

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 O₂ 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), K₂HPO₄ (1 g/l) and KH₂PO₄ (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 (*EcoRI*–*PstI* 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	<i>estA</i>	5'-GCTAAACCAGTA ^G GGTCTTCAAAA-3' 5'-CCCGGTACA ^G GCAGGATTACAACA-3'	[11]	147
SHIG	<i>ial</i>	5'-CTGGTAGGTATGGTGAGG-3' 5'-CCAGGCCAACAATTATTTCC-3'	[12]	320
bfpA	<i>bfpA</i>	5'-TTCTTGGTGCTTGCCTGTCTTTT-3' 5'-TTTTGTTTGTGTATCTTTGTAA-3'	[13]	367
EA	pCVD	5'-CTGGCGAAAGACTGTATCAT-3' 5'-CAATGTATAGAAATCCGCTGTT-3'	[14]	630
Set 2				
VT1	<i>vt1</i>	5'-GAAGAGTCCGTGGGATTACG-3' 5'-AGCGATGCAGCTATTAATAA-3'	[15]	130
VT2	<i>vt2</i>	5'-ACCGTTTTTCAGATTTT ^G CACATA-3' 5'-TACACAGGAGCAGTTTCAGACAGT-3'	[16]	298
Eae	<i>eaeA</i>	5'-CACACGAATAAACTGACTAAAATG-3' 5'-AAAAACGCTGACCCGCACCTAAAT-3'	[17]	376
Set 3				
ipaH	<i>ipaH</i>	5'-GCTGGAAAACTCAGTGCCT-3' 5'-CCAGTCCGTAAATTCATTCT-3'	[18]	423
LT	<i>eltB</i>	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 1:10¹³ (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 1:10³ for diarrhoeagenic to non-diarrhoeagenic *E. coli* was required for a positive

PCR result, while the minimum ratio for *vt2* was 1:10⁴ (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 $P > 0.05$.

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 *vt1* 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 *vt1* 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. 1a, 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

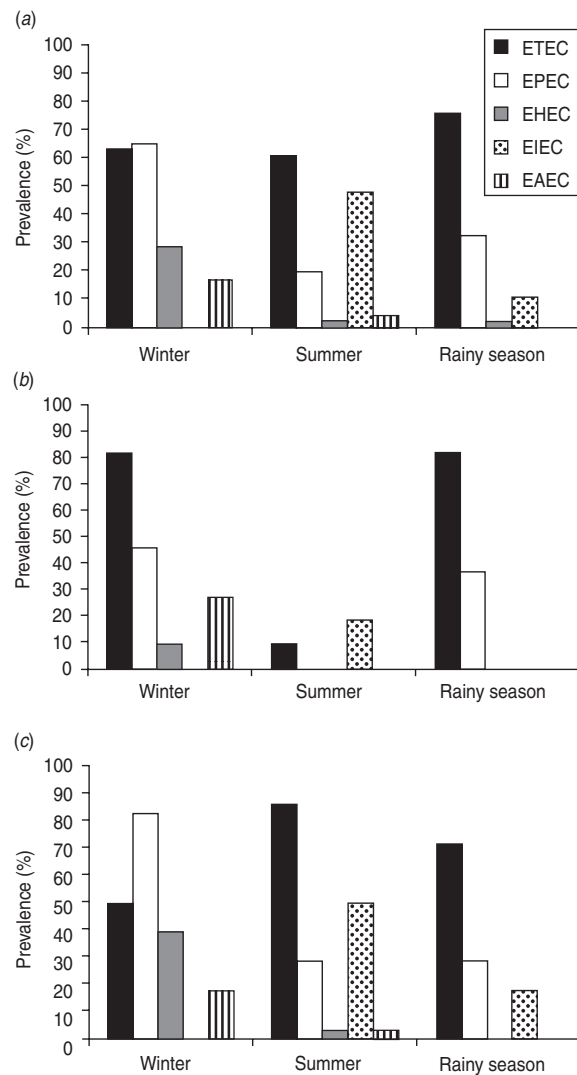


Fig. 1. The prevalence (in terms of proportion of positive sampling sites) of five different diarrhoeagenic *E. coli* virulotypes in the aquatic system of Bangladesh in three successive seasons. (a) Data compiled for all 46 sites, (b) in 11 marine sites, (c) in 35 freshwater sites.

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. 1a, 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 ($P > 0.05$). 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 physico-chemical 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 ($P > 0.05$) 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

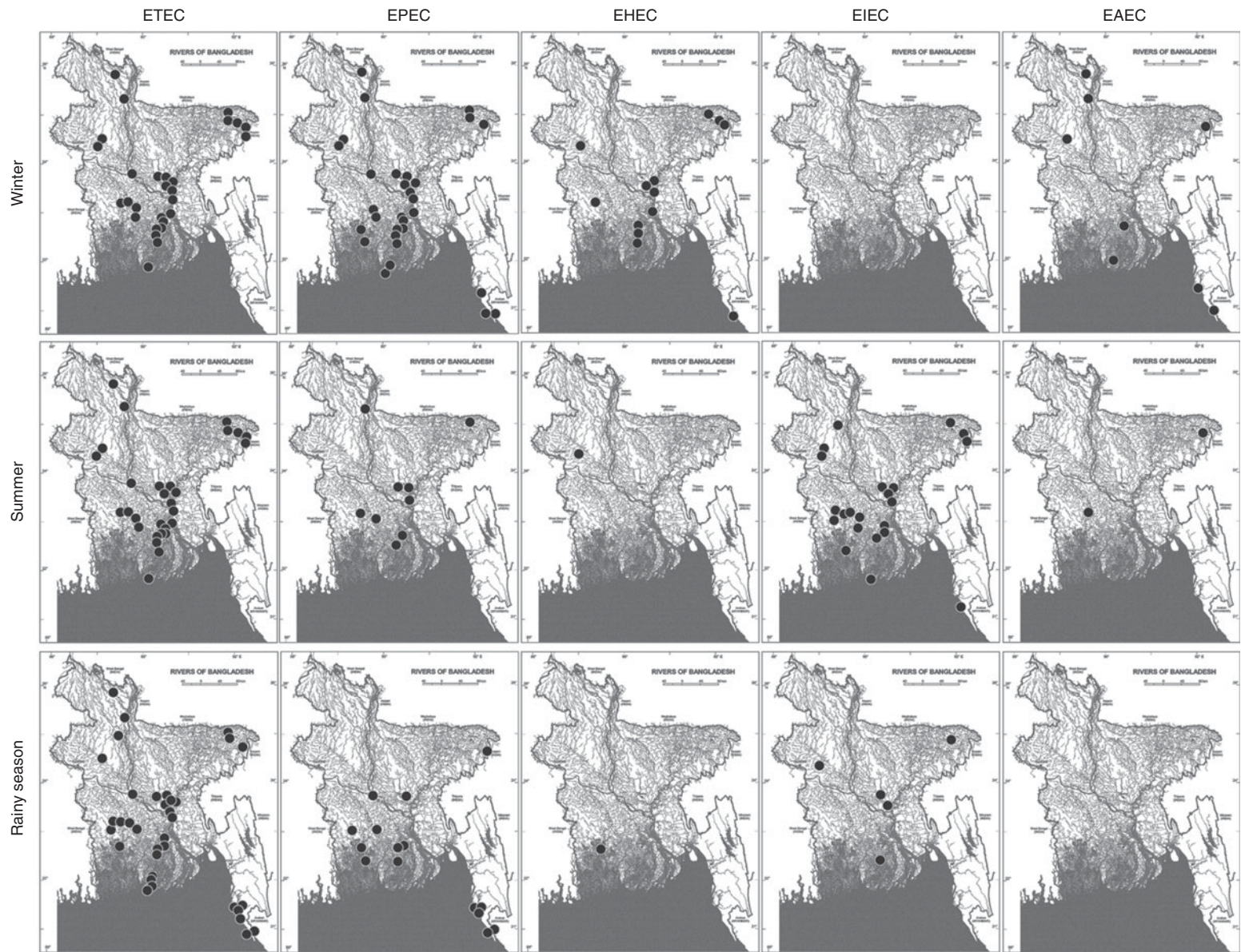


Fig. 2. The regional and temporal distribution patterns of five different diarrhoeagenic *E. coli* strains in three seasons in the aquatic system of Bangladesh. The black circles on the map of Bangladesh designate the presence of different diarrhoeagenic *E. coli* virulotypes in the corresponding geographical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

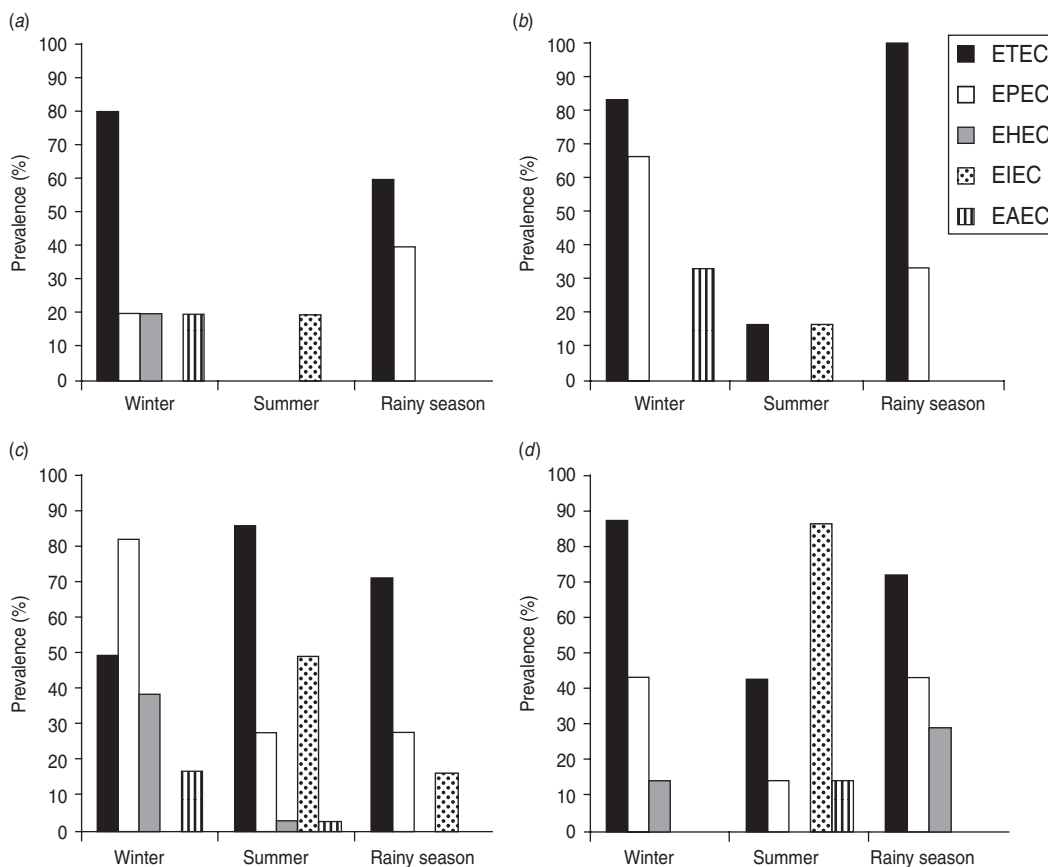


Fig. 3. The effect of seasonal fluctuations in salinity on the prevalence of diarrhoeagenic *E. coli* virulotypes in marine and freshwater sites. (a) The prevalence pattern of diarrhoeagenic *E. coli* in five marine sites where salinity did not fluctuate between seasons, (b) the prevalence pattern of 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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