Indian Journal of Medical Microbiology, (2016) 34(4): 483-488

Original Article

Flooding adds pathogenic *Escherichia coli* strains to the water sources in southern Khyber Pakhtunkhwa, Pakistan

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Abstract

Purpose: Seasonal rains in Pakistan result in heavy floods across the country, whereby faecal contaminants will be added to the water bodies and cause numerous food-borne outbreaks. The present study was aimed to determine the prevalence of diarrheagenic *Escherichia coli* (DEC) strains in the water sources. **Materials and Methods:** Two hundred water samples collected during (2011–2012) were processed for the isolation of *E. coli* (EC) strains. EC strains were further analysed for antibiotic susceptibility patterns, and pathogroups-specific virulence factors *stx1*, *stx2*, *stx2c*, *eae*, *tir*, *hlyA*, *bfpA*, *estA* and e*ltA* were detected using multiplex polymerase chain reaction. **Results:** Thirty-three percent of the water samples were contaminated with EC pathotypes. Fifty percent (33/66) of the DEC pathotypes were identified as enterotoxigenic EC (ETEC). Seventy-two percent (13/18) of the enteropathogenic EC (EPEC) strains were identified as typical EPEC and 28% (5/18) as atypical EPEC. Eleven percent (7/66) of the Shiga toxin EC (STEC) isolates carried a combination of *stx1* and *stx2* genes. Summer was found as a peak season with 47% (31/66) for EC pathogroups' activities. Eighty-nine percent of the strains showed resistance against tetracycline. **Conclusion:** ETEC and EPEC are the primary causes of water contamination in southern regions of Khyber Pakhtunkhwa province, Pakistan. Firm adherence to the prescribed drugs can decrease trends in antibiotic resistance.

Key words: *Diarrheagenic Escherichia coli, Escherichia coli, Shiga toxin-producing Escherichia coli*

Introduction

Escherichia coli (EC) pathotypes, especially enterotoxigenic and Shiga toxin-producing EC (ETEC and STEC), gained considerable importance resulting in a series of water- and food-borne diarrhoeal outbreaks. An ETEC outbreak due to salad consumption was documented in persons attending an international meeting in Puerto Vallarta, Mexico.[1] A serious food-borne outbreak involving entero-aggregative-haemorrhagic EC originating from sprouted seeds in several European countries highlights the importance of screening for diarrheagenic EC pathotypes (DEPs) in fresh vegetables.[2] In many countries, food-borne disease outbreaks originating in prepared raw green vegetable salads (ready-to-eat foods) were more likely to occur on commercial food service premises than outbreaks from other sources, with restaurants and hotels accounting for almost 75% of the outbreaks.[3] The consumption of cooked vegetable salads has increased in many countries; however, a greater consumption has led to a concurrent rise in the number of cooked vegetable salad-associated food-borne illness outbreaks, with at least 25 outbreaks reported in several countries.[4] As a consequence, the number of gastroenteritis outbreaks caused by food-borne pathogens, including DEPs, after the consumption of raw vegetable salads, has increased worldwide.[5-8]

Diarrhoeal complications may lead to life-threatening complications, such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). EC strains colonise intestinal epithelial lumen of humans and animals within hours of birth. A very small proportion (<1%) of EC pathogroups are harmful as they cause a variety of infections including diarrhoea, nausea and vomiting. EC-mediated diarrhoeal infection is either expressed as

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Received: 21-11-2014
Accepted: 25-08-2016

Access this article online

Quick Response Code:

Website: www.ijmm.org

DOI: 10.4103/0255-0857.195350

How to cite this article: Shah MS, Eppinger M, Ahmed S, Shah AA, Hameed A, Hasan F. Flooding adds pathogenic *Escherichia coli* strains to the water sources in southern Khyber Pakhtunkhwa, Pakistan. Indian J Med Microbiol 2016;34:483-8.

acute watery, persistent or bloody diarrhoea. The acute and persistent diarrhoeas are not distinct from each other, but generally represent two ends of a continuum. Acute watery diarrhoea resolves within a couple to 7 days, accounting for 80% of all cases of childhood diarrhoea with 50% mortality rate.[9] Persistent diarrhoea is a less frequent diarrhoeal form lasting for 2–4 weeks accounting for 10% of the diarrhoeal episodes and 35% all diarrhoeal deaths. More than half of these deaths are reported from South Asia.[10] Bloody diarrhoea is caused by STEC by invading mucosal epithelial cell lining, exerting visible or microscopic blood in the stools, due to local mucosal evasion and intestinal haemorrhage.[11] STEC infection may lead to HC or HUS. Estimated disease burden of STEC in developing countries is about 30%–40% of all diarrhoeal infections.[12]

Contamination of fresh and stored water bodies occurs through defecation by humans and domestic animals in or near catchment areas. Pathogenic strains of EC, for example, ETEC, enteropathogenic EC (EPEC) and STEC can also be transmitted through fresh fruits, raw vegetables (mesclun, a mixture of young leafy greens, containing young lettuces, used as salad and white radish sprouts) and their processed products.[13] These pathogroups are differentiated based on the presence or absence of characteristic virulence factors, for example, *eaeA* (mediates attachment to epithelial cells) and *bfpA* (bundle-forming pili) for EPEC; *stx1* and *stx2* for STEC; heat-stable (*estA*) and heat-labile (*eltA*) toxins for ETEC.[10]

Pathogenic EC strains are of major importance causing a series of water-borne diarrhoeal outbreaks where globally or in Pakistan only. Water-borne diarrhoeal outbreaks emerge after heavy rainfall and flooding situation, by adding sewage waste to the drinking water.[14] Diarrhoeal outbreaks due to diarrhaegenic EC strains have been reported globally, such as in the UK, the USA, Canada, Japan and Sweden to name.[15-18] EC O157:H7 has also been reported in sheep, goats, water, buffalo and deer during summer and winter seasons.[19]

Considerable measures have been taken to decrease the rate of food-borne disease outbreaks due to pathogenic EC strains, but are still regarded as extremely important pathogens due to the complications they may cause. Food-borne outbreaks due to pathogenic EC strains are linked to the warmer season of the year (May–September). Seasonality in the prevalence of EC pathogroups may be attributed to the increased housefly populations during the summer.[20]

The 2010 flood in Pakistan has affected about 78 districts, affecting 21 million people. According to the World Health Organization, this catastrophe was the biggest after the 2005 earthquake and left people of affected areas in a horrible situation. Of 5.3 million medical consultations, 708,891 (13%) were made for acute diarrhoea. During this natural disaster, faecal contaminants were added to the water bodies by different contaminated sources, as untreated or poorly treated sewage water, direct leakage of septic tanks and spillage from sanitary passages.

High morbidity and mortality rates of diarrhoeal infection were recorded during and after the flooding season that stress the importance of detecting DEPs among water bodies in the aftermath of such calamities. The present study was aimed to determine the prevalence, antibiotic susceptibility and to characterise molecular subgroups of diarrhaegenic EC in water sources in the southern parts of Khyber Pakhtunkhwa, Pakistan.

Materials and Methods

Criteria for water sample collection from the representative sites

In the present study (2011–2012), 200 water samples (pond, stream, sewage and tap) were collected from the study area (southern districts of KP), using sterile wide mouth plastic containers. Water samples were subjected to microbiological investigations aimed for the detection of DEPs.

To thoroughly investigate the samples, the strains were isolated and they were assigned into EC pathogroups (ETEC, EPEC and STEC) and the processing of water samples was limited to 200.

- Further, each individual isolate was assigned to a pathotype and characterised for critical virulence factors; therefore, the sample number was limited to 200
- This study was performed as part of PhD thesis with a limited labour workforce and in a limited period.

We believe that the characterisation of isolates in our collection yielded a robust dataset that provides a good representation of the EC isolates in the water bodies.

Representative sites were ensured based on the following reasons:

- Diarrhoeal disease cases were most frequently reported from these sites and thus addressed representative
- These representative sites were further prone to the faecal contamination due to lack of sewage system
- In the studied sites, water (prone to contamination) was utilised for the irrigation of agricultural farms.

Isolation and biochemical identification of diarrheagenic Escherichia coli pathogens

A 50 ml water sample was filtered through a 0.45 µm filter paper, inoculated in 10 ml of EC broth and incubated at 37°C for 4 h. Novobiocin was added to a final concentration of 20 µg/ml, and the enriched samples were incubated at 42°C for 18–24 h. A 50 µl aliquot of

the enriched culture was plated onto eosin methylene blue, Sorbitol MacConkey agar (SMAC; Oxoid) and cefixime-tellurite-SMAC (Oxoid) and incubated at 42°C for 24 and 48 h. Suspected colonies (clear to smoky grey) were tested for the O157 antigen by latex agglutination (EC O157 Dryspot test kit, Oxoid).

Molecular characterisation

Multiplex polymerase chain reaction assay

EC DNA was extracted using Qiagen Miniprep 50 kit (Holland) according to the manufacturer's instructions. Template DNA was used in multiplex polymerase chain reaction (PCR) using pathogroups-specific primers for the amplification of *eae*, *bfpA* (virulence factor EPEC), *eltA* and *estA* (enterotoxin genes of ETEC) and *stx1* and *stx2* of STEC. Multiplex PCR assays were conducted using a reaction volume of 22 µl containing 5 ml (20 picomole) of template DNA, 5 µl of (10×) PCR buffer, 5 µl of a Q solution (10× buffer), 0.25 µl of (250 U) of Taq-polymerase per ml (Sigma, USA), 0.75 µl (20 mM) of each primer (Sigma) and 7 µl of PCR water. Thermocycler conditions set up in a Gene Amp PCR system 9700 (AB Applied Biosystem) were as follows: 96°C for 5 min, 94°C for 30 s, 57°C for 30 s and 72°C for 1 min in 35 cycles, with a final 5 min extension at 72°C. Concentrated PCR products were diluted using Orange G dye. Diluted PCR products (10 µl) were confirmed by running 2% (weight/volume) (Sigma) agarose gel electrophoresis gel.

A multiplexed PCR assay was utilised to test for gene absence/presence. Access to sequencing facility was not available for this project, and we note that our focus was not to determine respective allelic states rather than to test the prevalence of these virulence factors in the strain collection. For EPEC and STEC/EHEC isolates, EC O157:H7 reference strain EDL933 was used as positive control in all PCR reactions.

For ETEC isolates, EC reference strain ATCC 35401 (ETEC; *lt*, *st* positive) was used as a positive control.

The non-pathogenic EC strain ATCC25922 (*stx, lt*, *st*, *eae*, *tir* and *hly*) negative was used as negative controls in all PCR reaction.

Results

In the present study, 33% (66/200) of the water samples were found contaminated with DEPs. EC pathogroups were isolated in 44% (29/66) and other bacterial contaminants in 56% (37/66). Sixty-four out of the 66 strains were identified belonging to different pathotypes. In the present study, 28% (14/50) of the stagnant pond water samples, 4% (2/50) of tap water, 62% (31/50) of sewage water and 38% of (19/50) stream water were contaminated with DEPs. Furthermore, 16% (5/31) of sewage and 15% (3/19) of stream water isolates were identified as EC O157:H7.

Molecular characterisation of DEPs for ETEC, EPEC and STEC pathogroups was carried out based on the presence or absence of characteristic virulence factors. Fifty-five percent (17/31) of DEPs isolated from sewage water samples were as identified as ETEC, 29% (9/31) as EPEC and 16% (5/31) as STEC strains. Fifty-nine percent (10/17) of the ETEC strains were carrying a combination of enterotoxin (e*ltA*/est*A)*, compared to the 41% (7/17) strains carrying e*ltA* alone. Seventy-eight percent (7/9) of the EPEC isolates were identified as tEPEC (carrying *eae* and *bfpA*) genes while 22% (2/9) were identified as aEPEC (carrying *eae* but lack *bfpA*). One hundred percent STEC isolates were harbouring a combination of *stx1* and *stx2*, whereas no *stx2c* gene was detected [Figure 1].

Thirty percent (19/64) of the DEPs isolated from stream water were identified as ETEC, 26% (5/19) as EPEC and 16% (3/19) as STEC. Sixty-four percent (7/11) of ETEC were harbouring a combination of *estA*/*eltA* compared to 36% (4/11) of the strains carrying *eltA* genes. Similarly, 80% (4/5) of the EPEC isolates were identified as tEPEC (carrying *eae* + *bfpA*) genes and 20% (1/5) were identified as aEPEC (carrying *eae* but lack *bfpA*) genes. Sixteen percent (3/19) of the DEPs were identified as STEC isolates, harbouring a combination of both *stx1* and *stx2*. No isolate was carrying *stx2c*.

Seasonal prevalence of diarrhaegenic Escherichia coli

Fifty-two percent (33/64) of EC pathogroups were isolated during the summer season (May–August). Eleven percent (7/64) of the DEPs were isolated during the winter season (October–January), 23% (15/66) during the spring season and 14% (9/64) during the autumn season [Figure 2].

Antibiotic susceptibility

Ninety percent (80/90) of the isolates were sensitive to imipenem, indicating its role as a highly effective drug;

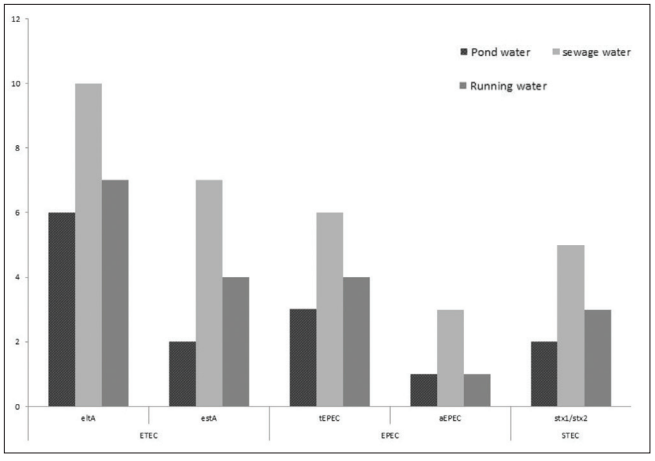

Figure 1: Prevalence of *Escherichia coli* serotypes (*Escherichia coli* O157:H7) among water samples

cefuroxime was the second most effective antibiotic (71.25%). The maximum resistance (97%) was observed against tetracycline followed by ciprofloxacin in 91%. In case of β-lactam antibiotics, high resistance (70%) was observed to cefotaxime and amoxicillin/clavulanic acid. Antimicrobial resistance was checked against the selected antibiotics based on the routine prescription/ self-medication and thereby posing emerging antimicrobial resistance, shown in Table 1.

EPEC isolates presented with two multi-resistance profiles, trimethoprim-sulfamethoxazole, tetracycline, ampicillin and chloramphenicol. With regard to ETEC, one isolate was resistant to tetracycline and ampicillin and the other to sulfamethoxazole-trimethoprim, tetracycline and amoxicillin.

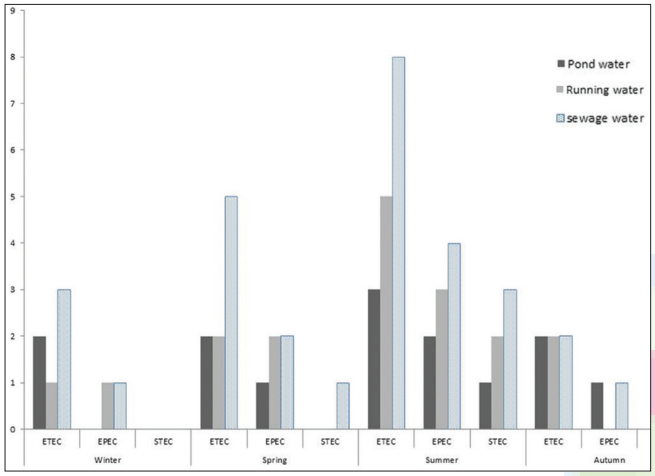

Figure 2: Seasonal prevalence of diarrhaegenic *Escherichia coli* pathotypes in water samples

Discussion

Microbes use water bodies including rivers, ponds and marshy places as nursery homes. Water contamination is getting more severe in remote areas of developing countries, where untreated sewerage water and industrial effluents are directly released to the water sources. In rural communities, waste water is not only used for irrigation purpose but also for drinking and watering of livestock.[21] Therefore, this study was specifically designed to evaluate the aetiology of DEPs in the water sources of southern Khyber Pakhtunkhwa, Pakistan.

During rainy season, flood runoff may lead to an increased transmission of microbial pathogens to the water bodies through mixing of animal and human faecal wastes present in the soil and other sources. In the present study, a maximum number of EC pathotypes of 61% (39/64) were obtained after heavy rainfall resulting in a flooding situation. A potential cause could be the addition of human and animal excreta from sewerage leakage and overflow, transmission of EC surviving in the soil sediments and other aquatic environment.[22] These findings are in accordance with the previously reported observation of several fold increase in faecal coliform numbers in the surface water bodies after storm events.[23] This study clearly highlights the importance of managing municipal waste water including sewerage leaks and overflows in aquatic environments.

Heavy rainfall and subsequent flooding are responsible for water-borne EC outbreaks. Food-borne outbreaks due to EC strains are observed in hot and humid seasons,[12] and bacterial survival rates are further strengthened by the hot and humid weather conditions. Fifty-two percent (33/64)

Table 1: Antibiotic resistance of the selected *E. coli* pathotypes

Class and antimicrobials	Phenotype and % Resistant			
	Total no of diarrheagenic *E. coli* *n*=33	ETEC (*n*=17)	EPEC (*n*=12)	EHEC (*n*=4)
Carbapenems				
Imipenem	3/33 (9%)	0/17 (0%)	3/12 (25%)	0/4 (0%)
Aminoglycoside				
Streptomycin	24/33 (73%)	13/17 (76%)	8/12 (67%)	3/4 (75%)
Quinolones				
Nalidixic acid	22/33 (67%)	12/17 (71%)	7/12 (58%)	4/4 (100%)
Ciprofloxacin	30/33 (91%)	15/17 (88%)	11/12 (91.6%)	4/4 (100%)
Sulfonamides				
Trimethoprim-Sulfamethoxazole	23/33 (69.9%)	13/17 (76.5%)	7/12 (58%)	3/4 (75%)
Tetracycline				
Tetracycline	32/33 (97%)	17/17 (100%)	11/12 (91.6%)	4/4 (100%)
β lactams				
Amoxicillin-Clavulanic acid	17/33 (52%)	10/17 (59%)	4/12 (33%)	3/4 (75%)
Cephalosporin				
Cefuroxime	21/33 (64%)	10/17 (59%)	8/12 (67%)	3/4 (75%)
Cefotaxime	23/33 (70%)	12/17 (70.6%)	7/12 (58%)	4/4 (100%)

of the EC pathogroups were isolated during the summer season (May–August). Eleven percent (7/64) of the DEPs were isolated during the winter season (October–January), 23% (15/66) during spring season and 14% (9/64) during the autumn season. Transmission- and season-specific prevalence of EC pathogroups are attributed to the increased housefly populations in the summer season, environmental factors (humidity and rainfall) and waste handling practices.[17] Previous studies have shown a link between environmental conditions (e.g., temperature, rainfall, humidity and wind) and enteric infections in calves. However, further studies are required to evaluate the seasonal shedding of pathogenic EC and in the tropical and subtropical regions.

Antibiotics are routinely prescribed for the treatment of infectious diseases in developing countries. Antibiotic resistance is attributed to inappropriate choice of drugs, self-prescription and lacking regulation on the use of antibiotics.[24] Inappropriate choice of antibiotics exerts selective pressures on the dissemination of resistant genes in clinical environments, waste water and water used for irrigation. In the present study, 98% of the DEPs strains showed resistance against tetracycline and 91% to ciprofloxacin. Seventy percent of the DEP strains showed resistance against β-lactam antibiotics including cefotaxime and amoxicillin/clavulanic acid. Ninety percent (80/90) of the isolates were sensitive to imipenem, making it the most effective drug in this study; cefuroxime was the second most effective antibiotic (71.25%). This suggests the importance of waste water discharges in the dissemination of antimicrobial resistance strains. About two-thirds of all EC isolates were resistant to ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol. The presence of antibiotic-resistant EC was also observed in other studies from human and animal faecal sources, waste water treatment plant and surface water.[25] Similar resistance levels were found in EC isolated from children and adults in Latin America, and the 53.2% and 57.7% of the isolates were resistant to ampicillin and trimethoprim-sulfamethoxazole, respectively.[26] The permanent influx of pollutants such as antimicrobial agents, detergents, disinfectants, heavy metals, livestock waste and watershed may contribute to the emergence of antibiotic-resistant bacteria in water sources of southern Khyber Pakhtunkhwa.

Molecular characterisation revealed that 100% of the EPEC strains, both tEPEC and aEPEC, were carrying the *eaeA* gene in combination with *bfpA* or alone. Mostly, tEPEC strains were carrying the LEE pathogenicity island, containing intimin (*eaeA*) and the plasmid-encoded bundle-forming pilus (*bfp*), which facilitate adhesion to intestinal epithelial cells.[27] Similar observations were reported in the previous studies, showing significantly higher prevalence of *eaeA* (up to 96%) in EPEC strains isolated from surface water.[28] A combination of pathogenicity mechanisms is adopted by DEPs, consisting of attachment and effacement, modification of host's cell surface, invasion of the brush border epithelial cells and secretion of toxins. Identification of virulence genes is critical in determining the pathogenic properties of given EC pathotypes.

In the present study, EHEC pathotypes were carrying a combination of *eae*, *hlyA* and *tir*, along with stx1 and stx2 toxin genes. Bloody diarrhoeal infections caused by EHEC pathotypes are leading to haemorrhagic colitis and HUS. *eaeA* gene is mostly found in combination with *stx*1 genes. This type of combination is often assumed to cause more severe diarrhoea in humans. This suggests that there is a wide prevalence of this gene in EC found in aquatic ecosystems. Similarly, a high prevalence of the *eaeA* gene in surface water has been reported in other studies.[28,29] Furthermore, this study was conducted in densely populated flood refugee camps, a setting that is often encountered in developing countries and that must be taken into account.[19] These results suggest a thorough evaluation of the waste water management in Khyber Pakhtunkhwa, Pakistan, and if necessary, a local waste water treatment to prevent the emergence of infectious outbreaks can be implemented.

Conclusion

This study highlights the need for partial treatment of water prior to its reuse for potable and non-potable purposes for public health risk mitigation. The present study underscores the importance of controlling sources of human faecal pollution, such as managing municipal waste water sources to reduce potential risks to human health.

Financial support and sponsorship

This study was partly supported by the International Research Support Initiative Program, Higher Education Commission of Pakistan.

Conflicts of interest

There are no conflicts of interest.

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