Prevalence, serodiversity and antibiotogram of enterotoxigenic *Escherichia coli* (ETEC) in diarrhoeic calves and lambs of Kashmir valley (J&K), India

Rusheeba Manzoor¹, Mohammad Irfan Shah², Asma-ul-husna¹, Shakil Ahmad Wani¹, Farhat Pandit¹, Pervaiz Ahmad Dar¹, Mohammad Iqbal Mir³

¹Division of Veterinary Microbiology and Immunology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology, Kashmir Shuhama Campus, Srinagar-190006, INDIA
²Division of Veterinary Pharmacology and Toxicology, IVRI, Izatnagar, Bareilly 243122, Uttar Pradesh
³Division of Fish Pathology, Faculty of Fisheries, Sher-e-Kashmir University of Agricultural Sciences & Technology, Kashmir, Rangil, Ganderbal 190006

*Corresponding author. E-mail: qadrijavid2008@gmail.com

Received: November 24, 2014; Revised received: May 26, 2015; Accepted: June 12, 2015

**Abstract:** Enterotoxigenic *E. coli* (ETEC) is the major cause of diarrhoea in neonatal animals. This study determined the prevalence, serological diversity, virulence gene profile and *in-vitro* antibiotogram of ETEC isolates from diarrhoeic faeces of calves and lambs. The prevalence rate of ETEC in lambs was recorded 18.46% with O8 as predominant serotype. However, in calves the prevalence rate was recorded 8.57% with O15 and O26 as predominant serotypes. The antibiotogram screening showed differential susceptibility pattern among ETEC isolates with highest resistance to ampicillin and highest sensitivity to enrofloxacin. In the present study, for the first time it was reported that the diarrhoea in calves and lambs occur due to virulent gene *est* not due to *elt* gene, which was absent in all the isolates.

**Keywords:** Calves, Diarrhoea, Lambs, PCR

**INTRODUCTION**

Neonatal diarrhoea during first few weeks of life is a leading cause of economic losses to the livestock industry all over the world. There are six different groups of *Escherichia coli* associated with neonatal diarrhoea in humans and animals including entero-pathogenic *E. coli* (EPEC), entero-aggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), entero-invasive *E. coli* (EIEC), diffusely-adherent *E. coli* (DAEC) and shiga-toxin producing *E. coli* (STEC) (Coxen et al., 2013). ETEC is the most common group associated with the neonatal diarrhoea in animals causing significant morbidity and mortality (Kolanda et al., 2015).

Like other *E. coli* strains, ETEC is serotyped based on the combination of 173 O (somatic) antigens, 80 H (flagellar) antigens and 56 K (capsular) antigens (Orskov and Orskov, 1992). Detection of enterotoxins by ELISA or their corresponding genes by PCR are extensively used to identify ETEC. ETEC mainly produce two enterotoxins namely heat stable (ST) and heat labile (LT), which are encoded by *est* and *elt* genes, respectively. These enterotoxins may be cytotoxic which damage mucosal cells or cytotoxic which induce secretion of water and electrolytes (Philip et al., 2010).

The present study was undertaken with the objectives to determine the prevalence and serodiversity of ETEC in diarrhoeic calves and lambs, to study the distribution of virulence genes (*est* and *elt*) and to determine the *in-vitro* antibiotogram of ETEC isolates.

**MATERIALS AND METHODS**

**Sampling and isolation of *E. coli* isolates:** A total of 200 faecal samples from diarrhoeic calves and lambs up to 16 weeks of age were collected from organized livestock farms and individual livestock owners of Kashmir Valley. The samples were collected by rectal swabbing and transferred to laboratory on ice. Faecal samples were immediately inoculated on MacConkey’s agar (Hi-Media, Mumbai, India) plates. After overnight incubation at 37°C, two to three rose pink colonies per plate were randomly picked up and subcultured onto Eosin methylene blue (EMB) agar plates to observe the characteristic metallic sheen of *E. coli*. Well separated colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological and biochemical tests as described by Buchanan and Gibbon (1994).

**Extraction of bacterial DNA:** *E. coli* isolates were grown in nutrient broth (Hi- Media) at 37°C overnight. Organisms from 1.5 ml growth were pelleted by centrifugation at 10,000 rpm for 10 min. The bacterial DNA was extracted using the Wizard® Plus SV Miniprep DNA Purification System (Promega Corporation, Madison, USA) according to the kit protocol.

**Antibiogram:** Extraction of bacterial DNA was carried out using the Wizard® Plus SV Miniprep DNA Purification System (Promega Corporation, Madison, USA). The DNA was then subjected to *in-vitro* antibiotic sensitivity testing. The antibiotic screening was performed using the Kirby-Bauer disc diffusion method on Nutrient Agar Slants (Hi-Media, Mumbai, India) as described by Buchanan and Gibbon (1994). The antibiotics used for the *in-vitro* antibiotic sensitivity testing were ampicillin (10µg), enrofloxacin (5µg), cefuroxime (30µg), cephalothin (30µg), tetracycline (30µg), streptomycin (25µg), kanamycin (30µg), neomycin (30µg), apramycin (10µg), neomycin (30µg), amikacin (30µg), and gentamicin (10µg) and each disc contained a standard concentration of the antibiotic.

**Serological detection of *E. coli* serotypes:** Serological detection of *E. coli* serotypes was carried out using the slide agglutination technique using *E. coli* O15 (0901), O26 (0902) and O8 (0908) specific antisera (Bharat Biotech International, Hyderabad, India) according to the kit protocol.
pellet was re-suspended in 150 µl of sterile distilled water. The bacteria were lysed and the supernatant was used directly as template for PCR.

Detection of virulence genes by PCR: E. coli isolates were subjected to PCR for detection of elt and est genes. PCR was performed on Gene Amp PCR System 2400 thermal cycler (Applied Biosystems, USA). The primers and the predicted length of PCR amplification products are listed in Table 1.

PCR reactions were carried out in a 25 µl volume as per the method of Itoh et al. (1992). Each reaction consisted of 2.0 µl of template DNA, 2.5 µl of 10X PCR buffer, 0.2 µl of 100mM dNTP mix, 20 pmol of 0.5 µl of forward primer and reverse primer, 2.0 µl of 25mM MgCl2, 0.2 µl of Taq DNA polymerase (1 Unit) and 17.1 µl of Nuclease Free Water (NFW). The PCR was carried for 25 cycles comprising of 2 min of initial denaturation at 94°C followed by denaturation of 30 sec at 94°C, annealing of 1 min at 47°C, extension of 1.5 min at 72°C and final extension of 5 min at 72°C. Amplified PCR products were analyzed by electrophoresis in 2% (w/v) agarose gel containing ethidium bromide (0.5µg/ml) (Sambrook and Russel, 2001). The products were visualized under UV illumination and documented with Gel Doc System (Ultracam Digital Imaging, Ultra Lum, Inc, Claremont, CA).

Serogrouping: The E. coli isolates were serogrouped on the basis of their O antigen by National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, H.P. 173204 (India).

Antibiotic sensitivity test: In-vitro antibiotic sensitivity pattern of the ETEC isolates to various antimicrobial agents was determined by standard disc diffusion method on Mueller-Hinton agar. The antibiotics used in the test included sulphadiazine (300 mcg), chloramphenicol (30 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), amikacin (30 mcg), amoxicillin-clavulanic acid (10 mcg), neomycin (30 mcg), tetracycline (30 mcg), streptomycin (10 mcg), ampicillin (10 mcg), enrofloxacin (10 mcg), ceftriozone (30 mcg), cefoperazone (75 mcg), norfloxacin (10 mcg). The interpretation of the isolates as sensitive, intermediate, and resistant was done as per manufacturer’s instructions.

RESULTS
A total of 200 diarrhoeic faecal samples from calves and lambs were collected (Table 2). Out of 200 E. coli isolates 30 were detected to be ETEC on basis of est amplification. Thus, the overall prevalence of ETEC was recorded as 15%.

Out of 130 E. coli isolates from lambs, 24 (18.46%) isolates carried est virulence gene and were designated as ETEC (Table 3). Twenty one of the ETEC isolates belonged to eight different serogroups (Table 4), 2 isolates were rough and the remaining one isolate was untypeable. Serogroup O8 was the most prevalent among the lamb isolates.

Of the 70 E. coli isolates from calves, 6 (8.57%) isolates carried est virulence gene and were detected as ETEC (Table 3). Four ETEC isolates belonged to two different serogroups and one isolate was rough and the remaining one isolate was untypeable (Table 4). Serogroups O15 and O26 of ETEC were the most prevalent among the calf isolates.

Table 1. Details of primers used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence 5’ → 3’</th>
<th>Amplicon size (bp)</th>
<th>Primer Conc.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>elt</td>
<td>AGCAGGTTCCACCCGGATCACCA GTCCTAGATTCGTTGTC</td>
<td>132</td>
<td>0.5µM</td>
<td>Itoh et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>TTTATTTCTGTATTGTCTTT ATTACAACACAGTTCACAG</td>
<td>171</td>
<td>1µM</td>
<td>Itoh et al. (1992)</td>
</tr>
</tbody>
</table>

Table 2. Prevalence of ETEC in Kashmir valley.
Altogether, 30 ETEC isolates from both diarrhoeic lambs and calves carried est gene but none of the isolate carried elt gene. The representative gene profile is depicted in Fig. 1.

The 30 ETEC isolated from diarrhoeic calves and lambs were tested against 14 different antimicrobials for their susceptibility (Fig. 2). Most of the ETEC isolates showed resistance to ampicillin (86.66 %), nalidixic acid (80%), cefoperazone (66.6 %), streptomycin (66.6 %) and tetracycline (66.6 %) but were sensitive to enrofloxacin (86.66%), sulphadiazine (83.33%), norfloxacin (83.33%), gentamicin (66.6%) and chloramphenicol (66.6 %).

**DISCUSSION**

In present study, the prevalence rate of ETEC was recorded 18.46 % and 8.57 % in diarrhoeic lambs and calves, respectively. Earlier investigation by Myers (1975) in Montana recorded a prevalence rate of 18% in diarrhoeic calves. A study by Darong et al. (2010) in China reported a prevalence rate of 15.89% in piglets. However, a study by Sivaswamy and Gyles (1976) reported a prevalence rate of 36% of ETEC in diarrhoeic calves which is much higher than recorded in our study. This variation in prevalence rate may be associated with different factors such as species, season of the study, geographical location, environment and hygienic conditions in the farm.

In lambs we recorded O8 ETEC as the predominant serogroup as 20% of the isolates were positive for O8 antigen. This finding is similar to earlier report by Wani et al. (2003), who also recorded O8 as the most dominant serogroup of ETEC in Kashmir Valley with 13.33% prevalence rate. However, in calf diarrhoea we recorded O15 and O26 as dominant serogroup with prevalence rate of 6.6%. They also recorded O15 and O26 as predominant serogroup in diarrhoeic calves of Kashmir Valley with prevalence rate of 3.33%. The predominance of O8 in diarrhoeic lambs (Blanco et al., 1996 and Wray and Thomlinson, 1991) and O15 and O26 in diarrhoeic calves of Kashmir Valley has been previously reported.

We also studied the ETEC isolates for the presence of virulence genes, est and elt, by PCR method. The est gene was detected in 30 out of 200 (15 %) E. coli isolates, whereas the elt was not detected in any of the isolates. This finding is in agreement with the study by Bradford et al. (1999), who were not able to detect elt gene in any E. coli isolate from the diarrhoeic calves but detected est gene in 8 out of 32 isolates (25%). This is more likely because only human and porcine ETEC strains are able to produce LT (Gyles, 1992).

The analysis of antibiotic resistance pattern showed that the ETEC isolates are resistant to ampicillin,
nalidixic acid, cefoperazone, streptomycin and tetracycline, and sensitive to enrofloxacin, sulphadiazine, norfloxacin, gentamicin and chloramphenicol. These findings are in agreement with findings of Wani et al. (2013) who reported highest percentage of ETEC being resistant to ampicillin and sensitive to chloramphenicol, enrofloxacin, norfloxacin and gentamicin. Earlier, Seh et al. (2000) also reported 90.9% sensitivity of ETEC isolates to ciprofloxacin and norfloxacin and complete resistance to ampicillin/cloxacillin, erythromycin and cefadroxil. This finding suggests that use of antibiotic for disease prevention and treatment in animals may be responsible for the resistant phenotype in ETEC isolates.

Conclusion
The prevalence of ETEC in lambs was recorded 18.46% with O8 as predominant serotypes, whereas in calves the prevalence was 8.57% with O15 and O26 as predominant serotypes. The virulence gene est was detected in all ETEC isolates, whereas elt gene was not detected in any of the isolate. Differential susceptibility pattern for antibiotics were observed among ETEC isolates with highest resistance to ampicillin and highest sensitivity to enrofloxacin. The present study leaves scope for similar investigation in human diarrhoeic samples to precisely elucidate the zoonotic significance of ETEC in this part of globe.

REFERENCES
Toxins, 2: 2213-2229.