**Epidemiological Report**

Prevalence of Virulence Genes of Diarrheagenic *Escherichia coli* in Fecal Samples Obtained from Cattle, Poultry and Diarrheic Patients in Bangladesh

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**SUMMARY:** Using multiplex real-time PCR, 960 fecal samples collected from poultry, cattle, and patients with diarrhea in Bangladesh were screened for diarrheagenic *Escherichia coli* (DEC). The invasion-related gene *virB* showed the highest prevalence in human patients (41%) and was shown to be positively correlated first with *afaB* with regards to diffuse adhesion and second with *aggR* with regards to aggregative adhesion. These three genes were specific to human patients. In contrast, the Shiga toxin genes *stx1* (57%) and *stx2* (40%) were prevalent in cattle samples. The *eae* gene, which is associated with attaching and effacing lesion formation, and the *elt* and *est* genes, which are associated with enterotoxins, were detected from all three sample sources. Heat map construction and hierarchical clustering assigned the samples into five different clusters, with the patient samples positive for *virB* and *afaB* being placed together in one cluster. Although the detection of virulence genes cannot be a direct indication of the distribution of diarrheagenic organisms, their detection suggests that *Shigella* spp. or enteroinvasive *E. coli* are the most prevalent diarrheagenic bacteria in Bangladesh and that diffusely adherent *E. coli* is concomitantly present with these bacteria. *eae*-possessing organisms in patients may come from cattle and poultry sources. The small number of *stx*-positive patients could be explained by the small number of animal samples that were positive for both *eae* and *stx*.

**INTRODUCTION**

*Escherichia coli* is a normal inhabitant of the intestinal tract of humans and warm-blooded animals; however, several groups of *E. coli* cause gastrointestinal diseases in their hosts and are referred to as diarrheagenic *E. coli* (DEC). Based on bacterial virulence determinants, enteroxin production, and epidemiological and clinical features produced by the pathogen, DEC strains have been classified into several pathotypes, including enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC) (1). The EPEC pathotype is a leading cause of diarrhea in children globally, especially in developing countries (2). The Shiga toxin-producing *E. coli* (STEC) pathotype is highly pathogenic and induces gastrointestinal illnesses by producing the Shiga toxins (*stx*), *Stx1*, and *Stx2*, and their variants (3). EPEC and STEC are likely related to each other since the STEC that possesses the intimin gene (*eae*) of EPEC is often recognized as enterohemorrhagic *E. coli* (EHEC). ETEC is the most common etiological agent of diarrhea in children and adults living in developing countries and is also the most frequent cause of traveler’s diarrhea (4); it is characterized by the capability to secrete and successfully deliver heat-labile (LT) and/or heat-stable (ST) enterotoxins to the epithelial receptors in the host. EIEC, another classic DEC, carries a plasmid harboring the regulatory gene *virB* and causes dysentery-like symptoms (5). EAEC is an emerging pathogen linked to acute diarrhea in children (6). The DAEC pathotype comprises heterogeneous groups of organisms with variable virulence, but its etiological role remains controversial (7). *astA*, which encodes the EAEC heat-stable enterotoxin 1 (EAST1), has originally been detected in EAEC (8), and subsequently, in other DEC pathotypes, including EPEC, ETEC, and STEC (9–11). Although the role of EAST1 in human disease remains unknown, we designated the *E. coli* that possesses *astA* but no other identifiable pathogenic properties as EAST1EC (12); in this study, we screened samples for *astA*.

In previous studies, 45% or 34% of cases of acute diarrhea in Bangladeshi children have been found to be caused by DEC (13,14), and 34% of all diarrheal episodes in Bangladesh have been attributed to DEC (15). In Bangladesh, Albert et al. (13) have examined
stool samples for DEC pathotypes and detected ETEC, EPEC, EAEC, and DAEC, while other researchers have isolated EPEC and ETEC (14) or only STEC (16). All these studies have been conducted only on hospitalized patients with diarrhea; stool samples from patients with diarrhea and domestic animals in Bangladesh have not been exhaustively examined for all DEC pathotypes. These pathogens may be transmitted through foods of animal origin or water contaminated with human or animal feces. Therefore, the reservoirs and pathways of all DEC pathotypes need to be reinvestigated using sensitive methods for their detection in samples from children with diarrhea and all possible reservoirs. A lack of knowledge that could be obtained by such studies will hinder the establishment of appropriate target-oriented prevention measures in this developing country.

Previously, we have developed a highly sensitive multiplex real-time polymerase chain reaction (PCR) protocol capable of detecting DEC virulence genes (17). To determine the possible sources and routes of transmission for DEC, we used this method for the exhaustive detection of the genes of DEC in fecal samples obtained from cattle, poultry, and human patients with diarrhea following the enrichment culture of these samples. We also analyzed the epidemiological associations among virulence genes by principal component analysis, correlation matrix analysis, and hierarchal clustering methods to predict the sources and routes of transmission of the DEC pathotypes in the study area.

MATERIALS AND METHODS

Samples: A total of 960 fecal samples were obtained by random sampling from different poultry farms, dairy farms, and hospitals (patients with diarrhea) in Bangladesh during 2016 and 2017. Fecal samples from 600 poultry were collected from 20 poultry farms across seven districts, and 200 fecal samples from cattle were collected from 10 different dairy farms. Finally, 160 fecal samples from pediatric patients experiencing diarrhea were collected from four different hospitals across three districts. Bacteriological sample collection media (pro-media FC-20, ELMEX, Tokyo, Japan) was used for culturing the fecal samples.

Enrichment culture of the fecal samples: The fecal samples were cultured in trypticase soy broth (Nissui Pharmaceutical Company, Tokyo, Japan) for 20 h at 37°C for bacterial enrichment. After the enrichment culture, an aliquot (0.5 mL) of each sample was centrifuged and the collected bacterial cells were used for DNA extraction and PCR screening.

DNA isolation from bacterial cells: DNA extraction was performed using a Genomic DNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Purified DNA was used as a template for real-time PCR.

Multiplex real-time PCR for the detection of DEC: Duplex or triplex PCR reactions were performed as reported in a previous study (17). The genes encoding (eae), Stx (stx1 and stx2), LT (elt), ST types h and p (est-h and est-p, respectively), EAEC transcription factor (aggR), EAST1 (astA), EIEC transcription factor (virB), and DAEC adhesion (afaB) were targeted for amplification. Triplex PCR was performed to simultaneously detect eae, which can be associated with EPEC, and stx1 and stx2 in STEC, followed by duplex PCR to distinguish between stx1 and stx2. Another triplex PCR reaction was performed for ETEC (elt, est-h, and est-p). Two duplex PCR analyses were

Fig. 1. Prevalence of virulence genes of DEC in fecal samples from poultry, cattle, and juvenile human patients with diarrhea.
performed for the simultaneous detection of EAEC (aggR) and EAST1EC (astA), in addition to EIEC (virB) and DAEC (afaB). All PCR reactions were performed using the QuantiTect Multiplex PCR solution (QIAGEN, Hilden, Germany) with the Step One Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR cycling conditions were as follows: denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 1 min and 60°C for 1 min.

**Bacterial strains:** The following representative bacterial strains were used as positive controls during the experiments: EPEC strain E2348 ⁄ 69; ETEC strains E5798 and 97-245-244; STEC strains 99-140-A, V-831; EIEC strain E35990; EAEC strain E59152; EAST1EC strain 96-127-23; and DAEC strain V-64, according to a previous study (17).

**Statistical analysis:** The open statistical program R version 3.5 was used for the statistical analysis (18). The R packages 'FactoMineR (19)' and 'factoextra'(20) were used to perform principal component analysis (PCA) and visualize the results. The ‘cor’ function was used to analyze correlations and the ‘cor.test’ function was used to determine the significance between variables. Significant correlations were visualized using the ‘corrplot’ function from the ‘corrplot’ package. Heat map representations were performed using the ‘heatmap.2’ function in the ‘gplot’ package.

**RESULTS**

**Prevalence of genes encoding the virulence of DEC:** Among the three sample sources, eae was the most prevalent gene; it was evenly distributed. Specifically, this virulence gene was the most prevalent in the stool samples of human patients (37%), followed by cattle feces (35%) and poultry feces (19%) (Fig. 1). Shiga toxin-encoding genes (stx) were detected in 131 samples, of which 126 and 95 samples were positive for stx1 and stx2, respectively; both genes were more prevalent in cattle feces (57% and 40%, respectively) than in feces from other sources (Fig. 1). In contrast, virB, afaB, and aggR, which can suggest the presence of EIEC or Shigella spp., DAEC, and EAEC, respectively, were predominant in the stool samples of human patients (Table 1, Fig. 1). Among the 160 stool samples obtained from the patients, 66 (41%), 48 (30%), and 34 (21%) samples were positive for virB, afaB, and aggR, respectively. The enterotoxin genes est and elt were detected in fecal samples from poultry, cattle, and human patients, but at low levels (Fig. 1); est was found in 5%, 10%, and 6% of the poultry, cattle, and human patient samples, respectively, and elt was found in 3%, 5%, and 8% of the poultry, cattle, and human patient samples, respectively. The most ubiquitous virulence gene in each sample group was astA, which was detected in 677 of the 960 fecal samples. The highest detection rate of this gene was found in samples from cattle (84%), followed by those from poultry (75%) and human patients (34%).

**Correlation of DEC genes in human**
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In this study, stx1 and stx2 were associated with each other, irrespective of the sample source (Figs. 2A, 3, and 4). However, virB, afaB, and aggR were correlated to one another in the patient samples (described above), and astA was correlated with virB, aggR, and elt in the patient samples (Figs. 3A and 4A). Although eae was prevalent among the patient samples, no association was found between eae and stx (Figs. 3A and 4A). However, elt was correlated with stx (Figs. 3A and 4A). The correlation between the enterotoxin genes (est and elt) was unexpectedly found to be slightly negative.

**Correlation of DEC genes in domestic animals:**
The genes eae, elt, and est appeared to be correlated in cattle (Fig. 3B); however, the correlation matrix analysis did not support this observation (Fig. 4B). Instead, astA was found to be positively correlated with stx and est, but negatively associated with afaB in cattle (Fig. 4B). eae was weakly associated with stx and astA in poultry (Figs. 3C and 4C).

**Hierarchical clustering of DEC virulence genes among the fecal samples:**
Heat map construction and hierarchical clustering of DEC genes in the fecal samples from poultry, cattle, and human patients in Bangladesh categorized all the 960 samples into five different clusters according to the presence and absence of the virulence genes (Fig. 5): (A) patient samples positive for virB and afaB, (B) aggR- or est-positive samples from patients and poultry, (C) cattle samples positive for stx, (D) virB- or eae-positive patient samples and eae-positive cattle and poultry samples, and (E) samples with no specific virulence genes (comprising 50% of all the samples), but including several stx1-positive cattle and astA-, afaB-, or elt-positive poultry samples.

**DISCUSSION**
This study sought to clarify the presence of virulence genes most commonly associated with DEC or Shigella in domestic animals and human patients, and to consider these findings within the context of the so-called ‘One Health’ approach to public health. While we were able to detect virulence genes, we have not yet managed to isolate the causative bacteria themselves. This is particularly important, given that the detection of the genes of interest does not necessarily indicate the presence of DEC or Shigella. For example, double-positive samples may indicate coinfection with different types of DEC, new chimeric types of DEC, and a mixture of DEC and other bacteria with similar genes. However, the data can be used to infer which DEC should be targeted in order to isolate the causative bacteria from specific target animals.

A total of 78% of hospitalized patients with diarrhea had at least one virulence gene of DEC, even if EAST1EC was excluded. This statistic is higher than that mentioned in previous reports regarding Bangladeshi patients with diarrhea (13,14). Our results imply a very high prevalence of DEC among patients; however, this difference may be attributable to the use of a sensitive multiplex real-time PCR protocol, and not because of an increase in the incidences of DEC infection over time. In addition, previous studies conducted in Bangladesh did not consider the virulence genes virB, afaB, and astA of the DEC pathotypes (14,16,21).

Based on the prevalence of virB (41%), the most predominant pathogens among the patients in this study could be EIEC or Shigella spp. In a study reporting the prevalence of DEC in Bangladesh, EIEC has not been detected in children with diarrhea or healthy children (13), whereas EIEC has been reported to be...
more prevalent (7.78%) than EPEC (4.79%) in children with diarrhea and healthy children from India (22). Both EIEC and Shigella spp. can express the virB gene (23); further, Shigella spp. is prevalent in south Asian countries, including Bangladesh (24). Whether the prevalence of virB in this study was due to the presence of Shigella spp. or EIEC should be clarified. In addition, most of the virB-positive patients in this study were positive for afaB, aggR, and astA. According to the correlation matrix analysis and PCA, the strongest association was recognized between virB and afaB. Since the diarrheagenic ability of DAEC is still controversial, these findings suggest that DAEC tends to be associated with EIEC or Shigella infection.

In this study, among children with diarrhea, eae was the second most prevalent gene (37%); this is higher than the statistic mentioned in previous reports regarding subjects with diarrhea in Bangladesh (13,14) and Japan (12). This gene was also highly prevalent in fecal samples from cattle (35%) and poultry (19%). However, since atypical EPEC strains are prevalent not only among patients with diarrhea, but also healthy children (25), further investigation is needed to clarify whether eae-positive organisms that did not play an etiological role were wrongly included (26). In our previous report, based on the molecular genotyping of the isolated organisms, cattle appeared to be the source of EPEC infection in Japanese patients (27,28).

Cattle and poultry are strongly considered as possible sources of astA in Bangladesh, since according to the real-time PCR analysis, 84% and 75% of fecal samples from cattle and poultry, respectively, were positive for this gene. About 34% of the patients with diarrhea included in this study were positive for astA; however, the role of this gene in diarrhea remains controversial. In this study, astA was positively correlated with eae.
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in poultry and *stx* in cattle, while *astA, aggR*, and *virB* were simultaneously observed in patients, reflecting different ecological niches and coinfection patterns of bacteria possessing these virulence genes in different hosts.

The findings showed that *afaB* is also common (30%) in Bangladeshi patients, but *afaB*-positive samples were very rare in samples from poultry (1%) and cattle (0.5%), which is consistent with the results from previous reports (12,29). DAEC has also been frequently isolated from human fecal samples, irrespective of enteric symptoms or diarrhea (30,31). Consequently, further investigations involving children without diarrhea in Bangladesh and other possible sources may be helpful to better understand the transmission route of DAEC.

*aggR* was the fifth most prevalent (21%) gene in the fecal samples obtained from Bangladeshi patients with diarrhea, while 1% of fecal samples from poultry and no fecal samples from cattle were positive for this gene. The detection rate of this gene in poultry and cattle reported in this study is consistent with that in our previous report regarding fecal samples from poultry and cattle in Japan; however, the detection rate of this gene in Japanese patients was very low (2%), compared to that in Bangladeshi patients (12). In this study, findings showing the absence of *aggR* in the fecal samples from cattle corroborate those from a previous study (32). Similar to the case for *virB* and *afaB*, it is likely that humans are the reservoirs for *aggR*-possessing bacteria in Bangladesh.

About 14% of the patients in this study were positive for ETEC enterotoxin genes; however, this pathogen has been previously detected to be the most prevalent (4) and second most prevalent (after EPEC) DEC pathotype in Bangladesh (14). Whether poultry and cattle could be sources of ETEC in humans is yet to be ascertained, as the *est* and *elt* genes were detected in fecal samples from these two sources in this study.

Cattle are considered a source of STEC worldwide. The detection rate of *stx* was consistent with that in previous studies conducted to isolate STEC in Dhaka city, Bangladesh (16,21). Furthermore, previous studies have reported a higher prevalence (43.33%) of STEC in cattle, based on rectal swabs (33) and contamination

"Fig. 5. (Color online) Heat map and hierarchical clustering of DEC pathotypes in fecal samples of poultry, cattle, and human patients in Bangladesh. Green indicates presence and red indicates absence of DEC genes. The bar above the heat map shows color-coding of sample source. Letters A, B, C, D, and E denote the five clusters formed by genotyping patterns of the analyzed samples. Hierarchical clustering was implemented by Wald’s method and a binary distance matrix."
of beef and raw milk samples in Bangladesh (34,35). However, the incidence of stx among patients with diarrhea was not as high as that of other virulence genes identified in this study. The fact that no association was observed between eae and stx in cattle in this study could be a reason for this occurrence.

In conclusion, cattle and poultry may be the sources of eae-positive organisms (EPEC and/or EHEC) in Bangladesh, as described for Japan based on previous studies (11,27). These two natural sources are also expected to act as reservoirs of astA-positive and enterotoxin gene-positive bacteria (EAST1EC and ETFC). Although the stx gene was rarely detected in patients and poultry, its high frequency in cattle has implications for human health. afaB, aggR, and virB (DAEC, EAEC, and EIEC/shigella, respectively) were prevalent in patients with diarrhea and were often found simultaneously, but the etiological significance of the coinflection with these organisms should be studied further.

Conflict of interest

None to declare.

REFERENCES