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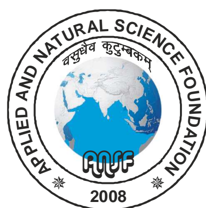

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Prevalence, serodiversity and antibiogram of enterotoxigenic *Escherichia coli* (ETEC) in diarrhoeic calves and lambs of Kashmir valley (J&K), India

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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* antibiogram 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 antibiogram 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), entero toxigenic *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 dia rhoea 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 enter toxins may be cytotoxic which damage mucosal cells or cytotonic 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* antibiogram of ETEC isolates.

MATERIALS AND METHODS

Sampling and isolation of *E. coli* isolates: A total of 200 faecal samples from diarrhoeic calves and lambs upto 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

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pellet was re-suspended in 150 µl of sterile distilled water. The bacteria were lysed by boiling for 10 min in a water bath. The lysate was centrifuged 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 $MgCl_2$, 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-clauvalanic acid (10 mcg), neomycin (30 mcg), tetracycline (30 mcg), streptomycin (10 mcg), ampicillin (10 mcg), enrofloxacin (10 mcg), ceftrizone (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 (Table3). 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 (Table3). 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.

Target gene	Sequence 5' → 3'	Amplicon size (bp)	Primer Conc.	Reference
elt	AGCAGGTTTCCCACCGGATCACCA	132	0.5µM	Itoh *et al.* (1992)
	GTGCTCAGATTCTGGGTCTC		0.5µM	
est	TTTATTTCTGTATTGTCTTT	171	1µM	Itoh *et al.* (1992)
	ATTACAACACAGTTCACAG		1µM	

Table 2. Prevalence of ETEC in Kashmir valley.

Source	Species	No. of samples	Positive samples for ETEC	
			No.	%
Sheep Husbandry, Department, Zakura	Ovine	10	3	30
Sheep Breeding Farm, Dachigam	Ovine	15	4	26.66
Mountain Research Centre for Sheep and Goat, Shuhama	Ovine	62	9	14.5
Mountain Livestock Research Institute, Mansbal	Bovine	16	1	6.25
Military Dairy Farm, Qamarwari	Bovine	2	0	0
Private owners	Ovine	43	8	18.6
Private owners	Bovine	52	5	9.61
Total		200	30	15

Table 3. Virulence gene profile of ETEC in diarrhoeic calves and lambs.

Number of Samples	No. of isolates with at least 1 virulence gene	Pathotype	Percentage of isolates
Dirarrhoeic lambs (130)	24	ETEC	18.46
Diarrhoeic calves (70)	6	ETEC	8.57

Table 4. Serogroup diversity of Enterotoxigenic *E. coli* from diarrhoeic calves and lambs.

Serogroup	No. of isolates from			Percentage
	Calves (6)	Lambs (24)	Total (30)	
O8	0	6	6	20.00
O15	2	0	2	6.66
O17	0	4	4	13.33
O26	2	0	2	6.66
O89	0	1	1	3.33
O91	0	1	1	3.33
O106	0	2	2	6.66
O109	0	1	1	3.33
O159	0	5	5	16.66
O192	0	1	1	3.33
Rough	1	2	3	10.00
Untypeable	1	1	2	6.66
Total	6	24	30	100.00

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 (Tripathi and Soni, 1984) 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 diarrhoreic 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,

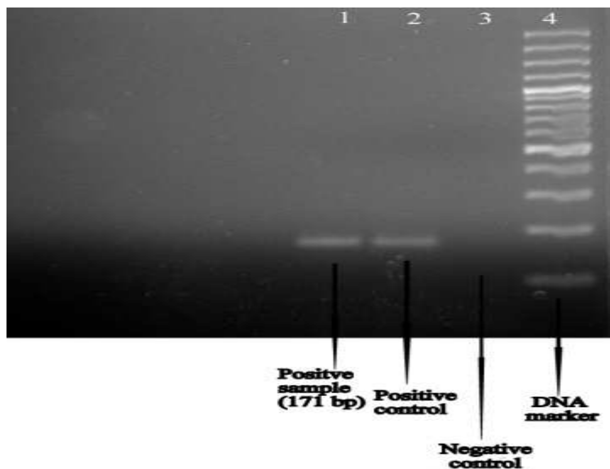

Fig. 1. Representative virulence gene profile of ETEC isolated from diarrhoeic calves and lambs using PCR. Lane 1, est positive. Lane 2, positive control. Lane 3, negative control. Lane 4, 100 bp DNA ladder.

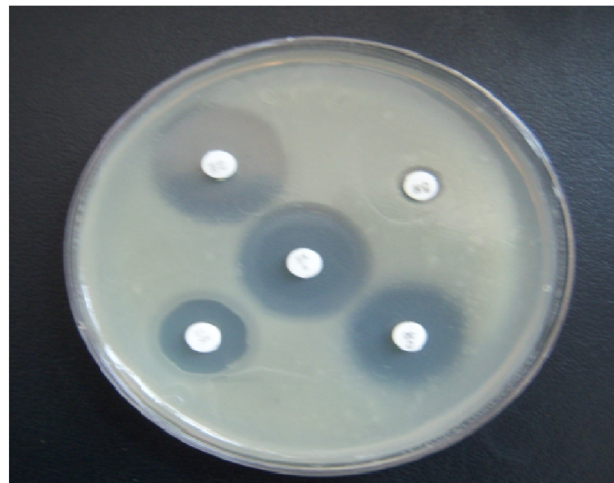

Fig. 2. Representative antimicrobial drug sensitivity pattern of ETEC isolates from diarrhoeic calves and lambs.

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.

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