Evolution of diarrheagenic *Escherichia coli* pathotypes in India

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Abstract:

CONTEXT: Diarrheagenic *Escherichia coli* (DEC) is the leading cause of infectious diarrhea in developing countries. On the basis of virulence and phenotypic characteristics, the DEC is categorized into multiple pathotypes. Each pathotype has different pathogenesis and geographical distribution. Thus, the proper management of disease relies on rapid and accurate identification of DEC pathotypes.

AIMS: The aim of the study was to determine the prevalence of DEC pathotypes in India.

MATERIALS AND METHODS: A cross-sectional study was carried out between January 2008 and December 2012 at Jawaharlal Nehru Medical College and KLES Dr. Prabhakar Kore Hospital and Medical Research Center, Belgaum (Karnataka), India. A total of 300 stool samples were collected from diarrhea patients with age >3 months. The DEC was identified by both conventional and molecular methods.

RESULTS: Of 300 samples, *E. coli* were detected in 198 (66%) and 170 (56.6%) samples by culture and polymerase chain reaction, respectively. Among DEC (*n* = 198) isolates, *eae* gene (59.5%) was the most prevalent followed by *stx* (27.7%), *east* (27.2%), *elt* (12.6%), *est* (10.6%), *ipaH* (5.5%), and *eagg* (1.5%) genes. On the basis of virulence genes, enteropathogenic *E. coli* (33.8%) was the most common pathotype followed by Shiga toxin-producing *E. coli* (STEC, 23.2%), enterotoxigenic *E. coli* (ETEC, 13.6%), enteroinvasive *E. coli* (5.5%), enteroaggregative heat-stable enterotoxin 1-harboring *E. coli* (EAST1EC, 4.5%), STEC/ETEC (3.5%), STEC/enteroaggregative *E. coli* (STEC/EAEC, 1.0%), and EAEC (0.05%).

CONCLUSIONS: The hybrid DEC is potentially more virulent than basic pathotypes. The pathotyping should be included in clinical settings for the proper management of DEC-associated diarrhea.

Key words:

Enteroaggregative *Escherichia coli*, enteroaggregative heat-stable enterotoxin 1-harboring *Escherichia coli*, enteroinvasive *Escherichia coli*, enteropathogenic *Escherichia coli*, enterotoxigenic *Escherichia coli*, Shiga toxin-producing *Escherichia coli*

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Introduction

Infectious diarrhea caused by bacteria, parasites, viruses, and fungi is the leading cause of morbidity and mortality worldwide.[1-3] Among these pathogens, *Escherichia coli (E. coli)* is one of the most common causes of bacterial diarrhea in developing countries.[3,4] On the basis of virulence and phenotypic characteristics, the diarrheagenic *E. coli* (DEC) are categorized into six main pathotypes including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC), and diffusely adherent *E. coli* (DAEC).[5] Each pathotype has a distinct geographical distribution and pathogenesis.[5,6] Thus, the proper management of the disease depends on the rapid and reliable identification of DEC pathotypes.

Identification and typing both are equally important for the proper management of DEC-associated diarrhea. Today,

numerous methods have been developed for the typing/identification of DEC such as serotyping, pulsed-field gel electrophoresis, multilocus satellite typing, multilocus variable-number tandem-repeat analysis, ribotyping, and multiplex polymerase chain reaction (mPCR).[7-11] However, none of these methods can be used for rapid and reliable typing/identification of DEC except mPCR. Moreover, only PCR can differentiate between pathogenic and commensal *E. coli*.

Several PCR-based studies have been published for the identification of DEC pathotypes.[8,12-14] However, we cannot neglect the evolution of DEC pathotypes with time because of genomic flexibility of *E. coli*. In the past few decades, multiple new DEC pathotypes have been reported worldwide.[15,16] For instance, the hybrid strains such as STEC/ETEC and STEC/ EAEC (O104:H4), these strains cannot be fit into the aforementioned six major pathotypes.[15,16] Moreover, the controversy exists regarding the role of EAEC heat-stable enterotoxin 1-harboring *E. coli* (EAST1EC) in diarrhea.[17-21] In this study, we used monoplex and multiplex PCRs with minimum set of virulence genes to determine the prevalence of existing and evolving DEC pathotypes.

Materials and Methods

Study population and clinical samples
A cross-sectional study was carried out between January 2008 and December 2012 at Jawaharlal Nehru Medical College and KLES Dr. Prabhakar Kore Hospital and Medical Research Center, Belgaum (Karnataka), India. A total of 300 stool samples were collected from diarrhea patients with age >3 months. Inform consent was obtained from each patient. The study was approved by the Institutional Ethics Committee.

Selection of cases
a. Inclusion criteria – Patients of >3 months of age, suffering from diarrhea
b. Exclusion criteria – Patients of <3 months of age, patients having HIV-associated diarrhea, or patients having diarrhea due administration of antibiotics and cases with already known enteropathogens were excluded from the study.

Isolation and identification of *Escherichia coli*
Stool samples were cultured on MacConkey agar and incubated at 37°C for 18-24 hours. The lactose-fermenting colonies were subjected to conventional and molecular identification. The samples positive for one of the enteric pathogens, namely *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Candida*, and trophozoites/cyst/ova of the parasites were excluded from the study.

Bacterial strains
The control strains of EPEC, ETEC, EIEC, enterohemorrhagic *E. coli* (EHEC), and EAEC were provided by Dr. Shanta Dutta, Scientist "F," of the National Institute of Cholera and Enteric Diseases, Kolkata. The reference strains were positive for virulence genes included in PCR, namely EPEC: *eae* gene, ETEC: *elt* and *est* genes, STEC: *stx* gene, EIEC: *ipaH* gene, and EAEC: *eagg* gene.

DNA extraction
DNA extraction was carried out using the cetyltrimethylammonium bromide (CTAB) method.[22] In brief, the culture was centrifuged at 8000 revolution per minute (rpm) for 5 minutes (min), and the supernatant was discarded and transferred the pellet to a vial containing 10 mg/mL lysozyme (Fermentas Inc., Maryland, USA). Following lysis of bacterial cells, the lysate treated with 10% sodium dodecyl sulfate (56 µL) and proteinase K (5 µL) (Fermentas Inc., Maryland, USA). For polysaccharide precipitation, 5 M sodium chloride (NaCl) and CTAB/ NaCl (64 µL) (Fermentas Inc., Maryland, USA) were added and incubated at 65°C for 30 min. After this, an equal amount of chloroform: isoamyl alcohol solution (Fermentas Inc., Maryland, USA) was added to the mix and centrifuged at 10,000 rpm for 15 min followed by the precipitation of DNA with ethanol. Finally, the DNA precipitate was resuspended in 40 µL Tris-EDTA (TE) buffer.

Polymerase chain reaction
The primer sequences used for DEC pathotype identification were retrieved from the previous publication.[23-27] The primers were synthesized by Sigma (Sigma-Aldrich Co., St. Louis, MO, USA) and specificity for targeted genes (*eae*, *astA*, *eagg*, *stx*, *ipaH*, *elt*, and *est* genes) confirmed by monoplex PCR [Supplementary Table 1]. The multiplex PCR assays were standardized by testing different PCR cycling conditions. The criteria used for the identification of DEC pathotypes are enlisted in Supplementary Table 2. The two pairs of primers were used in each mPCR because of non-specific amplification and primer-dimer formations; therefore, five multiplex PCRs were standardized for the identification of DEC.

- Multiplex 1: Identification EPEC (*eae* and *astA* genes)
- Multiplex 2: Identification ETEC (*elt* and *astA* genes)
- Multiplex 3: Identification ETEC (*est* and *astA* genes)
- Multiplex 4: Identification STEC (*stx* and *astA* genes)
- Multiplex 5: Identification EAEC (*eagg* and *astA* genes).

Each monoplex and multiplex PCRs were performed using 25 µL reaction mixtures. The master mix included 10X PCR buffer with $MgCl_2$: 2.5, dNTP mix: 2.0 µL (200 µM each), forward: 1.0 µL (400 nM), reverse primer: 1.0

µL (400 nM), template DNA: 2.5 µL (100 ng), sterile water: 15.8 µL, and Taq polymerase: 0.2 µL (0.4 U) (Fermentas Inc., Maryland, USA). For multiplex PCRs, the volume of water was adjusted, according to the number of primers. The PCR thermocycling conditions were as follows: initial denaturation 94°C for 5 min followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (primer dependent), extension (72°C for 30 seconds), and final extension (72°C for 10 min).[9]

Statistical analysis

All statistical analysis was done using Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (IBM Corp., Armonk, New York, USA). The patients were classified into multiple groups according to their age.

Results

Out of 300, 102 (34%) samples were positive for one of the enteric pathogens and the remaining 198 (66%) samples yielded growth of *E. coli* [Table 1]. Among *E. coli* (*n* = 198) isolates, PCR detected virulence genes in 170 (85.8%) isolates only. In DEC pathotypes, EPEC (33.8%) was the predominant pathotypes followed by STEC (23.2%), ETEC (13.6%), EIEC (5.5%), EAST1EC (4.5%), EIEC with *east* (1.7%), STEC/ETEC (3.5%), and STEC/EAEC (1.0%). In about 14.1% DEC, no virulence genes were detected [Figure 1].

In *E. coli* (*n* = 198), the *eae* gene (59.5%) was the most prevalent followed by *stx* (27.7%), *east* (27.2%), *elt* (12.6%), *est* (10.6%), *ipaH* (5.5%), and *eagg* (1.5%) [Figure 2]. The

overlapping of virulence genes was observed among DEC pathotypes. The *east* gene was detected in all DEC pathotypes [Figure 2].

The age of patients ranges from 4 months to 40 years. Eighty-three (*n* = 83, 41.9%) patients were <5 years of age and 115 patients (*n* = 115, 58%) were >5 years of age. Among different age groups, the maximum number of diarrhea cases was seen in children [Figure 3].

Discussion

DEC-associated outbreaks have risen in advanced and developing nations in recent decades, recognizing the significance of DEC globally.[5,6] In India, multiple studies have been published on the pediatric population but very few targeted adults.[1-3] Thus, we included both children and adults in this study. The prevalence of DEC in our study was much higher (66%) than previous studies.[2,3] The inclusion of the adult age group may be the reason of higher prevalence. On comparing the children and adults, the low prevalence of DEC was seen in adult. The low prevalence of DEC in adults may also be due to mucosal immunity, loss of receptors, and acquired immunity.[5]

Among DEC pathotypes, EPEC (33.8%, 67/198) was the most common pathotype, corresponding to the studies from other developing countries. Although EPEC frequently isolated from persistent infection in children, we detected the cases in both children and adults, suggesting that EPEC is not limited to children only. On the basis of virulence genes (*eae* and *bfp*), EPEC is classified into typical (*eae*) and atypical EPEC (*bfp*).[6] In
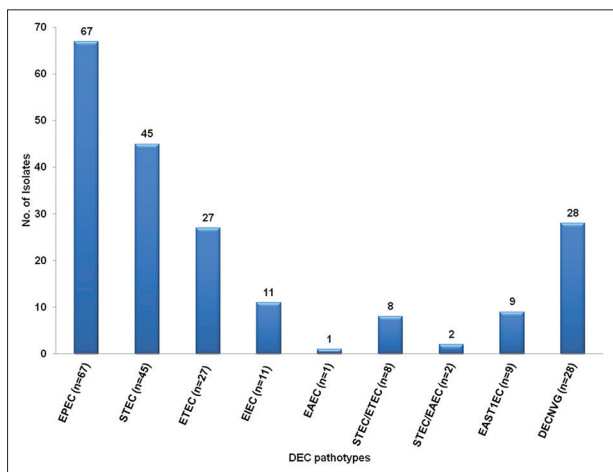
Figure 1: Prevalence of DEC pathotypes (*n* = 198). *E. coli* = *Escherichia coli*, DEC = Diarrheagenic *Escherichia coli*, EPEC = Enteropathogenic *Escherichia coli*, ETEC = Enterotoxigenic *Escherichia coli*, EIEC = Enteroinvasive *Escherichia coli*, EAEC = Enteroaggregative *Escherichia coli*, STEC = Shiga toxin-producing *Escherichia coli*, STEC/EAEC = *Escherichia coli* with *stx* and *eagg* genes, STEC/ ETEC = *Escherichia coli* with *stx* and *elt*/*est* or both genes, EAST1EC = EAEC heat-stable enterotoxin 1-harboring *Escherichia coli* (possess no other diarrheagenic characteristics), DECNVG = Diarrheagenic *Escherichia coli* with no virulence genes
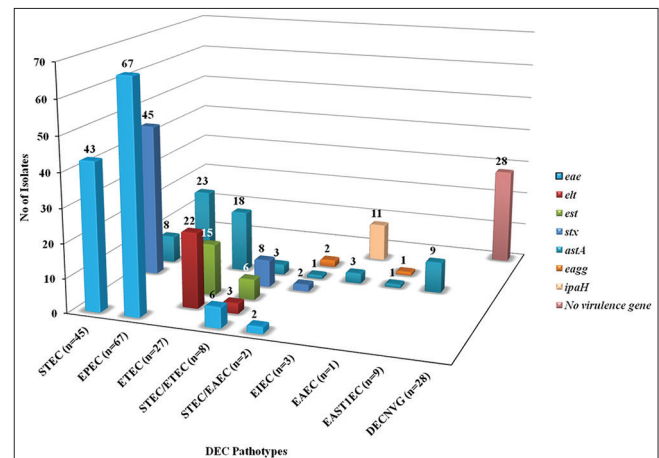
Figure 2: Distribution of virulence genes among DEC (*n* = 198), *E. coli* = *Escherichia coli*, DEC = Diarrheagenic *Escherichia coli*, EPEC = Enteropathogenic *Escherichia coli*, ETEC = Enterotoxigenic *Escherichia coli*, EIEC = Enteroinvasive *Escherichia coli*, EAEC = Enteroaggregative *Escherichia coli*, STEC = Shiga toxin-producing *Escherichia coli*, STEC/ EAEC = *Escherichia coli* with *stx* and *eagg* genes, STEC/ETEC = *Escherichia coli* with *stx* and *elt*/*est* or both genes, EAST1EC = EAEC heat-stable enterotoxin 1-harboring *Escherichia coli* (possess no other diarrheagenic characteristics), DECNVG = Diarrheagenic *Escherichia coli* with no virulence genes

atypical EPEC, multiple alleles of *bfp* gene exist, which cannot be targeted by single PCR.[28] Thus, we targeted only typical EPEC in this study.

Verocytotoxigenic *E. coli*/STEC/Shiga-like toxin-producing *E. coli*/EHEC represents one group of DEC.[5] The STEC terminology was used in this study. Most of the studies reported the low prevalence of STEC in India, whereas we observed the high prevalence of STEC (46, 23.2%), suggesting poor hygiene and sanitation in this region.[6] ETEC is characterized by the expression of *elt* or *est* gene only or both.[5,6] The isolates which possessed either of *elt/est* or both the genes were identified as ETEC pathotypes in this study. The prevalence of ETEC varies from 10% to 30%.[29,30] Similarly, we observed a 13% prevalence in this study.
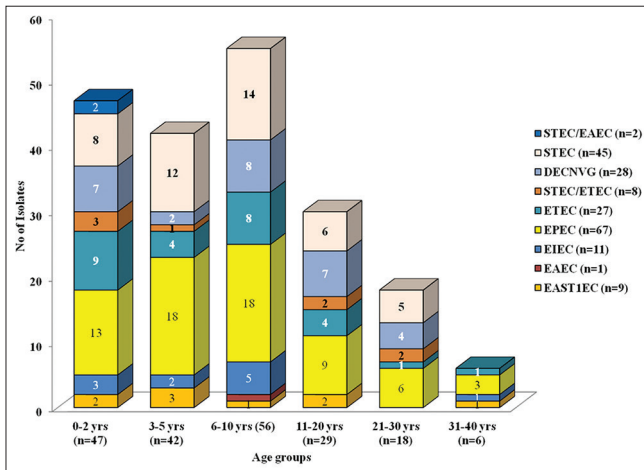
EIEC is characterized by *ipaH* gene expression.[5,31] However, *ipaH* gene sequence cannot differentiate between *Shigella* and EIEC.[5] The EIEC isolates may also

be missed or misidentified as *Shigella* species by this PCR. To exclude the *Shigella*, only lactose-fermenting, indole-producing *E. coli* colonies were subjected to PCR in this study. Despite this, the possibility of missing some lactose negative, nonmotile, and lysine decarboxylase-negative EIEC strains cannot be rule out. In addition, EIEC are missed in clinical laboratory because of suspecting them only in stool sample with mucus and blood, suggesting that the actual incidence of EIEC may also be slightly higher than the result derived in our study.[5]

EAEC is characterized by the absence of heat-labile/heat-stable enterotoxin and the presence of aggregative adherence.[17,32] The *eagg*, *aap*, *aggR*, and *shf* genes were targeted for identification of EAEC on the basis of previous studies.[23,26] EAEC is considered as emerging DEC pathotypes.[26,33] However, a very low prevalence of EAEC was found in this study. In addition, *stx* gene was also detected in two EAEC (*n* = 3). Hence, we cannot draw any conclusion regarding the pathogenicity of EAEC with this small number of isolates.

Among DEC, the *astA* gene (EAST1 toxin) has been reported in multiple DEC pathotypes: ETEC, STEC, and EPEC.[18-21,34] Similarly, the *astA* gene expression was detected in EPEC, ETEC, STEC, EIEC, and EAEC in this study. Moreover, we also identified the nine isolates (4.5%) with *astA* gene only, correlating with the findings of Nishikawa *et al.* 1999 and 2002.[35,36] This suggests that some unrecognized DEC pathotypes exist, which can cause diarrhea even in the absence of typical virulence factors. However, few studies reported no role of *astA* gene in diarrhea.[19,20] Further, the study with large number of isolates may provide insight within the exact role of *astA* gene in diarrhea.

Figure 3: Distribution of DEC pathotypes among different age groups (*n* = 170), *E. coli* = *Escherichia coli*, DEC = Diarrheagenic *Escherichia coli*, EPEC = Enteropathogenic *Escherichia coli*, ETEC = Enterotoxigenic *Escherichia coli*, EIEC = Enteroinvasive *Escherichia coli*, EAEC = Enteroaggregative *Escherichia coli*, STEC = Shiga toxin-producing *Escherichia coli*, STEC/EAEC = *Escherichia coli* with *stx* and *eagg* genes, STEC/ETEC = *Escherichia coli* with *stx* and *elt/est* or both genes, EAST1EC = EAEC heat-stable enterotoxin 1-harboring *E. coli* (possess no other diarrheagenic characteristics), DECNVG = Diarrheagenic *Escherichia coli* with no virulence genes

In the past few decades, multiple hybrid strains of DEC have been reported worldwide.[15,16,37,38] Similarly, we also detected STEC/ETEC (7, 2.3%) and STEC/EAEC (2, 0.6%) in this study. The high prevalence of STEC may also be the reason of emergence of hybrid strains. The hybrid strains contain multiple virulence genes, making

Table 1: Distribution of pathogenic microorganism in acute diarrhea (*n*=300)

Serial number	Pathogens isolated/observed	Number of cases (%)	Remarks (%)
1	*E. coli*	198 (66)	
2	Other pathogens		
A	Other bacteria (*Vibrio cholerae*, *Salmonella spp.*, and *Shigella* spp.)	36 (12)	102 (34) cases was not associated with DEC
B	Parasites (protozoan parasite and helminths)	49 (16.3)	
C	*Candida* spp. (*Candida* was presumed as causative pathogen on the basis of the presence of large number of budding yeasts resembling *Candida* in morphology in the stool samples and the absence of any other known diarrheagenic pathogen. The *Candida* isolates were differentiated by germ tube test into *albicans* and non-*albicans* spp. All isolates were *C. albicans*)	17 (5.6)	
	Total	300	

E. coli=*Escherichia coli*, *C. albicans*=*Candida albicans*
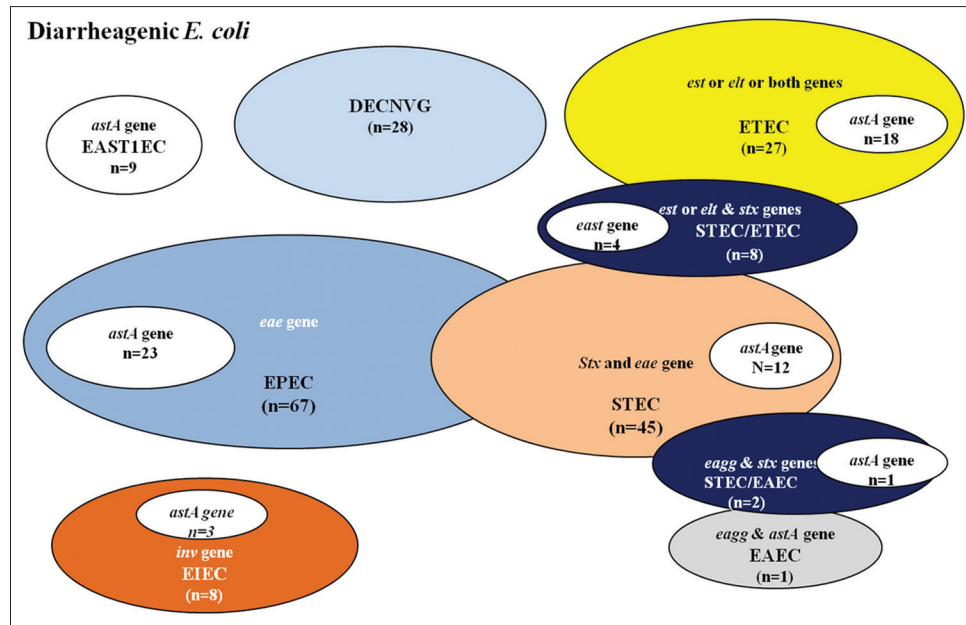
Figure 4: Model for overlapping virulence genes among DEC pathotypes. *E. coli* = *Escherichia coli*, DEC = Diarrheagenic *Escherichia coli*, EPEC = Enteropathogenic *Escherichia coli*, ETEC = Enterotoxigenic *Escherichia coli*, EIEC = Enteroinvasive *Escherichia coli*, EAEC = Enteroaggregative *Escherichia coli*, STEC = Shiga toxin-producing *Escherichia coli*, STEC/EAEC = *Escherichia coli* with *stx* and *eagg* genes, STEC/ETEC = *Escherichia coli* with *stx* and *elt/est* or both genes, EAST1EC = EAEC heat-stable enterotoxin 1-harboring *Escherichia coli* (possess no other diarrheagenic characteristics), DECNVG = Diarrheagenic *Escherichia coli* with no virulence genes

them more virulent and pathogenic.[37,38] The emergence of these hybrid strains may pose a serious threat to public health.

Among DEC, 28 (14.1%) isolates were with no known diarrhea-associated virulence genes, suggesting the existence of some unknown virulence genes and DEC pathotypes, which cannot be detected by targeting common virulence genes such as DAEC and cell-detaching *E. coli*.[39] Further studies may define the role of these DEC in diarrhea.

Conclusions

In brief, the DEC is evolving in India, indicated by the presence of hybrid strain [Figure 4]. The combination of phenotypic and genotypic methods should be used in clinical setting for the reliable identification of typical and emerging DEC pathotypes. Moreover, the surveillance system should be established to monitor DEC-associated outbreak.

Conflicts of interest
There are no conflicts of interest.

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Supplementary Table 1: The primer sequences and their corresponding references

Gene	Primer sequence (5`-3`)	Amplicon size (bp)	Annealing temperature (°C)	Reference number
eae	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCCTCTGC	454	54	24
elt	GCGACAAATTATACCGTGCT CCGAATTCTGTTATATATGT	708	55	24
est	ATTTTTA/CTTTCTGTATTA/GTCTT CACCCGGTACAA/GGCAGGATT	190	55	24
stx	CAG TTA ATG TGG TTG CGA AG CTG CTA ATA GTT CTG CGC ATC	895	55	25
ipaH	TGG AAA AAC TCA GTG CCT CT CCA GTC CGT AAA TTC ATT CT	423	60	27
eagg	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	54	23
astA	CACAGTATATCCGAAGGC CGAGTGACGGCTTTGTAG	94	55	24
aggR	CTAATTGTACAATCGATGTA ATGAAGTAATTCTTGAAT	308	55	26
aap	CTTTTCTGGCATCTTGGGT GTAACAACCCCTTTGGAAGT	232	54	26
shf	ACTTTCTCCCGAGACATTC CTTTAGCGGGAGCATTCAT	613	53	26

Supplementary Table 2: Criteria for identification of diarrheagenic *Escherichia coli* pathotypes

Criteria (gene detected)	Interpretation
eae and/or *astA* but no *stx/est/elt/ipaH/eagg*	EPEC
eae and/or *stx* and/or *astA*	STEC
est and/or *elt* and/*astA*	ETEC
ipaH and/or *astA*	EIEC
eagg and/or astA	EAEC
stx and elt/est and/or *astA*	STEC/ETEC
stx and eagg and/or *astA*	STEC/EAEC
None of the above genes detected but only *astA*	EAST1EC
None of the above genes detected	DECNVG

E. coli=*Escherichia coli*, EPEC=Enteropathogenic *E. coli*, ETEC=Enterotoxigenic *E. coli*, EIEC=Enteroinvasive *E. coli*, EAEC=Enteroaggregative *E. coli*, STEC=Shiga toxin-producing *E. coli*, STEC/EAEC=*E. coli* with *stx* and *eagg* genes, STEC/ETEC=*E. coli* with *stx* and *elt/est* or both genes, EAST1EC=EAEC heat-stable enterotoxin 1-harboring *E. coli* (possess no other diarrheagenic characteristics), DECNVG=Diarrheagenic *Escherichia coli* with no virulence genes