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Escherichia coli Pathotypes in Pakistan from Consecutive Floods in 2010 and 2011

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Abstract. This study compares *Escherichia coli* pathotypes circulating among children in Pakistan during the floods of 2010 and 2011 and from sporadic cases outside flood affected areas. Using multiplex polymerase chain reaction 115 of 205 stool samples (56.29%) were positive for diarrheagenic *E. coli* from specimens taken during the floods compared with 50 of 400 (12.5%) stool samples being positive for sporadic cases. The *E. coli* pathotypes were categorized as Enteropathogenic *E. coli* 33 (28.69%) and 13 (26%), Enterotoxigenic *E. coli* 29 (25.21%) and 15 (30%), Enteroggregative *E. coli* 21 (18.2%) and 18 (36%), Enterohemorrhagic *E. coli* 5 (4.34%) and 1 (2%) from flood and sporadic cases, respectively. Furthermore, patients co-infected with more than one pathotype were 26 (22.60%) and 3 (6%) from flood and sporadic cases, respectively. The study shows an unexpectedly high rate of isolation of *E. coli* pathotypes suggesting Pakistan as an endemic region that requires active surveillance particularly during flood periods.

INTRODUCTION

Diarrheagenic *Escherichia coli* (DEC) are one of the predominant causes of diarrheal disease globally and a leading cause of childhood mortalities.^{1,2} The DEC can be categorized as pathotypes on the basis of gene-specific sequences. The six known pathotypes include Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enteroggregative *E. coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), and Diffusely adherent *E. coli* (DAEC).^{3,4}

The severity of DEC is well documented in developing countries and the association of different pathotypes determines the outcome of the clinical manifestation of the disease, particularly among children.^{5,6} The recent availability of the full genome sequences of the DECs has allowed the identification of genetic markers that can be used to determine the etiological agents.^{7–9}

In this study, we collected stool samples from hospitalized patients and patients admitted to makeshift camps from the three main flood affected provinces of Pakistan during 2010 and 2011 Khyber Pakhtunkhwa (KPK), Punjab, and Sindh, as well as from sporadic diarrheal cases outside flood areas. Using the genetic sequences *eae*, *bfp*, *vt*, *aggR*, *lt*, *st*, *daaE*, *ipaH*, *virF*,^{2,10–12} we were able to determine the pathotype of the DECs by polymerase chain reaction (PCR) and to map the geographic distribution of the pathotypes in Pakistan. Furthermore, the identified pathotypes were subjected to rapid and cost-effective Enterobacterial repetitive intergenic consensus (ERIC) PCR for fingerprinting. Based on highly conserved short (~126 bps) noncoding sequence strains were clustered based on banding patterns of ERIC-PCR products on agarose gels.^{13,14}

MATERIALS AND METHODS

Surveillance of *E. coli* diarrhea in Pakistan. In 2010–2011, based on the active surveillance of flood affected and non-flood affected localities in North of Pakistan, i.e., KPK: (population >

20 million), west of Pakistan, i.e., Province Sindh: (population > 35 million), and province Punjab: (population > 80 million), respectively, we identified 605 potential patients (age > 15) with clinical diarrhea-like symptoms. From these 605 patients stool specimens from 205 and 400 from flood and non-flood affected cases, respectively, were analyzed. The samples were stored in blue capped sterile plastic tubes containing Cary-Blair transport media and transported in containers filled with ice bags within 24–48 hours after collection, from field to the laboratory.

Samples were collected from 10 districts from mainly three provinces and upon arrival at the laboratory, a loopful of fecal material was collected from the specimen using a sterile inoculating loop and was streaked on MacConkey agar. The plates were incubated at 37°C for 24 hours and pure cultures were prepared after restreaking single colonies on MacConkey agar for the isolation of *E. coli*. For further identification, colonies indicative of *E. coli* were then subjected to routine biochemical assays, i.e., Catalase test, Oxidase test, Methyl red test, Indole test, Hydrogen sulfide production, Motility test, Citrate test, and Urease test. Pure *E. coli* cultures were preserved in 15% glycerol and stored at –80°C.

Multiplex PCR for pathotype screening. The DNA was extracted using a previously established method.¹⁵ All clinical DEC isolates were screened for respective *E. coli* pathotypes using multiplex PCR, in which two sets of primers were used as explained previously.^{2,10–12} The PCR1 contained primers (Table 1) designed on intimin (*eae*), bundle-forming pilus structural subunit (*bfp*), verotoxin (*vt*), and regulatory gene (*aggR*) to distinguish between EPEC, EHEC, and EAEC, respectively. The PCR2 was composed of primers designed for heat-labile toxin (*lt*), heat-stable toxin (*st*), diffuse adherence structural subunit gene (*daaE*), invasion plasmid antigen H (*ipaH*), and virulence invasion factor (*virF*) to distinguish between ETEC, DAEC, and EIEC, respectively.^{2,10–12} Plasmids containing target genes (gift from Oscar G. Gomez-Duarte, International Enteric Vaccines Research Program [IEVRP], University of Iowa Children's Hospital, Iowa City, IA) were used as positive controls as listed in Supplemental Table 1. The DNA from *E. coli* DH5 α was used as a negative control.

ERIC-PCR. Genotyping was performed using the ERIC fingerprinting assay, which uses one 22-bp primer designed against the conserved ERIC region.^{16,17} The PCR was performed in a final volume of 25 μ L mixture containing 100 ng DNA, 25 pmol of ERIC2 primer (5-AAGTAAGTGACTGGGTGAGCG-3).¹⁶

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TABLE 1
Escherichia coli pathotypes identified by multiplex polymerase chain reaction (PCR)

| Pathotype | Genes | Fragment size (bps) | Primer sequences | References |
|-----------|----------------|---------------------|---|------------|
| EAEC | <i>aggR</i> | 254 | GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC | 10 |
| EPEC | <i>eae</i> | 482 | TCAATGCAGTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG | 11 |
| | <i>eae+bfp</i> | 482 + 327 | GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTAGT | 11, 12 |
| ETEC | <i>lt</i> | 218 | GCACACGGAGCTCCTCAGTC TCCTTCATCCTTTCAATGGCTTT | 2 |
| | <i>st</i> | 147 | GCTAAACCAGTAGAG(C)TCTCAAAA CCCGGTACAG(A)GCAGGATTACAACA | 2 |
| | <i>lt+st</i> | 218 + 147 | | 2 |
| EHEC | <i>vt</i> | 518 | GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT | 10 |
| | <i>vt+eae</i> | 518 + 482 | | 10, 11 |
| EIEC | <i>ipaH</i> | 933 | CTCGGCACGTTTTAATAGTCTGG GTGGAGAGCTGAAGTTTCTCTGC | 11 |

The PCR amplification products of different pathotypes (EPEC, ETEC, and EAEC) were visualized by electrophoresis on 1.5% agarose gels with 100-bp DNA ladder (Fermentas, Cambridge, UK).

Statistical analysis. The ERIC-PCR fingerprints were converted to binary codes based on the presence and absence of the ERIC-PCR fragments. Because of the limited number of

ERIC-PCR bands (ranging from 3 to 17 DNA bands) all *E. coli* isolates were analyzed using Gel Compare-II software (Applied Maths, Inc., Austin, TX). Tiff images of ERIC-PCR results were standardized. Briefly, the amplification profiles obtained using ERIC primer for EPEC, ETEC, and EAEC were subjected to cluster analyses using the Pearson correlation coefficient based on densitometric readings of the banding

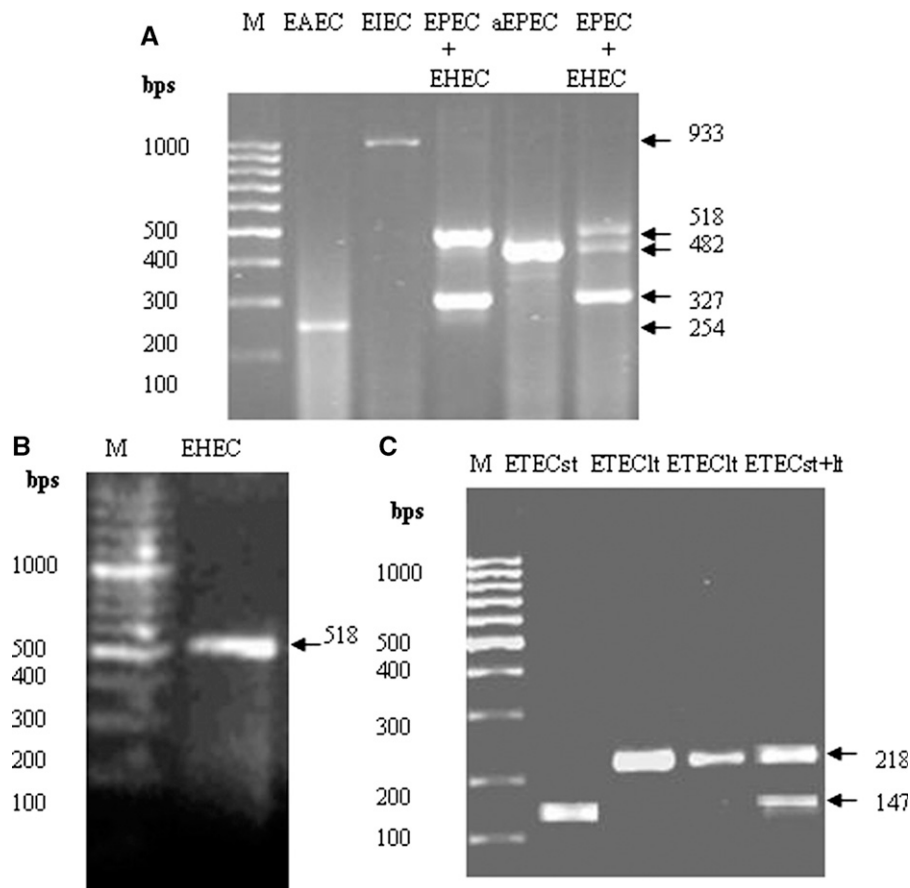


FIGURE 1. Screening of *Escherichia coli* pathotypes by multiplex polymerase chain reaction (PCR) from flood affected diarrhea patients. (A) Lane M: 100 bp DNA ladder, different lanes are labeled with the respective pathotypes identified as EAEC (254 bps-*aggR*), EIEC (933 bps-*ipaH*), EPEC + EHEC (typical: 482 bps-*eae*, 327 bps-*bfp* + 518 bps-*vir*), atypical EPEC (482 bps-*eae*), EPEC + EHEC (typical: 482 bps-*eae*, 327 bps-*bfp* + 518 bps-*vir*). (B) Lane M: 100 bp DNA ladder, EHEC (518 bps-*vir*). (C) Lane M: 100 bp DNA ladder, ETEC (one band of 147 bps (*lt*), ETEC (one band 218 bps (*lt*), ETEC (one band 218 bps (*lt*), second band 147 bps (*st*)).

TABLE 2
Distribution of diarrheagenic *Escherichia coli* (DEC) pathotypes during 2010 and 2011 floods

| Province | Districts | Months isolated Jun–Sept | No. of samples | <i>E. coli</i> n (%) | EPEC Type | | | | | EIEC n (%) | EHEC n (%) | ETEC n (%) | EAEC n (%) | Atypical n (%) | Mix combinations n (%) | Unknown† |
|-----------------|-------------|-----------------------------|-------------------|-------------------------|---------------|------------------|-------------------|---------------|---------------|---------------|---------------|---------------------------------|--|-------------------|---------------------------|----------|
| | | | | | EPEC n (%) | Typical n (%) | Atypical n (%) | EAEC n (%) | ETEC n (%) | | | | | | | |
| Punjab | Muzafargarh | // | 25 | 24 | 10 (45) | 1 (10) | 9 (90) | 1 (4) | 3 (13) | 0 | 0 | 0 | 10* (45) | 0 | 1 | |
| | D.G. Khan | // | 2 | 2 | 1 (50) | 0 | 1 (100) | 1 (50) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | KhairPur | // | 36 | 34 | 1 (3) | 0 | 1 (100) | 17 (50) | 14 (41) | 0 | 0 | 0 | 1** (2.94), 1*** (2.94) | 0 | 2 | |
| Sindh | Shikarpur | // | 6 | 1 | 0 | 0 | 0 | 0 | 1 (17) | 0 | 0 | 0 | 0 | 0 | 5 | |
| | Sukkur | // | 6 | 3 | 0 | 0 | 0 | 0 | 2 (40) | 0 | 1 (20) | 0 | 0 | 0 | 3 | |
| | Jamshoro | // | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | |
| | Nowshera | // | 20 | 12 | 3 (20) | 1 (33.3) | 2 (66.7) | 0 | 0 | 0 | 2 (13.3) | 0 | 2*** (16.6), 3**** (25), 2***** (16.6) | 0 | 8 | |
| Pakhtun khwa | Charsada | // | 40 | 8 | 7 (28) | 0 | 7 (100) | 0 | 1 (4) | 0 | 0 | 0 | 0 | 0 | 32 | |
| | D.I. Khan | // | 60 | 31 | 11 (22.9) | 4 (36.36) | 7 (63.63) | 2 (4.1) | 8 (16.7) | 3 (6.25) | 0 | 5***** (16.1), 2***** (6.45) | 0 | 29 | | |
| Total | - | - | 205 (100%) | 115 (56.3%) | 33 (28.6%) | 6 (18.1%) | 27 (81.8%) | 21 (18.2%) | 29 (25.2%) | 5 (4.34%) | 1 (0.8%) | 26 (22.6%) | 90 (43.7%) | | | |

Mix Combinations = * EPEC/EAEC, ** EAEC/EHEC, *** EPEC/EHEC, **** EPEC/EIEC, ***** EPEC/EHEC; aEPEC = Atypical EPEC
(*aEPEC = 7, *EPEC = 3); (**aEPEC = 3); (***)aEPEC = 2, ****aEPEC = 1, *****aEPEC = 1, *****aEPEC = 1, *****aEPEC = 1).
† Unknown = Diarrheal infections other than *E. coli*.

patterns strain. Isolates in each group (EPEC, ETEC, and EAEC) were analyzed by clustering trends.

RESULTS

In this study, we tested diarrheal patient samples from the Sindh, KPK, and Punjab Provinces, and Islamabad that included 58 and 85, 120 and 85, 27 and 195 samples from flood and non-flood areas, respectively. Of these samples, 39.60% (240) were positive by culture for *E. coli*. Furthermore, 27.27% (165) were confirmed as *E. coli* pathotypes by biochemical assays and multiplex PCR during the months (June–September) of the surveillance period (2010–2011) from the total of 605 samples collected.

Pathotype screening. A total of 115 (56.29%) samples out of 205 stool samples from flood affected areas were culture positive and were confirmed by conventional biochemical assays and multiplex PCR results for the DEC using appropriate positive controls (Supplemental Table 1). They were subdivided into five *E. coli* pathotypes where 33 (28.6%) were EPEC, 29 (25.2%) were ETEC, 21 (18.2%) were EAEC, and 5 (4.34%) were EHEC based on multiplex PCR results. However, only a single EIEC (0.86%) and no DAEC strains were identified (Figure 1, Table 2).

Patients co-infected with two DEC isolates from flood affected areas were designated mixed diarrheagenic *E. coli* (MDEC). The MDEC were found with an overall prevalence of 22.60%. In these patients MDEC infection was caused by ETEC/EPEC and EAEC/EPEC, 10 (38.46%) each, was more frequent as compared with other combinations including EPEC/EHEC 4 (15.38%), EAEC/EHEC 1 (3.84%), and ETEC/EAEC 1 (3.84%), respectively (Table 2).

By contrast among 400 isolates collected from sporadic diarrheal cases, only 50 (12.5%) were identified belonging to four different *E. coli* pathotypes. The study identified that 18 (36%) were EAEC, 15 (30%) were ETEC, 13 (26%) were EPEC, and 1 (2%) was EHEC and the remaining 3 (6%) were MDEC infections (2 were EPEC/ETEC and 1 was EPEC/EAEC) (Table 3).

We identified and investigated three predominant *E. coli* pathotypes, i.e., EPEC, ETEC, and EAEC for their genetic relatedness using ERIC-PCR. A similar matrix of each *E. coli* pathotype based on their band patterns was identified by clustering analysis. The study includes clustering and then analysis of differential banding patterns of *E. coli* strains from geographically diverse Pakistan cities during 2010 and 2011, including flood areas and from sporadic cases outside flood affected areas.

Sixty-three and 46 (Tables 2 and 3) *E. coli* human strains isolated from flood and sporadic diarrheal cases outside flood periods, including 33 EPEC, 29 ETEC, and 21 EAEC isolates as well as 13 EPEC, 15 ETEC, and 18 EAEC from the different cities of Pakistan were used for the ERIC-PCR DNA fingerprinting assay, respectively. A maximum of 17 and minimum of 3 quality DNA bands were generated by the ERIC-PCR. An average of nine bands per isolate ranging from 100 to > 1,500 bps was observed (Supplemental Figure 1).

The ERIC-PCR fingerprints of the total 58 EPEC, 46 ETEC, and 20 EAEC isolates from diverse geographic regions clustered together with similarity coefficients among intrapathotypes was at least 75%, 85%, and 95%, respectively, suggesting that the community structure of the pathotypes

TABLE 3
Distribution of diarrheagenic *Escherichia coli* (DEC) pathotypes from sporadic cases during 2010 and 2011

| Province | Districts | Month Isolated Jun-Sept | No. of samples | <i>E. coli</i> n (%) | EPEC Type | | EPEC n (%) | EPEC n (%) | EHEC n (%) | ETEC n (%) | EAEC n (%) | ETEC n (%) | EHEC n (%) | EIEC n (%) | Mix Combinations n (%) | Unknown† |
|-----------------------------|------------|----------------------------|-------------------|-------------------------|------------------|-------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------------------|----------|
| | | | | | Typical n (%) | Atypical n (%) | | | | | | | | | | |
| Punjab | Rawalpindi | // | 125 | 17 | 2 (11.76) | 0 | 2 (100) | 9 (52.9) | 3 (17.6) | 1 (5.88) | 0 | 0 | 0 | 0 | 2* (11.76) | 108 |
| | Multan | // | 50 | 5 | 1 (20) | 0 | 1 (100) | 3 (60) | 1 (20) | 0 | 0 | 0 | 0 | 0 | 0 | 45 |
| | Faisalabad | // | 13 | 3 | 1 (33.3) | 1 (33.3) | 0 | 1 (33.3) | 1 (33.3) | 1 (33.3) | 0 | 0 | 0 | 0 | 0 | 10 |
| Sindh | Murree | // | 7 | 1 | 1 (14.2) | 1 (14.2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| | Hyderabad | // | 30 | 4 | 0 (0) | 0 | 0 | 2 (50) | 1 (25) | 0 | 0 | 0 | 0 | 0 | 1** (25) | 26 |
| | Karachi | // | 40 | 5 | 1 (20) | 1 (20) | 0 | 1 (20) | 3 (60) | 0 | 0 | 0 | 0 | 0 | 0 | 35 |
| | Rohri | // | 15 | 1 | 0 | 0 | 0 | 0 | 1 (6.66) | 0 | 0 | 0 | 0 | 0 | 0 | 14 |
| | Peshawar | // | 29 | 3 | 0 | 0 | 0 | 2 (66.6) | 1 (33.3) | 0 | 0 | 0 | 0 | 0 | 0 | 26 |
| Khyber Pakhtun khawa | Swat | // | 15 | 2 | 1 (6.66) | 0 | 1 (100) | 0 | 1 (6.66) | 0 | 0 | 0 | 0 | 0 | 0 | 13 |
| | Abbottabad | // | 20 | 2 | 0 | 0 | 0 | 0 | 2 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 18 |
| | Swabi | // | 10 | 1 | 1 (100) | 0 | 1 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 |
| Islamabad (Capital City) | Waziristan | // | 10 | 3 | 3 (100) | 1 (33.3) | 2 (66.6) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |
| | - | // | 36 | 3 | 2 (66.6) | 1 (50) | 1 (50) | 0 | 1 (33.3) | 0 | 0 | 0 | 0 | 0 | 0 | 33 |
| Total | - | - | 400 | 50 (12.5%) | 13 (26%) | 5 (38.1%) | 8 (61.5%) | 18 (36%) | 15 (30%) | 1 (2%) | 0 (0.0%) | 3 (6%) | 0 | 0 | 350 (87.5%) | |

Mix Combinations = * aEPEC/EAEC; ** EPEC/ETEC; aEPEC = Atypical EPEC
† Unknown = diarrheal infections other than *E. coli*.

TABLE 4
Age-wise distribution of different *Escherichia coli* pathotypes

| Pathotype | Population studied floods cases sporadic cases | | | | | |
|-----------|--|-------------|---------------|-------------|---------------|-------------|
| | Total samples | Average age | Total samples | Average age | Total samples | Average age |
| EAEC | 39 | 4.96 | 21 | 3.09 | 18 | 6.83 |
| EPEC | 46 | 6.60 | 33 | 2.96 | 13 | 10.25 |
| ETEC | 44 | 7.27 | 29 | 3.94 | 15 | 10.6 |
| EHEC | 6 | 5.95 | 5 | 5.8 | 1 | 6 |
| EIEC | 1 | 2.5 | 1 | 2.5 | 0 | 0 |
| Mixed | 29 | 6.44 | 26 | 4.88 | 3 | 8 |
| Total | 165 | 5.62 | 105 | 3.86 | 50 | 8.3 |

can be monitored using ERIC-PCR during natural disasters such as floods.

The EPEC isolates were run on three gels (EPEC A, EPEC B, and EPEC C), which were classified into different clusters based on a similarity coefficient threshold derived from the banding patterns (Supplemental Table 2). Similarly, ETEC isolates were run on three gels (ETEC D, ETEC E, and ETEC F), which were classified into different clusters based on banding patterns (Supplemental Table 2). However, the EAEC isolates were divided into only two clusters as shown in (Supplemental Table 2).

Moreover, EPEC pathotypes from flood affected cases as well as from sporadic cases were further scrutinized on the basis of the presence of both *eae* and *bfp* or *eae*, respectively (Figure 1A). The study suggested that 6 (18.18%) and 5 (38.18%) were typical EPEC from both flood and sporadic cases, respectively, whereas 27 (81.82%) and 8 (61.53%) atypical EPEC were identified from flood and sporadic cases, respectively. In addition, 29 ETEC from flood affected cases were further subdivided into three categories 1) producing both heat labile toxin (LT) and heat stable toxin (ST), 4 (13.79%); 2) producing only LT, 24 (82.75%); 3) producing only ST, 1 (3.44%). However, 15 ETEC from sporadic cases were divided into two categories, producing both heat LT and heat ST, 8 (53.33%) or producing only LT, 7 (46.66%) (Figure 1C); and 4) children younger than 5 years of age are more often affected by EAEC than older children in both flood affected and non-flood affected areas (Table 4).

DISCUSSION

Diarrhea is a major cause of mortality in children of developing countries and is endemic in South Asia caused by the lack of potable water, poor sanitation, and poverty. During natural disasters in areas where diarrhea is endemic, the situation is exacerbated. To date few systematic field studies on diarrheal pathogens have been undertaken in Pakistan. Pakistan faced a sudden increase in diarrheal cases during the floods in 2010 and 2011. This study shows that this is largely a result of different *E. coli* pathotypes compared with sporadic cases from the non-flood affected areas in the same provinces during the similar time periods.

Out of a total 205 isolates investigated, a high prevalence of DEC 115 (56.29%) pathotypes from flood affected cities was noted and the trend of their occurrence was EPEC>ETEC>EAEC>EHEC>EIEC (Table 4). However, low prevalence of diarrhea, i.e., 12.5% was recorded from sporadic cases and the trend of different pathotypes were EAEC>ETEC>EPEC>EHEC from mainly inland cities (Table 4).

Out of a total 115 *E. coli* pathotypes during the floods (Table 2); interestingly, EAEC and ETEC were the predominant types from Khairpur, Sindh situated close to the river Indus, atypical EPEC was seen in Muzaffargarh, Punjab situated close to the rivers Chenab and Indus, and from Charsada, KPK situated close to the rivers Swat and Kabul. However, we have seen all types circulating in the D.I. Khan district of KPK, in an area adversely affected by floods, where most of the previously mentioned rivers converge. Only five cases of EHEC were detected and they were from Nowshera and D.I. Khan districts of KPK.

Similar trends have already been reported for the occurrence of different DEC such as atypical EPEC, ETEC, EIEC, and DAEC.^{18–22} On the other hand typical EPEC, a well-recognized cause of infantile diarrhea,⁴ contributed much less during the current study compared with atypical EPEC in previous studies.^{23,24} Similar results have been observed in Bangladesh where the majority of ETEC producing LT, followed by LT + ST and ST producing were predominantly associated with flood associated diarrheal cases²⁵ compared with previous studies from China, India, Mexico, Myanmar, and Pakistan where strains predominantly produced ST.²⁶ The association of EAEC with diarrhea has not been frequently reported,²⁷ however in our study EAEC is one of the most frequently isolated pathotype in sporadic cases, whereas EPEC is from flood affected cases. Furthermore, younger children (< 5 years of age) are more often affected by EAEC than older children in both the flood affected and non-flood affected areas (Table 4). On the other hand, contrary to a

previous study,²⁸ EHEC was detected at a very low level from both flood affected and sporadic cases in this study.

The ERIC-PCR results are generally comparable to the results of a previous report where three to five ERIC-PCR bands were detected in each of several mastitis *E. coli* strains and further validate the identification of pathotypes in this study.^{29,30} Because many isolates studied were gathered from flooded areas where a large number of livestock casualties were also reported, hence there is a strong possibility that many of these isolates can be of different animal origin leading to perhaps diverse intrapathotype fingerprinting patterns (Supplemental Table 2).

Five of the six DEC pathotypes were identified during floods (excluding DAEC) and EAEC, ETEC, EPEC, and EHEC were identified from sporadic cases during 2010 and 2011. We showed that 1) the majority of diarrhea cases were surprisingly *E. coli* during the floods in contrast to sporadic controls; 2) all strains of ETEC isolated from the flooded cities possessed either *lt + st* or *lt* except one isolate that had *st* alone, whereas only the first two categories were present in sporadic cases; 3) the presence of the major four pathotypes of *E. coli* were prevalent in city of D.I. Khan during floods and in Rawalpindi from sporadic cases; and 4) the geographic information of the diverse areas of Pakistan with reference to the cases of diarrhea associated with particular pathotype(s) was reported.

The large number of unknown infectious agents in sporadic infection is apparent in this study. This could be caused by Rotaviruses and Caliciviruses, which are principal agents

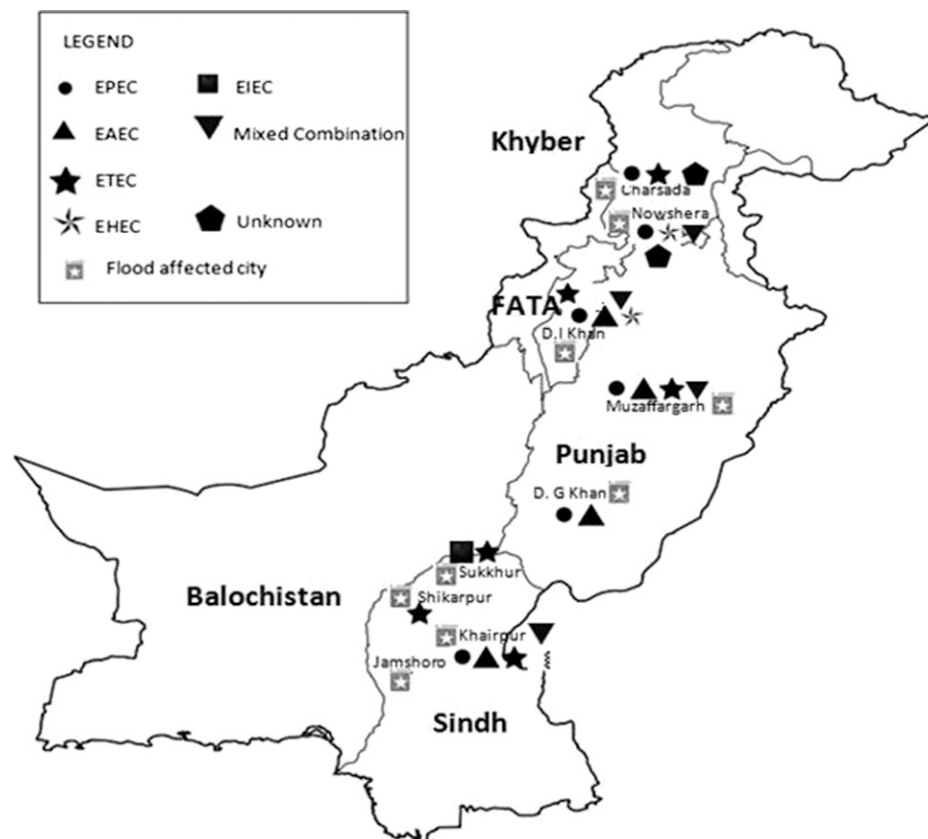


FIGURE 2. Geographic distribution of *Escherichia coli* pathotypes in various districts of Punjab, Khyber Pakhtunkhwa, and Sindh during the floods in 2010 and 2011.

causing infrequent and sporadic diarrheal disease in many regions of the world, including developing countries.^{31–33} Furthermore, parasites are also an important cause of diarrhea in children.³⁴ It is possible that in “crowded urban settings” from where most of the sampling for sporadic cases was undertaken, these infectious agents may contribute significantly. Our results also showed the presence of *Vibrio cholerae*, *Shigella* sp., *Campylobacter* sp., and *Salmonella* sp. (unpublished results, Shah MA, Akram M, Akhtar S, Siddiqui F, Bokhari H, Wren B) diarrheal samples in children that may also add to the non-*E. coli* diarrheal disease burden in these settings.

In Thailand a geographical information system (GIS)-based analysis was used to map urban canals that were predominant sources of pathogens including *E. coli*.³⁵ We have also done a preliminary investigation using the GIS to manually map point the location of diarrheal pathogens collected from flood affected areas of Pakistan (Figure 2). During the last 2 years (2010 and 2011) abrupt climatic changes caused by an average increase of rainfall and rise in temperature combined with storms intensified the transport of fecal and wastewater sources, which led to the spread of diarrheal outbreaks across the country as reported previously³⁶ and are expected to further increase during 2013. Exceptional trends are seen in the months of August, September, and especially in October. It is believed that because of contamination by flood water, samples are severely affected by multiple DEC such as ETEC, EAEC, EPEC with variable distribution.

Thus, we conclude that DEC pathogens are likely to flourish with conducive climatic parameters, such as rainfall intensity and temperature, and thereby play a major role in diarrheal episodes in Pakistan during floods. This study emphasizes that ETEC, EPEC, and EAEC can be a major source of diarrhea in epidemics caused by floods and determining the geographic location of diarrheal pathogen in Pakistan may be useful for public health officials to trace the sources and routes of DEC infection. We also conclude that EAEC bacteria are involved in a significant proportion of diarrhea cases among children.

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REFERENCES

- Cohen MB, Nataro JP, Bernstein DI, Hawkins J, Roberts N, Staat MA, 2005. Prevalence of diarrheagenic *Escherichia coli* in acute childhood enteritis: a prospective controlled study. *J Pediatr* 146: 54–61.
- Nguyen TV, Van PL, Huy CL, Gia KN, Weintraub A, 2005. Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. *J Clin Microbiol* 43: 755–760.
- Iruka IN, 2009. Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status, uncertainties and necessities. *J Infect Dev Ctries* 3: 817–842.
- Nataro JP, Kaper JP, 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11: 142–201.
- Albert MJ, Rotimi VO, Dhar R, Silpikurian S, Pacsa AS, Molla AM, Szucs G, 2009. Diarrheagenic *Escherichia coli* are not a significant cause of diarrhea in hospitalized children in Kuwait. *BMC Microbiol* 9: 62.
- Ibenyassine K, Aitmhand R, Karamoko Y, Ennajim M, 2007. A simple and rapid detection by PCR of enteropathogenic *Escherichia coli* in naturally contaminated vegetables. *J Rap Meth Auto Microbiol* 16: 113–121.
- Robins-browne RM, Hartland EL, 2002. *Escherichia coli* as a cause of diarrhea. *J Gastro Hepatol* 17: 467–475.
- Kaper JB, Nataro JP, Mobley HL, 2004. Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2: 123–140.
- Croxen MA, Finlay BB, 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nature* 8: 26–38.
- Aranda KR, Fabbriotti SH, Fagundes-Neto U, Scaletsky IC, 2007. Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. *FEMS Microbiol Lett* 267: 145–150.
- Vidal M, Kruger E, Duran C, Lagos R, Levine M, Prado V, 2005. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. *J Clin Microbiol* 43: 5362–5365.
- Gómez-Duarte OG, Bai J, Newell E, 2009. Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae* and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. *Diagn Microbiol Infect Dis* 63: 1–9.
- Hulton CS, Higgins CF, Sharp PM, 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Mol Microbiol* 5: 825–834.
- Sharlps GJ, Lloyd RG, 1990. A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Res* 18: 6503–6508.
- Hai-Rong C, Ning J, 2006. Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol Lett* 28: 55–59.
- Johnson JR, O'Bryan TT, 2000. Improved repetitive-element PCR fingerprinting for resolving pathogenic and nonpathogenic phylogenetic groups within *Escherichia coli*. *Clin Diagn Lab Immunol* 7: 265–273.
- Manges AR, Johnson JR, Foxman B, O'Bryan T, Fullerton KE, Riley LW, 2001. Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* clonal group. *N Engl J Med* 345: 1007–1013.
- Vieira MA, Andrade JR, Trabulsi LR, Rosa AC, Dias AM, Ramos SR, Frankel G, Gomes TA, 2001. Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry EAE and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. *J Infect Dis* 183: 762–772.
- Jenkins C, Lawson AJ, Cheasty T, Willshaw GA, Wright P, Dougan G, Frankel G, Smith HR, 2003. Subtyping intimin genes from enteropathogenic *Escherichia coli* associated with outbreaks and sporadic cases in the United Kingdom and Eire. *Mol Cell Probes* 17: 149–156.
- Bouckenooghe AR, Jiang ZD, DeLaCabada FJ, Ericsson CD, DuPont HL, 2002. Enterotoxigenic *Escherichia coli* as cause of diarrhea among Mexican adults and US travelers in Mexico. *J Travel Med* 9: 137–140.
- Hien BT, Scheutz F, Cam PD, Serichantalergs O, Huong TT, Thu TM, Dalsgaard A, 2008. Diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in a hospital case-control study in Hanoi, Vietnam. *J Clin Microbiol* 46: 996–1004.
- Qadri F, Svennerholm AM, Faruque ASG, Sack RB, 2005. Enterotoxigenic *Escherichia coli* (ETEC): epidemiology, microbiology,

- and clinical features in developing countries. *Clin Microbiol Rev* 18: 465–483.
23. Dulguer MV, Fabbriotti SH, Bando SY, Moreira-Filho CA, Fagundes-Neto U, Scaletsky IC, 2003. Atypical enteropathogenic *Escherichia coli* strains: phenotypic and genetic profiling reveals a strong association between enteroaggregative *E. coli* heat-stable enterotoxin and diarrhea. *J Infect Dis* 188: 1685–1694.
 24. Yatsuyanagi J, Saito S, Sato H, Miyajima Y, Amano K, Enomoto K, 2002. Characterization of enteropathogenic and enteroaggregative *Escherichia coli* isolated from diarrheal outbreaks. *J Clin Microbiol* 40: 294–297.
 25. Harris AM, Chowdhury F, Begum YA, Khan AI, Faruque AS, Svennerholm AM, Harris JB, Ryan ET, Cravioto A, Calderwood SB, Qadri F, 2008. Shifting prevalence of major diarrheal pathogens in patients seeking hospital care during floods in 1998, 2004, and 2007 in Dhaka, Bangladesh. *Am J Trop Med Hyg* 79: 708–714.
 26. Huilan S, Guang ZL, Mathan MM, Mathew MM, Olarte J, Espejo R, Khin MU, Ghafoor MA, Khan MM, Sami Z, Sutton RG, 1991. Aetiology of acute diarrhea among children in developing countries: a multicentre study in five countries. *Bull World Health Organ* 69: 549–555.
 27. Gunzburg ST, Chang BJ, Elliott SJ, Burke V, Gracey M, 1993. Diffuse and enteroaggregative patterns of adherence of enteric *Escherichia coli* isolated from aboriginal children from the Kimberley region of Western Australia. *J Infect Dis* 167: 755–758.
 28. Nguyen T, Van PL, Huy CL, Gia KN, Weintraub A, 2005. Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. *J Clin Microbiol* 43: 755–760.
 29. Prabhu S, Vivek Isloor S, Balu M, Suryanarayana VV, Rathnamma D, 2010. Genotyping by ERIC-PCR of *Escherichia coli* isolated from bovine mastitis cases. *Indian J Biotechnol* 9: 298–301.
 30. Lipman LJ, de-Nijs A, Lam TJ, Gaastra W, 1995. Identification of *Escherichia coli* strains from cows with clinical mastitis by serotyping and DNA polymorphism patterns with REP and ERIC primers. *Vet Microbiol* 43: 13–19.
 31. Shamim A, Ansari V, Springthorpe S, Sattar SA, 1991. Survival and vehicular spread of human rotaviruses: possible relation to seasonality of outbreaks. *Rev Infect Dis* 13: 448–461.
 32. Glass RI, Kilgore PE, Holman RC, Jin S, Smith JC, Woods PA, Clarke MJ, Ho MS, Gentsch JR, 1996. The epidemiology of rotavirus diarrhea in United States: surveillance and estimates of disease burden. *J Infect Dis* 174: S5–S11.
 33. Peasey AE, Ruiz-Palacios GM, Quigley M, Newsholme W, Martinez J, Resales G, Jiang X, Blumenthal UJ, 2004. Sero-epidemiology and risk factors for sporadic norovirus/Mexico strain. *J Infect Dis* 189: 2027–2036.
 34. Kaur R, Rawat D, Kakkar M, Uppal B, Sharma VK, 2002. Intestinal parasites in children with diarrhea in Delhi, India. *Southeast Asian J Trop Med Public Health* 33: 725–729.
 35. Tripathi NK, Anceno AJ, 2009. GIS-based analysis of the fate of waste related pathogens, *Cryptosporidium parvum*, *Giardia lamblia* and *Escherichia coli* in tropical canal network. *J Water Health* 7: 133–143.
 36. Rose JB, Epstein PR, Lipp EK, Sherman BH, Bernard SM, Patz JA, 2001. Climate variability and change in the United States: potential impact on water and food-borne diseases caused by microbiologic agents. *Environ Health Perspect* 109: 211–221.