INVESTIGATION OF PATHOGENIC *ESCHERICHIA COLI* FROM DIARRHEIC CALVES IN SELECTIVE AREA OF BANGLADESH

A. K. M. A. Islam1, M. Rahman1, A. Nahar1, A. Khair1 and M. M. Alam1*

1Department of Medicine, 2Department of Microbiology and Hygiene, Faculty of Veterinary Science
Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

**ABSTRACT**

Molecular technique was used to investigate the prevalence of virulent diarrheic genes in pathogenic *Escherichia coli* and their antibiotic sensitivity patterns. A hundred samples from 100 different diarrheic calves from mid-north-western part of Bangladesh were screened for the presence of virulence factors associated with diarrhea. Following isolation and identification on the basis of cultural, morphological and biochemical properties, the presence of the virulence genes such as *eaeA*, *bfpA*, *elt*, *est*, *stx1* and *stx2* were examined using PCR. Antimicrobial susceptibility of 57 *E. coli* was determined by agar disk diffusion method for 8 antimicrobial agents. Out of 100 samples 57 (57%) were found to be positive for *E. coli* and their distribution rates according to their age, breed and sex were 66.7% (6 days old), 85.7% (Sahiwal breed) and in 64.2% (female calves) respectively. Among 57 *E. coli* isolates, only 16 isolates were analyzed for the detection of the said genes. Among them, only *eaeA* gene was detected in 2 *E. coli* isolates (12.5%). Antibiotic resistance patterns revealed that Oxacillin, Rifampicin and Penicillin were 100% resistant followed by Erythromycin which was more than 80% resistant. In case of Amoxicillin and Tetracycline, about 59.65% and 61.40% were found to be resistant respectively whereas all 57 *E. coli* isolates showed moderately susceptible (30%) to Cefuroxime, a second generation Cephalosporin. Therefore, none of the eight antimicrobials studied can not be recommended as single best therapeutic agent for the treatment of neonatal calf diarrhea. In addition, this study indicated that diarrhea in calves in these locations can be ascribed to mainly *Enteropathogenic E. coli (EPEC)* which was atypical (only contained the *eaeA* genes but not *bfpA*). However, further studies are necessary to characterize the isolated *eaeA* gene positive *E. coli* by serotyping, tissue culture assay and other molecular techniques to find out the potentiality of those virulent genes contributing pathogenicity of *E. coli* causing diarrhea in calves.

**Key words:** Prevalence; polymerase chain reaction; virulence genes; antibiotic sensitivity; atypical EPEC

**INTRODUCTION**

Infectious diseases, especially diarrhoea, are among the most important disorders in calves (Sivula et al., 1996; Bendali et al., 1999a; Svensson et al., 2003). Diarrhoea in young calves is a syndrome of great aetiological complexity that causes economic losses directly through mortality and indirectly from poor growth. In addition to the influence of various environmental, managemental, nutritional and physiological factors, the infectious agents capable of causing diarrhoea in the neonatal calf are numerous.

Bacterial infections are an important cause of morbidity and mortality in large animal neonates (Fecteau et al., 1997). In addition to economic losses, diarrhoea in livestock is very important because of the public health implications. Numerous infectious agents causing diarrhoea in animals are zoonotic and have been associated with food-borne diseases (Trevejo et al., 2005). The diarrheal cause may be bacteria, virus, parasites and other etiological agents while *E.coli* is getting recognized as leading cause. *E. coli* produces septicaemia and diarrhoea in a wide range of hosts including man, avian and animals such as cattle, piglet, goat lings, foals, lambs and buffalo. Calves are most vulnerable to *E. coli* infection where age group appears to be of mostly 1-3 days of age. The pathogenicity of *E. coli* is associated with a number of virulence factors, including Shiga toxin 1 (encoded by the *stx1* gene), Shiga toxin 2 (encoded by the *stx2* gene), intimin (encoded by the *eaeA* gene), bundle forming pilus (encoded by *bfp* gene), and enterohaemolysin (encoded by the *Ehly* gene) (Kang et al., 2004). The strains inducing gastroenteric disease are known as Diarrheagenic *E. coli* (DEC). DEC are subdivided in different pathotypes based on their virulence properties (Nataro et al., 1998). Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (*ETEC*), Enterohemorrhagic *E. coli* (*EHEC*), Enteroinvasive *E. coli* (*EIEC*), Enteroaggregative *E. coli* (*EAEC*), and Diffusely Adherent *E. coli* (*DAEC*).

Enteropathogenic *Escherichia coli* (EPEC) are defined as diarrheagenic *E. coli* that produce a characteristic histopathology known as attaching and effacing (A/E) on intestinal cells through encoding intimin, but that do not produce Shiga, Shiga-like or verocytotoxins.

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*Corresponding e-mail address: asamahbub2003@yahoo.com

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These are major causes of diarrhea among children & neonatal animals in developing countries (Carneiro et al., 2006). The primary virulence factor in EPEC is the eae gene that encodes intimin, located in the locus of enterocyte effacement (LEE). The LEE genes facilitate intimate adherence to host cells and the formation of the characteristic attaching and effacing (A/E) lesions (Bolton, 2011). Human EPEC also carry a plasmid called the EPEC adherence plasmid (pEAF) which includes the bfp gene, encoding the bundle-forming pilus required for adherence and clustering on cultured epithelial cells (Orden et al., 2002; Hornitzky et al., 2005). However, not all EPEC are pEAF positive, and many nonhuman isolates lack this plasmid. The former (pEAF positive) are referred to as typical (tEPEC) and the latter as atypical EPEC (aEPEC). Typical EPEC are transmitted from human to human via the faecal-oral route, and as countries become more industrialized, the relative incidence of tEPEC infection decreases, probably as a result of improved sanitation. However, the epidemiology of aEPEC is different; associated diarrhoeal disease remains a public health issue even in developed countries (Blanco et al., 2006). Although a strong association between aEPEC and endemic diarrhoea has not been demonstrated, large outbreaks have been reported (Moller-Stray et al., 2012). Sporadic cases or large STEC outbreaks in humans are associated with the consumption of raw or undercooked meat of food animals and other foods contaminated by animal faeces, and also by contact with STEC-positive animals or with their environment (Paton and Paton, 1998b). Furthermore, the emergence of aEPEC in Europe, the United States of America and other industrialized countries is a cause for concern as aEPEC more readily acquire the bacteriophage-mediated verocytotoxin genes, thus developing the ability to cause more serious illness including enterohaemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Trabulsi et al., 2002). Despite this, aEPEC are not as well characterized as tEPEC, and research is now required to address this deficit (Dulguer et al., 2003; Blanco et al., 2006; Abe et al., 2009). Numerous studies in several countries have shown that cattle are implicated as the principal reservoir of STEC in their gastrointestinal tract, the organism has also been reported in sheep, goats, water buffalos, and deer (Wieler et al., 1998; Osek et al., 2000). However, the organism did not appear to be pathogenic in older calves and adults (Kang et al., 2004).

Antibiotic resistance to bacteria is a serious and growing phenomenon and has emerged as one of the pre-eminent public health concerns of the 21st century. In Bangladesh complete understanding on the occurrence of antimicrobial resistance in E.coli is largely unknown. The choice of which antibiotic is likely to be most effective requires knowledge of potential resistance. The practice of under dosing, over dosing as well as indiscriminate usage of drugs are not uncommon in Bangladesh. As a result, bacterial strains are being developed which are multi-drug resistant and new types of antibiotics are required for the prevention and control of diseases. Considering the above questions this study was aimed to investigate the presence of E. coli causing diarrhea in calves and to detect their virulence factors as well as the study of their antibiotic sensitivity patterns.

MATERIALS AND METHODS
The study was conducted from January 2014 to June 2014 on the calves of Bathan regions in Sirajgonj district. Diarrheic samples from this region were brought to the Laboratory of Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh as soon as possible after collection for further examinations. Bathans are considered the most important production zone of dairy cattle in Bangladesh where calves are reared in clusters.

Collection of samples
A hundred diarrheic faecal samples were collected from the Sirajgonj district of Bangladesh. Each sample was aseptically collected in a sterile stool pot and transported to the laboratory of department of Medicine as soon as possible for further bacteriological examinations. Samples were processed within 24–48 h after reception.

Isolation of the bacteria
For the isolation and identification of E. coli standard methods as described by Cowan, 1985 were followed. Nutrient broth (NB) was used for primary culturing of E. coli organisms that were present in the collected faecal samples. For the differentiation of the bacteria MacConkey (MC) agar medium was used followed by Eosin Methylen blue (EMB) agar medium which was used as a selective medium and these are used according to the methods as described by Cheesbrough, 1984.
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Identification and characterization of the bacteria
The isolated bacteria was confirmed with their distinctive cultural characteristics, morphology with Gram’s staining and biochemical tests according to Cowan (1985) and Cheesbrough (1984).

Detection of virulent genes by PCR
Among 57 E. coli isolates, only 16 E. coli isolates were subjected to PCR for detection of six diarrheic pathogenic genes (eaeA, bfpA, stx1, stx2, est and elt).

Bacterial DNA extraction was done through boiling methods. For the extraction of genomic DNA of E. coli, a single colony of E. coli was taken in 1000 µl of TE buffer in Eppendorf tube. The mixture was then vortexed and boiled at 100°C for 10 minutes. After boiling the tubes were immediately placed on ice for 5 minutes followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was collected and stored at -20°C which was used as template DNA. The base sequences, PCR conditions and predicted sizes of the amplified products for the specific oligonucleotide primers used in the study were shown in the Table 1.

Analysis of the PCR products was then carried out by agar gel electrophoresis method at 50 Volt for 60 minutes using 1% agarose gel stained with ethidium bromide. Finally the PCR products were visualized under UV transluminator.

Antimicrobial susceptibility study
Susceptibility to different classes of antimicrobial agents was evaluated for E. coli using standard disc-diffusion method in Mueller Hinton agar using the inhibition-zone patterns. Antibiotic susceptibility and resistance patterns of E. coli against different antibiotics were measured according to the Kirby-Bauer method (Bauer et al., 1996). The zone diameter interpretative criteria of E. coli were used to classify isolates as susceptible, intermediate or resistant based on the standard interpretation table updated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). The discs containing the following amount of antibiotics were used: Penicillin-G 10 µg, Amoxycillin 30 µg, Rifampicin 5 µg, Gentamicin 10 µg, Cefuroxime 30 µg, Erythromycin 15 µg, and Oxacillin 1 µg.

RESULTS AND DISCUSSION
Prevalence
The overall prevalence of E. coli causing diarrhea in calves of Bathan area was 57%. However, several authors reported prevalence of E. coli associated diarrhea in calves which varied from 25.0% to 49.8% published from 2002 to 2014 (Malik et al., 2013; Ansari et al., 2014). But Nazir (2007) reported relatively higher (60%) prevalence. Samples positive to E. coli were grouped according to age, breed and sex (Table 2). A higher prevalence of E. coli causing diarrhea in calves (66.7%) were detected in calves of 6 days old, in calves of Sahiwal breed (85.7%) and in female calves 64.2%.

Table 1. The primers used in PCR for detection of 6 pathogenic genes in E. coli

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence from 5’ to 3’</th>
<th>Amplicon size (bp)</th>
<th>Target Gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAE 1</td>
<td>AAACAGGTGAAAACCTTGGCC</td>
<td>454</td>
<td>eaeA</td>
<td>Yuluo et al., 2010</td>
</tr>
<tr>
<td>EAE 2</td>
<td>CTCCTCAGATTAAACCTTGC</td>
<td>324</td>
<td>bfpA</td>
<td>Hinenoya et al., 2009</td>
</tr>
<tr>
<td>BfpA-f</td>
<td>AATGGGTGCTTTGCGTGCTTGC</td>
<td>110</td>
<td>stx2</td>
<td>Yuluo et al., 2010</td>
</tr>
<tr>
<td>BfpA-r</td>
<td>GCCGGTTTTATCCAACCTTGA</td>
<td>190</td>
<td>elt</td>
<td>Hinenoya et al., 2009</td>
</tr>
</tbody>
</table>
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Table 2. Percentages of *E. coli* isolates in diarrheic calves according to different category of age, breed and sex.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Category level</th>
<th>No. of observation n = 100</th>
<th>No. of samples positive to <em>E. coli</em> (%)</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>6 days</td>
<td>6</td>
<td>4 (66.7)</td>
<td>22.3-95.7</td>
</tr>
<tr>
<td></td>
<td>Above 6 days to 1 month</td>
<td>61</td>
<td>39 (63.9)</td>
<td>50.6-75.8</td>
</tr>
<tr>
<td></td>
<td>Above 1 month to 2 months</td>
<td>32</td>
<td>14 (43.8)</td>
<td>26.4-62.3</td>
</tr>
<tr>
<td></td>
<td>2 months 15 days</td>
<td>1</td>
<td>0 (00)</td>
<td>0-97.5</td>
</tr>
<tr>
<td>Breed</td>
<td>Holstein Friesian cross</td>
<td>93</td>
<td>51 (54.8)</td>
<td>44.2-65.2</td>
</tr>
<tr>
<td></td>
<td>Sahiwal cross</td>
<td>7</td>
<td>6 (85.7)</td>
<td>42.1-99.6</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>47</td>
<td>23 (48.9)</td>
<td>34.1-63.9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>53</td>
<td>34 (64.2)</td>
<td>49.8-76.9</td>
</tr>
</tbody>
</table>

CI=Confidence Interval

Detection of PCR products

Most EPEC strains have both bundle-forming pilus gene (*bfpA*) and *eaeA* gene, but in this study, the EPEC strains isolated were atypical in that they only contained the *eaeA* gene. PCR detection of 16 *E. coli* isolates showed that the detection rate of *eaeA* genes was 12.50% (Fig. 1, Table 3) which is correlated with Hur et al., 2013 in Korea who reported 13-17% (from diarrheic calves) of the same gene. From other studies the reported range of *eaeA* gene was 1.2% to 9.8% (Yuluo et al., 2010; Nguyen et al., 2010; Salehi et al., 2011). Reasons for higher prevalence may be due to the sample size or number of experimented isolates, time of collections, age of the samples and age of the animals.

The differences of prevalence of virulence genes might be due to season, farm size, and number of animals on the farm, hygienic status, farm management practices, variation in sampling, variation in types of samples evaluated, and differences in detection methods.

![Fig. 1. Amplification of *eaeA* gene in *E. coli* isolates from diarrheic calves, M, Marker (1000 bp DNA ladder) P, positive control (*E. coli* O157 Sakai strain) and N, negative control (*E. coli* C600), Lane no 1-16 indicates sample no. (PCR products prepared from *E. coli* isolates).](image)

Table 3. Detection rate of 6 pathogenic genes in *E. coli* isolates in diarrheic calves

<table>
<thead>
<tr>
<th>Total no. of <em>E. coli</em> isolates used for PCR</th>
<th>Name of genes</th>
<th>Number of detected genes (n=16)</th>
<th>Percentage of 6 pathogenic genes (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td><em>eaeA</em></td>
<td>2.0</td>
<td>12.50</td>
</tr>
<tr>
<td></td>
<td><em>bfpA</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><em>stx1</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><em>stx2</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><em>est</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><em>elt</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
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**Antimicrobial susceptibility testing**

The antimicrobial susceptibility study of all the isolates against eight antibiotics used in this study revealed that most of the isolates were multidrug resistant and Oxacillin, Rifampicin and Penicillin has been shown to be 100% resistant followed by Erythromycin which is 80% resistant (Plate 1). Among all 8 antibiotics, Cefuroxime, although second generation of Cephalosporin, has shown to be moderately susceptible (30%) followed by Tetracycline (22%) and Gentamicin (12%) in this study. However, 2 eaeA gene containing E. coli showed 100% resistance to all antibiotics.

Hundred percent resistances to Penicillin was also reported by Malik et al., 2013 and the same in case of Erythromycin was reported by Nazir (2007) and Malik et al. (2013) in diarrheic calves. Malik et al. (2013) also reported Rifampicin as 100% resistant.

Rifampicin and Oxacillin are old drugs and is probably not used as veterinary drugs but it is still found resistant because of the fact that genomic plasticity of E. coli is very high. It can change its virulence properties very frequently. In addition, E. coli carry plasmid DNA and mobile genes and it can infect both human and animals. Due to its zoonotic potency these two drugs may have got resistance from human population. Acquisition of quick capability of transferring mobile genes from surrounding resistant strains of bacteria may occur through insertion, conjugation, transformation, transduction or other mechanisms, often facilitating the incorporation of the multiple resistance genes into the host’s genome or plasmids.

In case of Amoxycillin and Tetracycln 59.65% and 61.40% resistance were found respectively. Although 100% resistance in case of Tetracycln was reported by Malik et al. (2013) and Ansari et al. (2014) reported more than 80%. On the contrary, 100% sensitivity to tetracycline was documented by Hossain et al. (2012). Higher resistance to amoxycillin was reported by Abd-Elrahman et al. (2011) and Ansari et al. (2014). Hossain et al. (2012) found Gentamicin as 100% resistant. While Malik et al. (2013) has found Gentamicin as moderately sensitive in this research Gentamicin was found moderately resistant Cefuroxime was found as moderately susceptible in this study which is supported by Orden et al. (1999) and Mahanti et al. (2014). But none of the drugs used in this study could be termed as single best in treating the E. Coli causing diarrhea in calves.

This study stresses the importance of prevalence survey on the diarrheic E. coli isolated in diarrheic calves in Bathan region. So further investigation of the rest of the E. coli isolates is needed to detect any of the pathogenic genes to find out the real scenario of the prevalence of pathogenic genes existing in diarrheic calves.
REFERENCES


10. CLSI (2007). Clinical and Laboratory Standards Institute (CLSI) guidelines. 27: Wane, PA, USA.


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29. Salehi ZT, Badouei A, Mahdi, Brujeni N, Gholamreza, Madadgar and Omid (2011). Occurrence and characterization of enterohemorrhagic isolates *Escherichia coli* from diarrhoeic calves, department of microbiology, faculty of veterinary medicine, University of Tehran, Tehran, Iran.


