrichment	Bile salt enrichment	Streptomycin enrichment	Total samples positive for each gene
eltB	0	72	41	26	139
estA	0	11	18	10	39
pCV*D*	**0**	0	1	9	10
vt1	0	5	3	2	10
vt2	0	2	9	1	12
eaeA	**0**	20	45	11	76
bfpA	0	0	0	0	0
ial	0	0	0	0	0
ipaH	0	8	0	24	32
Total samples positive per enrichment	**0**	**118**	**117**	**83**	**318**
Total samples assessed	**138**	**138**	**138**	**138**	**552**

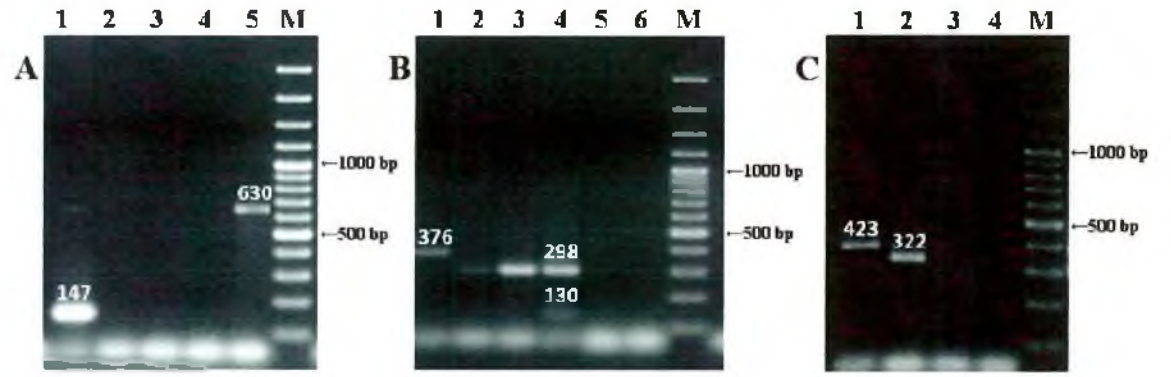


Fig. S3.2 Representative agarose gels (1.5%) stained with ethidium bromide, showing 100-bp DNA ladder (Fermentas) molecular weight markers (lane M), PCR amplified product of *estA* (147 bp) and pCVD (630 bp) in A, PCR amplified product of *eaeA* (376 bp), *vt2* (298 bp) and *vt1* (130 bp) in B and PCR amplified product of *ipaH* (423 bp) and *eltB* (322 bp) in C.

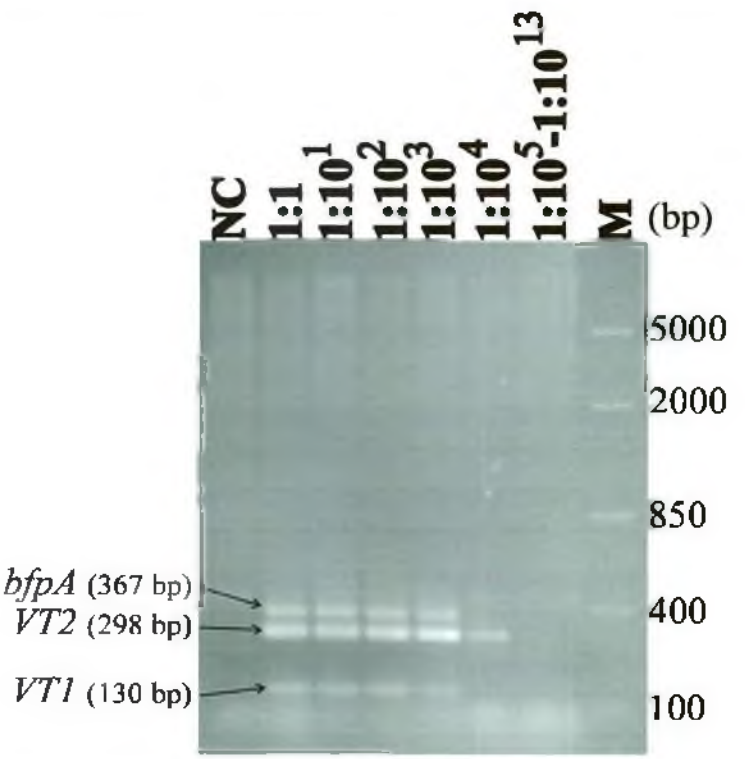


Fig. S3.3 Agarose gel (1.5%) stained with ethidium bromide, showing the detection limit of the multiplex PCR reaction. A series of mixed cultures were prepared having virulent (*vt1* and *vt2* possessing MMLA strain and *bfpA* possessing B171 strain) to avirulent (DH5α) *E. coli* cell ratios (determined by CFU/ml) of 1:1 to $1:10^{13}$. DNA was extracted and multiplex PCR was performed as done for the test DNA samples. The sensitivity of the assay was defined as the lowest ratio of diarrheagenic to non-diarrheagenic *E. coli* that yielded positive result. In case of *bfpA* and *vt1*, at least $1:10^3$ was the ratio of virulent to avirulent cells needed for a positive PCR reaction, while for *vt2* the ratio was $1:10^4$.

Table S3.5 Distribution of different types *cdt* genes in the sampling sites.

Sites	*cdtIA*	*cdtIIA*	*cdtIII*	*cdtIVB*	*cdtV*	*cdtVA*	*cdtVB*	*cdtVC*
Eagle Ghat	+	–	–	–	–	+	–	–
Teknaf	–	–	–	–	–	–	–	–
Noakhali	+	–	–	–	–	+	–	–
Shilkhali	+	+	–	+	–	–	–	–
Enani	+	+	–	+	–	–	–	–
Himchori	+	+	–	+	–	+	–	–
Laboni point	+	+	–	–	–	–	–	–
Kolatoli point	+	+	–	+	–	–	–	–
Kuakata	+	–	–	+	–	+	–	–
Mohipur	+	–	–	–	–	+	–	–
Hazipur	–	+	–	–	–	–	–	
Khepupara	+	–	–	–	–	–	–	–
Lebukhali	+	+	–	–	–	+	–	–
Dobdobia	+	+	–	–	–	–	–	+
Patuakhali Ghat	+	–	–	+	–	+	–	–
Barishal Ghat	+	+	+	–	–	–	–	–
Arial kha	+	+	–	–	–	–	–	+
kalabodor	+	+	–	–	–	–	–	–
Meghna	–	+	–	–	–	+	–	–
Dakatia	–	–	–	–	–	–	–	–
Dholessori	–	+	–	–	–	–	–	–
Buriganga	+	–	+	–	–	+	–	+
Afra	+	–	–	–	–	–	–	–
Rupganj	–	–	–	–	–	–	–	–
kalna	+	+	–	–	–	–	–	–
Haridashpur	+	–	–	–	–	–	–	–
Mollar hat	+	+	–	–	–	–	–	+
Mongla Port	+	–	–	+	–	–	–	–
Rupsha	–	–	–	–	–	–	–	–
Nowapara	+	–	–	–	–	–	–	–
Aricha	+	+	+	–	–	–	–	–
Narayanganj	+	+	–	–	–	–	–	–
Kanchpur	+	+	–	–	–	+	+	+
Boidderbazar	+	+	–	–	–	–	–	–
Nangolbondh	–	+	–	–	–	–	+	+
Narod	+	–	–	–	–	–	–	–
Singra	–	+	–	–	–	–	–	+
Jamuna	–	–	+	–	–	–	–	–
Kortoa	+	+	+	–	–	–	–	–
Tista	+	+	–	–	–	–	–	–
Dharala	–	+	–	–	–	–	–	–
Shurma	+	+	–	+	–	–	–	+
Srigayan	–	+	–	+	–	–	–	+
Kushiara	+	+	–	–	–	+	–	+
Sonai	+	+	–	–	–	–	–	+
Madhabkunda	+	+	–	–	–	–	–	–

Note: The '–' sign stands for absence and the '+' sign stands for the presence of the specific gene.

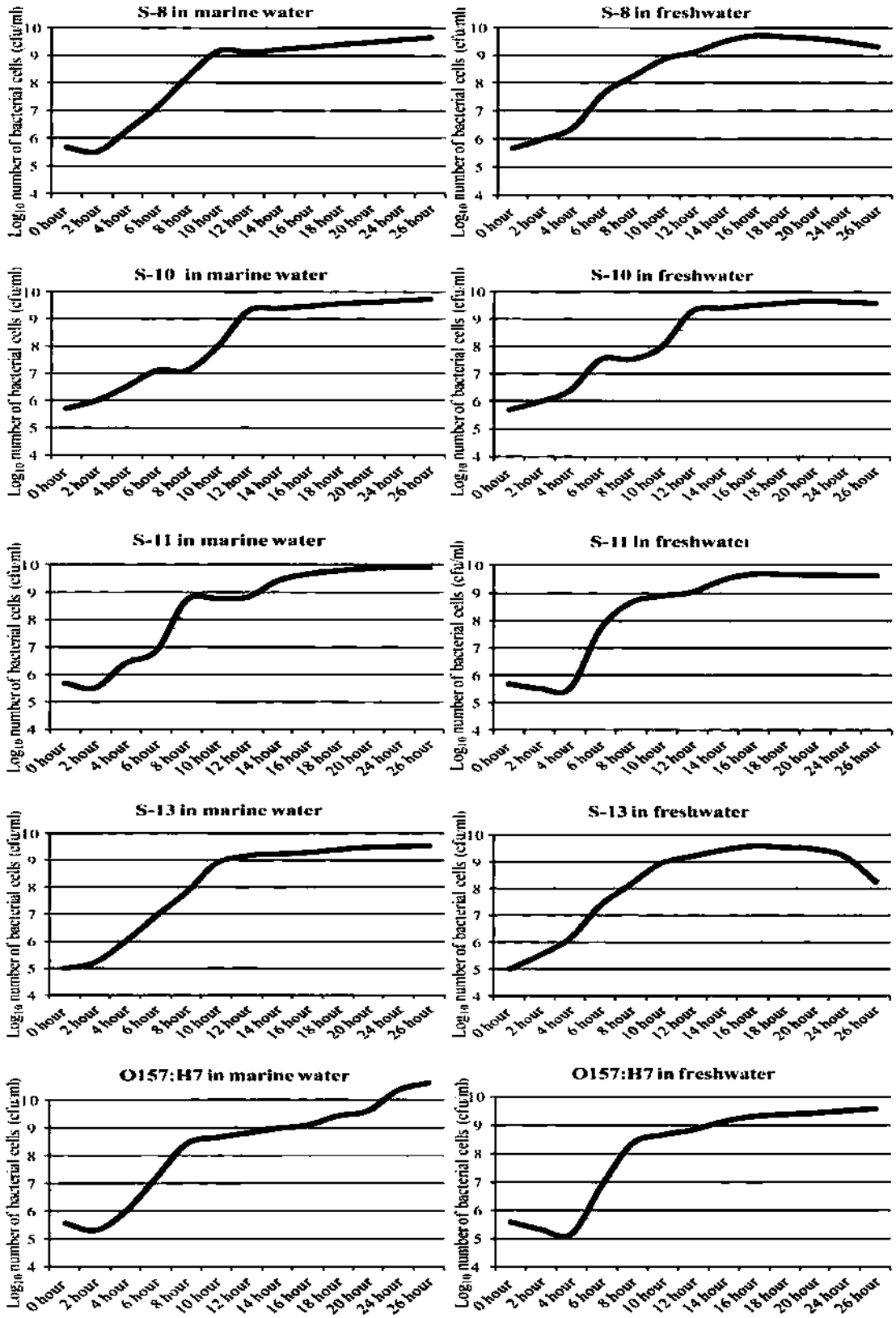


Fig. S3.4 Growth curves of the salt tolerant isolates in LB broth prepared by both marine water and freshwater.

Table S3.6: *E. coli* isolates from environmental water samples showing cross-reactivity with different *Shigella* spp. antisera (Denka Seiken, Japan).

Isolates cross-reactive to *Shigella dysenteriae* Polyvalent A antisera		Isolates cross-reactive to *Shigella dysenteriae* Polyvalent A1 antisera		Isolates cross-reactive to *Shigella flexneri* Polyvalent B antisera		Isolates cross-reactive to *Shigella boydii* Polyvalent C antisera	
Isolate code	Western blot	Isolate code	Western blot	Isolate code	Western blot	Isolate code	Western blot
β 4/2	+++	γ 3/8	+	γ 3/8	++	s 4/11	+
r 2/10	++	γ 7/12	+	γ7/12	–	θ 2/7	–
r 5/6	–	α 9/1	–	θ 2/7	+	β 4/2	–
s 4/9	–	r 2/10	–	β 4/2	+++	r 2/10	+
β 8/8	++	α 2/12	+++	α 9/1	+	α 1/7	+
β 8/11	–	α 1/7	–	r 2/10	++	s 1/5	+
α 1/8	–	α 14/19	+	α 1/7	+++	β 1/3	++
α 14/9	–	s 4/1	++	S 1/5	+	α 13/4	+++
α 6/7	+	α 9/6	+++	α 7/6	–	α 3/5	–
α 6/2	+	α 2/8	+++	α 2/3	–	γ 4/9	+++
α 6/11	–	γ 2/10	+	α 6/1	–	α 5/4	+
S- 10	+++	s 1/6	+	γ 2/14	+	β 8/8	++
S-12	+++	γ 7/5	–	S-8	–	γ 2/10	–
		α 7/9	+	83	+	γ 2/14	+++
		β 8/11	–	2	+	β 8/11	–
		S-5	–	1	+++	s 1/6	++
		6	+			γ 6/4	++
						γ 7/5	–
						α 5/6	++
						α 10/2	+++
						S-5	–
						S-10	–
						83	+
						2	–
						66	–
						11	++
						85	++

Table 3.6 (*continued*): *E. coli* isolates from environmental water samples showing cross-reactivity with different *Shigella* spp. antisera (Denka Seiken, Japan).

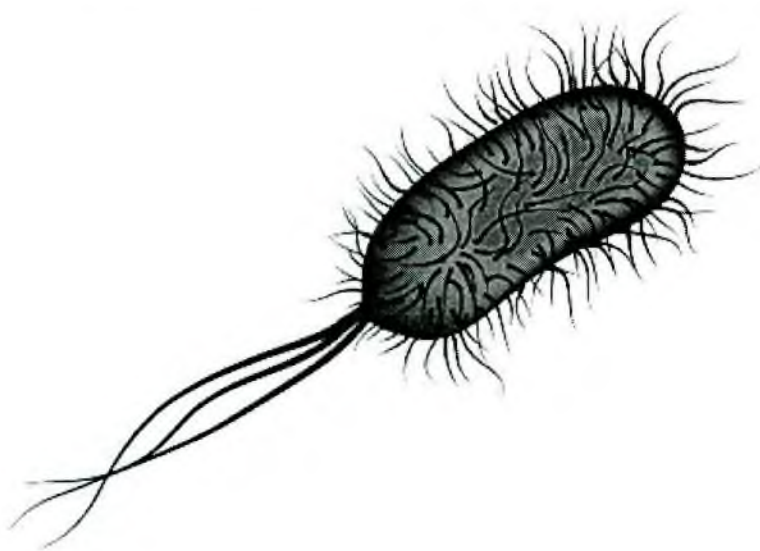
Isolates cross-reactive to *Shigella boydii* Polyvalent C1 antisera		Isolates cross-reactive to *Shigella boydii* Polyvalent C2 antisera		Isolates cross-reactive to *Shigella boydii* Polyvalent C3 antisera		Isolates cross-reactive to *Shigella sonnei* Polyvalent D antisera	
Isolate code	Western blot	Isolate code	Western blot	Isolate code	Western blot	Isolate code	Western blot
r 5/6	+++	γ 7/12	+	α 2/3	++	α 10/2	+++
r 4/4	++	r 2/10	+	r 5/3	+++	α 6/1	+
γ 2/14	–	β 10/3	–	β 8/8	++	β 9/1	–
s 1/6	++	s 1/1	++	α 6/7	++	α 4/3	+++
85	++	s 1/5	–	β 10/7	+	β 10/7	+++
78	++	α 7/6	–	81	+	α 5/6	–
S-1	+++	r 4/4	+	91	+++	74	–
Iso-2	+++	0 1/3	+++			30	–
		β 3/8	?			5	–
		r 5/6	+++				
		S-3	–				
		S-1	+++				

APPENDIX IV:
DNA SEQUENCED IN THE STUDY

This section contains a number of DNA sequences of fliC PCR products amplified from environmental *E. coli* isolates as well as in DNA extracted from pre-enrichment (PE), bile salt enrichment (BSE) and streptomycin enrichment (SE) cultures of different aquatic samples. The section also contains 16s rRNA gene sequence. This section along with other components of Appendices are available at http://vedlegg.uib.no/?id=2d2d43faed979b0fe24b311102f8285b.

8. PUBLICATION

Based on the observations of this study, one of the research article entitled 'Prevalence and distribution of different diarrhoeagenic *Escherichia coli* virulotypes in major water bodies in Bangladesh' has been published in the journal, *Epidemiology and Infection*, which have been annexed in this section. The other two articles entitled 'Survival potential of marine *Escherichia coli* isolates in high salt concentrations' and 'Serologically cross-reactive *Escherichia albertii* from aquatic environment: an avenue to search probable vaccine candidates for shigellosis' have been submitted in *Indian journal of Microbiology* and *Current Microbiology* respectively. The findings have also been presented in a number of international conferences.



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Prevalence and distribution of different diarrhoeagenic *Escherichia coli* virulotypes in major water bodies in Bangladesh

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SUMMARY

Escherichia coli, a prominent waterborne pathogen, causes a variety of gastrointestinal and extraintestinal infections that depend on virulence determinants. To monitor natural aquatic systems for virulence-associated genes of *E. coli*, multiplex PCR was used in a survey covering 46 major natural water bodies in Bangladesh. DNA was extracted directly from water samples as well as from pre-enriched and enriched cultures during three successive seasons and assessed for *E. coli* virulotype distribution. From the five virulotypes, genes from the enterotoxigenic (ETEC), enteropathogenic (EPEC), and enterohaemorrhagic (EHEC) virulotypes were detected consistently, but genes from the enteroinvasive (EIEC) and enteroaggregative (EAEC) virulotypes were traced only occasionally. ETEC was the most prevalent virulotype, followed by EPEC. However, EIEC and EAEC virulotypes could not be detected in winter or the rainy season, respectively. Specific regional distribution patterns of different *E. coli* virulotypes and their temporal fluctuations were identified. These observations may assist with assessing seasonal risk and identifying vulnerable areas of the country prone to *E. coli*-associated outbreaks.

Key words: Bacterial contamination, *Escherichia coli* virulotypes, water hygiene.

INTRODUCTION

Three to five billion cases of diarrhoea occur annually, with about 2·6 million diarrhoea-related deaths worldwide, of which 1·5 million deaths occur in children aged <5 years [1]. Global estimates indicate that each year, more than 120 million cases of gastrointestinal diseases are caused by swimming and bathing in wastewater-polluted coastal waters [2]. Thus, monitoring aquatic systems for faecal pollution is an important task. *Escherichia coli* is an abundant and normally harmless representative of the normal mammalian gut microflora. However, certain strains have virulent properties that cause a variety of gastrointestinal infections depending on specific arrays of pathogenic determinants, most of which are believed to be acquired by lateral gene transfer [3]. It has been suggested that the evolution of *E. coli* as a gastrointestinal pathogen is comparatively recent [3] and that it does not originate from a single ancestor. The physicochemical parameters greatly differ between primary (intestine) and secondary (extra-intestinal) habitats, necessitating adaptation of the discharged cells to the extra-intestinal environment. The differences in the selective forces acting in these two different environments have led to the evolution of a dual

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regulatory system in *E. coli*, which may also involve the emergence of distinct pathotypes [4]. Virulent *E. coli* strains are now considered as major diarrhoeagenic pathogens and are classified into several virulotypes according to their virulence mechanisms [5], e.g. enteropathogenic (EPEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohaemorrhagic (EHEC) *E. coli* strains. Diarrhoeagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed [6]. Multiplex PCR amplification has been adopted to detect almost all of these virulotypes in a single reaction by using primer pairs that target specific virulence genes and yield PCR products of different sizes that can be distinguished by gel electrophoresis [7]. In this study, we used multiplex PCR for environmental monitoring of *E. coli* virulotypes in aquatic systems in Bangladesh. The distribution of different *E. coli* virulotypes in surface water and their temporal fluctuations were observed during three successive seasons, which could help in assessing the seasonal risk for communities that are vulnerable to waterborne diseases as well as in understanding the environmental factors driving these fluctuations.

MATERIALS AND METHODS

Sample collection

Water samples were collected from 46 different natural aquatic locations in Bangladesh between December 2009 and January 2010 (winter season), in April 2010 (summer season) and in July 2010 (rainy season). The sampling sites included 35 freshwater and 11 marine/brackish sites covering a large part of the country (Supplementary Fig. S1). The sampling sites were chosen so that they included major rivers and sea beaches located in regions with higher human activity, economic importance, and tourist interest. The sites were divided into different groups as indicated in Supplementary Table S1, e.g. tourism sites at or near sea beaches or freshwater sites, highly populated commercial areas, industrial city locations, and sites at remote villages at big rivers. Physicochemical parameters, e.g. the temperature of, salinity of, conductivity of, and total dissolved solids (TDS) in the water samples, were measured by a sensION5 conductivity meter (Hach Company, USA), and pH and dissolved O_2 were measured by a CyberScan pH/DO Meter (Eutech Instruments, Singapore). The GPS coordinates were recorded by an eTrex Legend HCx personal navigator (Garmin Corporation, Taiwan). All the parameters were measured on site and are listed in Supplementary Table S1.

Enrichment

Sixty millilitres of each water sample were added to 20 ml of concentrated pre-enrichment (PE) broth to achieve the following final concentrations (pH 7): Lab-Lemco powder, 0·5 g/l; yeast extract, 1·0 g/l; peptone, 2·5 g/l; and NaCl, 2·5 g/l. After 8 h incubation at ambient temperature, 1 ml of pre-enriched culture was transferred to 50 ml of two different enrichment broths containing peptone (3 g/l), NaCl (1 g/l), K_2HPO_4 (1 g/l) and KH_2PO_4 (1 g/l) at pH 7, supplemented either with 0·15% bile salts (BSE) or with 50 μg/ml streptomycin (SE). The enrichment cultures were incubated for 16 h at 37 °C with shaking at 120 rpm.

PCR template preparation

DNA was extracted from pellets collected from direct water filtrate, from PE culture, BSE culture, and SE culture of each sample as described previously [8]. DNA extracted from direct water filtrate was further purified using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, Germany).

Multiplex PCR

Detection of virulence marker genes was performed by multiplex PCR [9, 10] with nine primer pairs (Table 1) targeting the following virulence-related genes: *eaeA* (intimin of EHEC and EPEC strains), *bfpA* (bundle-forming pilus of EPEC strains), *vt1* and/or *vt2* (Shiga toxins 1 and 2 of EHEC strains), *eltB* and/or *estA* (enterotoxins of ETEC strains), *ial* (invasion-associated locus in EIEC and *Shigella* spp.), *ipaH* (in the large invasive plasmid and chromosomes in *Shigella* spp. and EIEC strains), and pCVD (*Eco*RI–*Pst*I DNA fragment of pCVD432 of EAEC strains). Multiplex PCR was performed in three different sets by choosing primer pairs that generated PCR products that had different sizes and could easily be separated and distinguished by agarose gel electrophoresis [20]. PCR was performed in a 20-μl reaction mixture containing 1 μl template DNA, 0·2 μl of 2 U/μl DyNAzyme™ DNA polymerase, 2 μl of 10× buffer for DyNAzyme, 0·4 μl of a mixture of

Table 1. *List of oligonucleotide primers used in the multiplex PCR assay*

Primer	Target gene	Primer sequence	Ref.	Product size (bp)
Set 1				
ST	*estA*	5′-GCTAAACCAGTA$^{G}_{A}$GGTCTTCAAAA-3′ 5′-CCCGGTACA$^{G}_{A}$GCAGGATTACAACA-3′	[11]	147
SHIG	*ial*	5′-CTGGTAGGTATGGTGAGG-3′ 5′-CCAGGCCAACAATTATTTCC-3′	[12]	320
bfpA	*bfpA*	5′-TTCTTGGTGCTTGCGTGTCTTTT-3′ 5′-TTTTGTTTGTTGTATCTTTGTAA-3′	[13]	367
EA	pCVD	5′-CTGGCGAAAGACTGTATCAT-3′ 5′-CAATGTATAGAAATCCGCTGTT-3′	[14]	630
Set 2				
VT1	*vt1*	5′-GAAGAGTCCGTGGGATTACG-3′ 5′-AGCGATGCAGCTATTAATAA-3′	[15]	130
VT2	*vt2*	5′-ACCGTTTTTCAGATTTT$^{G}_{A}$CACATA-3′ 5′-TACACAGGAGCAGTTTCAGACAGT-3′	[16]	298
Eae	*eaeA*	5′-CACACGAATAAACTGACTAAAATG-3′ 5′-AAAAACGCTGACCCGCACCTAAAT-3′	[17]	376
Set 3				
ipaH	*ipaH*	5′-GCTGGAAAAACTCAGTGCCT-3′ 5′-CCAGTCCGTAAATTCATTCT-3′	[18]	423
LT	*eltB*	5′-TCTCTATGTGCATACGGAGC-3′ 5′-CCATACTGATTGCCGCAAT-3′	[19]	322

deoxynucleoside triphosphates (25 mM each), and 0·5 μl of 25 μM solution of each primer (Sigma-Aldrich, Germany). The thermocycler conditions used with a PTC 200 Peltier thermal cycler (MJ Research, USA) were as follows: initial denaturation at 96 °C for 4 min, 30 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, with a final extension at 72 °C for 7 min. PCR products (10 μl) were evaluated by electrophoresis on a 1·5% (w/v) agarose gel at 70 V for 30 min. The DNA bands were visualized and photographed under a UV illuminator (Bio-Rad, USA) after the gel was stained with ethidium bromide (Supplementary Fig. S2).

To determine the detection limit of the multiplex PCR, a series of mixed cultures were prepared with cell ratios (determined in terms of c.f.u./ml) ranging from 1:1 to 1:10^{13} (with tenfold dilutions) of an avirulent laboratory control strain (*E. coli* DH5α) and two virulent laboratory strains designated as MMLA (possessing *vt1* and *vt2*) and B171 (possessing *bfpA*). DNA was extracted and multiplex PCR was performed in the same way as for the test DNA samples. The sensitivity of the assay was defined as the lowest ratio of diarrhoeagenic to non-diarrhoeagenic *E. coli* that yielded a positive result. In the case of *bfpA* and *vt1*, a minimum ratio of 1:10^{3} for diarrhoeagenic to non-diarrhoeagenic *E. coli* was required for a positive PCR result, while the minimum ratio for *vt2* was 1:10^{4} (Supplementary Fig. S3).

Data analysis

A χ^2 test was used to assess the significance of the data. The relative standard used in the research was $P > 0{\cdot}05$.

RESULTS

Prevalence of virulence genes of diarrhoeagenic *E. coli*

Forty-six sampling sites covering a large part of Bangladesh were monitored for the presence of diarrhoeagenic *E. coli* in three consecutive seasons. Four different DNA templates, i.e. from direct water filtrate, PE culture, BSE culture, and SE culture of each water sample, were analysed to detect the presence of pathogenic genes. Using three sets of multiplex PCR assays, we analysed the distribution and seasonal fluctuations of nine different pathogenic genes possessed by five different *E. coli* virulotypes. The detailed results of the analysis have been given in Supplementary Table S2 and summarized in Supplementary Table S3. The use of a number of PE and enrichment media designed for this experiment

enabled enrichment of different virulotypes of *E. coli*. The PE medium was designed to resuscitate stressed or injured environmental bacteria, while the BSE medium was designed to enrich the enteric bacterial population, and streptomycin was added for enrichment of streptomycin-resistant *E. coli*. The following data were pooled from Supplementary Table S2 and have been compiled in Supplementary Table S4. A total of 138 DNA samples were evaluated per enrichment. No genes could be amplified from DNA extracted from direct water filtrate. Both *eltB* and *vtl* genes were mostly detected in PE cultures; 72 PE cultures were positive for *eltB*, but only 41 BSE cultures and 26 SE cultures were found to harbour this gene. Similarly, five PE cultures were positive for the *vtl* gene, but only three BSE cultures and two SE cultures were positive for this gene. Bile salt enabled enrichment for *E. coli* harbouring the *estA*, *vt2*, and *eaeA* genes. The *estA* gene was detected in 18 BSE cultures, 11 PE cultures, and 10 SE cultures. The *vt2* gene was detected in nine BSE cultures but only in two PE cultures and one SE culture. The *eaeA* gene was detected in 45 BSE cultures but only in 20 PE cultures and 11 SE cultures. SE enrichment medium successfully enriched EAEC and EIEC virulotypes in the water samples. The pCVD gene was detected in nine SE cultures, one BSE culture, and none of the PE cultures. The *ipaH* gene was detected in 24 SE cultures, eight PE cultures, but in none of the BSE cultures, indicating that bile salt suppresses EAEC and EIEC virulotypes. The presence of different virulence-associated genes in the three different enrichments was used to identify virulotypes harboured by a particular sample in a season.

In this investigation, ETEC was found to be the most prevalent virulotype among the five diarrhoeagenic *E. coli* virulotypes tested (Fig. 1*a*, Supplementary Table S3) from the sampling sites. The prevalence of the ETEC virulotype in winter, summer, and the rainy season was 63%, 61%, and 76%, respectively. Of the ETEC-containing samples, *eltB* was present in most cases, i.e. in an average of 60% of the sampling sites (Supplementary Table S3). *estA* was more prevalent during winter (34%) than during summer (8·6%) and the rainy season (6·5%). In most cases, *estA* was present along with *eltB*; only 4% of the sampling sites harboured *estA* alone (Supplementary Table S3). A total of six sites tested positive for *estA* alone, but with seasonal variations; sites 4 and 9 in winter, sites 7 and 8 in the rainy season and sites 10 and 24 in summer. Except for site 24, all these sites

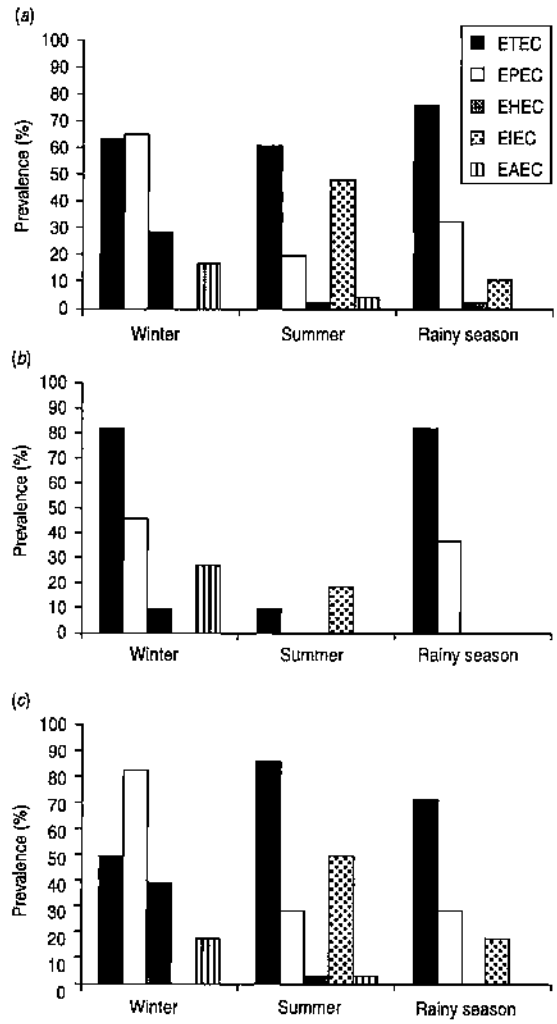

Fig. 1. The prevalence (in terms of proportion of positive sampling sites) of five different diarrhoeagenic *E. coli* virulotypes in the aquatic system of Bangladesh in three successive seasons. (*a*) Data compiled for all 46 sites, (*b*) in 11 marine sites, (*c*) in 35 freshwater sites.

were adjacent to southern sea beaches with marine or brackish salt concentrations.

EPEC was the second most prevalent type found. The prevalence of EPEC was 65% in winter, which was greater than that of ETEC in that season. In summer and the rainy season, only 19% and 32% of the sampling sites, respectively, showed positive results for the EPEC virulotype (Fig. 1*a*, Supplementary Table S3). In all the EPEC-positive sites, only the *eaeA* (no *bfpA*) gene was detected. The *eaeA* signal could also be due to the presence of EHEC strains. To aid interpretation, all *eaeA*-positive samples were considered as EPEC-positive samples,

and *vt1*- and/or *vt2*-positive samples were considered as EHEC-positive samples, regardless of the presence of *eaeA*. Like EPEC, the prevalence of EHEC (28%) and EAEC (17%) was also higher in winter (Fig. 1*a*). In summer, the prevalence of EHEC and EAEC was 2% and 4%, respectively, and in the rainy season, the prevalence of EHEC was also 2% but EAEC was absent. Unlike the other virulotypes, EIEC was relatively more prevalent in summer (48%). In the rainy season, EIEC was found in 11% of the sampling sites and was absent in winter. Although EPEC and ETEC virulotypes were almost evenly distributed throughout the country, we observed regional distribution patterns for certain virulotypes with distinct temporal variations (Fig. 2).

Significant differences were observed in the virulotype prevalence in freshwater and marine samples ($P>0{\cdot}05$). In marine samples (Fig. 1*b*), the ETEC virulotype was found to be the most prevalent virulotype both in winter (82%) and in the rainy (82%) season, but in summer its prevalence dropped to 9%. EPEC was the second most prevalent virulotype in winter (46%) and in the rainy (37%) season, but it could not be detected in summer. EHEC and EAEC virulotypes were detected only in winter, in 9% and 28% of the sampling sites, respectively, and EIEC was detected only in summer in 19% of the sites. In freshwater sites (Fig. 1*c*), the most prevalent virulotype was the ETEC virulotype, both in summer (86%) and the rainy (72%) season, but in winter the EPEC virulotype was the most prevalent virulotype (83%). Similar to the results for the marine samples (Fig. 1*b*), EIEC was also prevalent in freshwater samples (Fig. 1*c*) only in summer, but it had higher prevalence in freshwater samples (50%) than in marine samples (19%). Neither the EHEC nor EAEC virulotype was detected in the rainy season in the freshwater samples (Fig. 1*c*). In summer, the prevalence of EHEC and EAEC virulotypes in freshwater samples was still low (4%), but it increased to 29% and 18%, respectively, in winter.

Effect of seasonal variations on water quality and virulotype prevalence

Seasonal variations were found to affect the physicochemical parameters of natural water bodies in Bangladesh (Supplementary Table S1). Water temperature varied between seasons. In winter, the temperature ranged between 16 °C and 25 °C. Water temperature minutely differed between summer and the rainy season and ranged between 26 °C and 32 °C. The salinity of the water changed peculiarly with seasons in both fresh and marine sampling sites. In summer, the marine water samples possessed the highest salinity (~37‰), followed by that in winter (~34‰). In the rainy season, the salinity decreased due to rainwater runoff in certain sampling sites. From 11 marine sites, marked decreases in salinity were observed at six sites in the rainy season, while the salinities at the rest of the sites were rather stable. Significant ($P>0{\cdot}05$) differences were observed in virulotype prevalence in these two groups of marine sites in the rainy season (Fig. 3*a*, *b*). In both groups, only ETEC and EPEC virulotypes were detected in the rainy season, but their prevalence differed between the groups. In the group of marine sites where salinity was rather stable, the prevalence of ETEC and EPEC virulotypes was 60% and 40%, respectively (Fig. 3*a*). In the marine sites where salinity dropped in the rainy season, the prevalence of ETEC and EPEC was 100% and 26%, respectively (Fig. 3*b*).

The salinity of freshwater samples (rivers) distant from the sea rarely varied with season. However, the salinity of rivers closer to the sea increased in summer. Because of the lack of rain and blockage of the river flow (Farakka dam and Tista barrage) from the source, the water level decreased in rivers during summer, allowing the seawater to enter. The salinity varied with the distance from the sea. In the rainy season, the situation was reversed because of considerable water both from monsoon rainfall and the opening of the river barrages. Conductivity and TDS were found to be proportional to the salinity of the water.

From 35 freshwater sites, the salinity at 28 sites was mostly stable between seasons. The other seven sites had significantly increased salinity in summer. The pattern of the prevalence of virulotypes differed in these two groups of freshwater sites (Fig. 3*c*, *d*). In winter, the prevalence of the EPEC virulotype was the highest in the salinity-stable freshwater sites (Fig. 3*c*), whereas that of the ETEC virulotype was the highest in the salinity-increased freshwater samples (Fig. 3*d*). The EAEC virulotype was absent in those samples. In summer and the rainy season, the virulotype prevalence patterns also differed in these two groups of freshwater sites (Fig. 3*c*, *d*).

DISCUSSION

Multiplex PCR assays for detecting diarrhoeagenic *E. coli* have been used earlier for clinical specimens

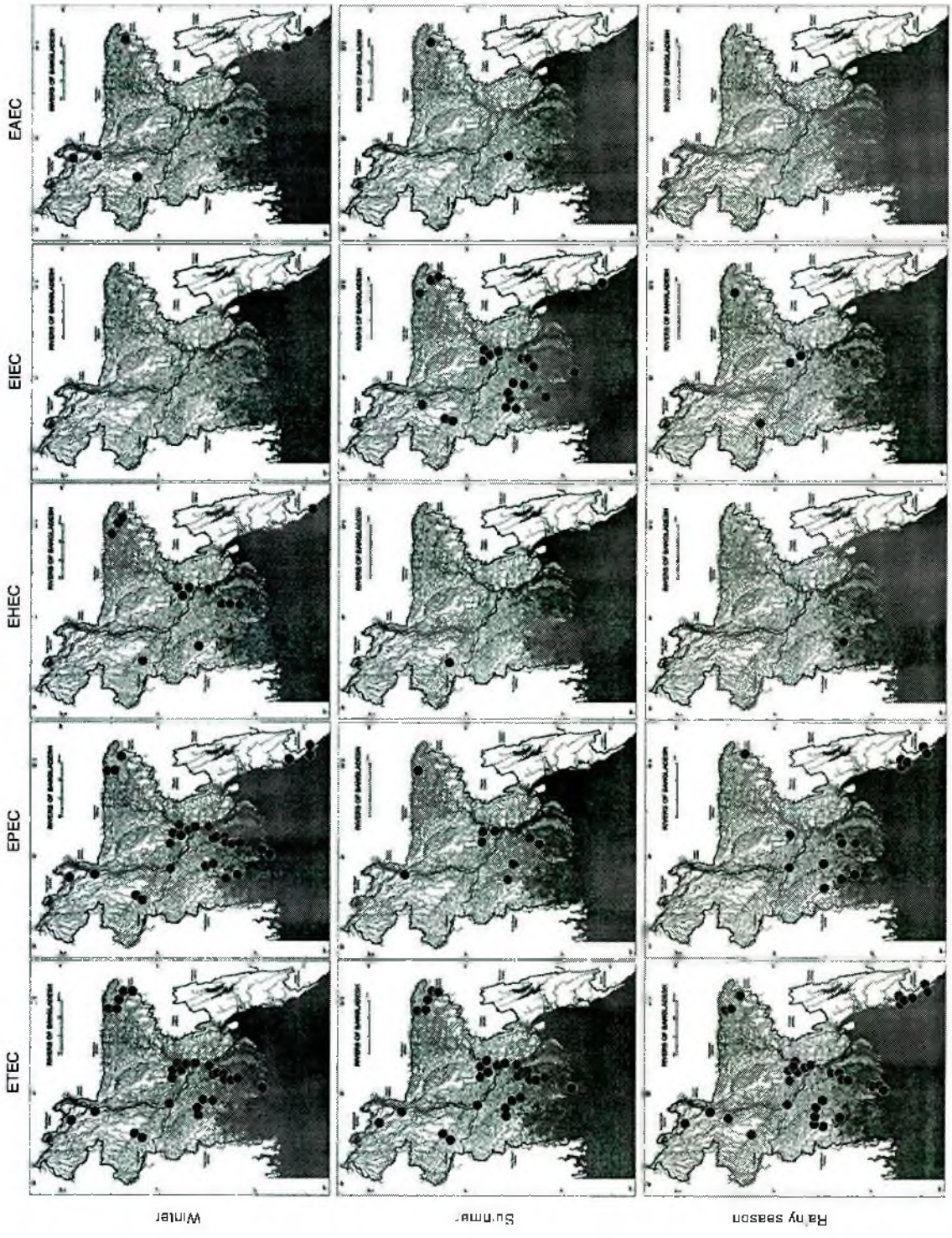

Fig. 2. The regional and temporal distribution patterns of five different diarrhoeagenic *E. coli* strains in three seasons in the aquatic system of Bangladesh. The black circles on the map of Bangladesh designate the presence of different diarrhoeagenic *E. coli* virulotypes in the corresponding geographical location. (*Source*: Banglapedia database [34], reproduced with permission.)

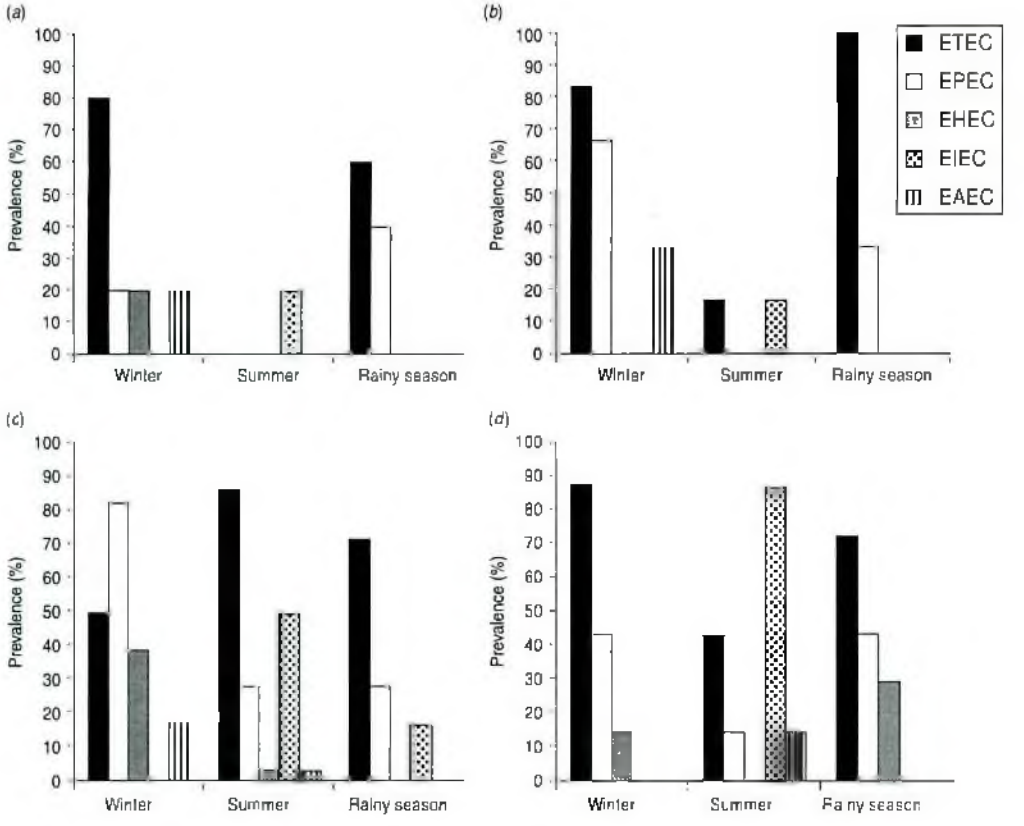

Fig. 3. The effect of seasonal fluctuations in salinity on the prevalence of diarrhoeagenic *E. coli* virulotypes in marine and freshwater sites. (*a*) The prevalence pattern of diarrhoeagenic *E. coli* in five marine sites where salinity did not fluctuate between seasons, (*b*) the prevalence pattern of diarrhoeagenic *E. coli* in six marine sites where salinity significantly dropped in the rainy season, (*c*) the prevalence pattern of diarrhoeagenic *E. coli* in 28 freshwater sites where salinity did not fluctuate between seasons, and (*d*) the prevalence pattern of diarrhoeagenic *E. coli* in seven freshwater sites where salinity significantly increased in summer.

[9] and environmental isolates. This work demonstrates the use of multiplex PCR for the environmental monitoring of *E. coli* virulotypes that evade the traditional isolation procedure and offers a prompt, easy, and reliable method to estimate water-related health risks. DNA extracted from direct water filtrates were assessed for the presence of nine virulence-associated genes of *E. coli*, but none of the samples were found to be positive. The concentration of pathogenic *E. coli* in the water samples might be below the detection limit of the PCR method; therefore, three different enrichments were used to enrich the *E. coli* virulotypes in the water samples. PE culture medium that did not contain any selective agent was more effective for *E. coli* harbouring *eltB* and *vt1*, while bile salt and streptomycin suppressed them. *E. coli* harbouring *estA*, *vt2*, and *eaeA* genes was selectively enriched in BSE medium. The reduction in the number of positive samples for these three genes in PE cultures could be explained by overgrowth of non-enteric bacteria present in water samples, which reduced the proportion of *E. coli* in the culture to below the PCR detection limit. In this study, we checked the detection limit of the multiplex PCR assay and found that the ratio of virulent to avirulent *E. coli* strains must be at least $1{:}10^4$ to obtain positive PCR results (Supplementary Fig. S3). The pCVD and *ipaH* genes were mostly detected in SE cultures. It can be assumed that EAEC and EIEC virulotypes (or *Shigella* spp.) are mostly streptomycin resistant and thus selectively enriched in SE cultures. These *E. coli* strains might grow slowly and be outcompeted in PE and BSE cultures.

E. coli is the most commonly isolated pathogen in travellers with diarrhoea [21]. In *E. coli* virulotypes, the EPEC and ETEC virulotypes are the most

important in terms of the total diarrhoeal episodes globally [22]. The ETEC virulotype is an important cause of diarrhoea in the developing world, where there is inadequate clean water and poor sanitation [23], and the ETEC virulotype has already been reported to cause endemic diarrhoeal disease in Bangladesh [24, 25]. Surface water sources in Bangladesh, in both rural and urban areas, are highly contaminated with the ETEC virulotype. In another study, ETEC strains were isolated from clinical samples as well as from 32% of water samples obtained from the ponds, rivers, and lakes around the clinical field sites [26]. In our study, we found that more than 60% of the surface water samples were contaminated with the ETEC virulotype. The differences might simply be due to increased contamination in recent years or because rivers and sea beaches are more vulnerable to contamination than isolated ponds and lakes. We can also predict that the enrichment protocol used in our study has successfully resuscitated more targets to the detectable limit. We found that *estA*-harbouring EPEC strains were mostly found in cities and industrial areas. All five industrial sites harboured both *estA* and *eltB* gene of ETEC. Out of nine sea beaches, four sites harboured *estA* alone and two sites harboured both *estA* and *eltB*. *estA*-harbouring ETEC were also prevalent in highly populated areas as 8/11 sites harboured the *estA* and *eltB* gene pair. EAEC is reported to cause sporadic diarrhoea in Bangladesh, particularly in children [23], as well as in Mexico, Chile, and Iran [27–30]. However, this virulotype was the least prevalent type found in the experiment, although an increase in the prevalence of EAEC was observed in winter. EAEC was found both in highly populated commercial and tourism sites as well as remote villages. EHEC strains are not common pathogens in Bangladesh in hospitalized patients with diarrhoea. These strains were found in 9/410 children in a hospital study in Bangladesh [31]. Some research findings are available on diarrhoeal epidemics in the country, but most of those are from regional and endemic studies performed in a few specialized hospitals. The information from these studies could help determine some trends in the prevalence of these strains in the country, but that would be a superficial picture.

In Bangladesh, the ETEC virulotype shows a very characteristic biannual seasonality with two separate peaks: one peak at the beginning of summer and the other just after the rainy season, but it remains endemic throughout the year [25, 32–34]. In our study of surface water samples, the ETEC virulotype did not follow any clear seasonal peak variation, but it showed greater prevalence in the rainy season. Prevalence data for other virulotypes in the country are not available, except for some regional epidemic studies. In our study, other virulotypes were found to be more prevalent in winter, except for the EIEC virulotype. The low water temperature might have contributed to the extended survival of these pathogens in water, but the increased prevalence of the EIEC virulotype in summer could not be explained. We found that the aquatic systems in the country harboured different virulotypes of diarrhoeagenic *E. coli* persistently throughout the year and showed a temporal fluctuation in the prevalence patterns of different virulotypes. ETEC and EPEC virulotypes were distributed throughout the country in three seasons (Fig. 2). The consistent presence and distribution of these virulotypes in the natural waters might be due to their extended survivability in water or the high level of ETEC- and/or EPEC-contaminated faecal discharge throughout the year. The virulotypes maintained regional distribution patterns that had temporal fluctuations. ETEC strains were absent from sea beaches in winter and summer but were present in the rainy season (Fig. 2). The decreased salinity of the water close to the seashore because of large amounts of runoff from the rivers in the rainy season might have an effect on the temporal occurrence of the ETEC virulotype in that region. In summer, the salinity was highest close to the seashore, which could be responsible for the absence of *E. coli* virulotypes in the region. The EHEC, EIEC, and EAEC virulotypes showed a concrete temporal pattern of occurrence, e.g. the EHEC and EAEC virulotypes were prevalent in winter and the EIEC virulotype in summer. Because of the lack of proper healthcare facilities in remote areas and the occurrence of many unreported home-treated cases where hospitalization would not be useful, a comparative analysis with clinical cases could not be performed in the relevant regions. However, our observations of the distribution and prevalence of *E. coli* virulotypes may assist with assessing seasonal risk and identifying vulnerable areas of the country prone to *E. coli*-associated outbreaks.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268813000320.

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DECLARATION OF INTEREST

None.

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