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Molecular characterization and antimicrobial profiling of *Escherichia coli* isolates from diarrheic calves

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ABSTRACT

Diarrhea due to *Escherichia coli* is one of the major economic concerns of bovine industry in the first few weeks after birth. The present study was carried out for characterization of virulence traits of *Escherichia coli* associated with diarrhea in calves less than 3 months of age and their antimicrobial profiling. *Escherichia coli* isolates (700) were recovered in this study, which belonged to 25 different serogroups out of which O2 and O6 were most predominant. The isolates were screened for 7 virulence genes, viz. *sta, stx1, stx2, lt, f5, f41* and *eae*. Out of 700 isolates, 65 (9.2%) carried virulence genes either alone (*f5, eae, f41, sta, stx1* and *stx2*) or in different combinations (*f41/sta, sta/lt, stx1/eae* and *f5/stx2*). Based on the presence of virulence factors, these isolates were classified as Shiga-toxin producing *E. coli* (27), enterotoxigenic *E. coli* (20), enteropathogenic *E. coli* (15) and 3 were unclassified. These 65 isolates were resistant to commonly used antibacterial agents like amoxicillin, ceftriaxone, cephalexin, cloxacillin, enrofloxacin and gentamicin. Multidrug resistance was also observed in 84.6% of them.This study reveals that PCR based detection of *E. coli* virotypes can be used in diagnosis and epidemiological studies. Also increasing multidrug resistance for most of the commonly used antibiotics is a matter of concern.

Key words: Antimicrobial assay, Diarrhea, Virulence genes of *E. coli,* PCR, Serogroup, Virotype

Diarrhea in young pre-weaned calves is one of the most important causes of calf morbidity and mortality. *E. coli* has been frequently implicated as the primary bacterial cause in calf diarrhea (Arya *et al*. 2008, Nguyen *et al*. 2011). Diarrhegenic *Escherichia coli* was divided into 5 categories based on virulence factors, epidemiological and clinical features (Hegde *et al*. 2012). It includes enterotoxigenic *E.coli* (ETEC), shiga toxin-producing *E.coli* (STEC), enteropathogenic *E.coli* (EPEC), enteroaggregative *E.coli* (EAEC) and enteroinvasive *E.coli* (EIEC) (Shahrani *et al*. 2014).

Diagnosis of *E. coli* infection currently relies on the phenotypic differentiation of pathogenic strains from non-pathogenic commensals using bioassays or immunoassays for toxins and fimbriae. These tests are time-consuming, require skilled technicians and are therefore not routinely used in many clinical laboratories. Polymerase chain reaction (PCR) is a useful diagnostic tool because it is quick, specific, sensitive, and relatively inexpensive.

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The proliferation of antibiotic resistance is currently outpacing the development of novel antibiotics due to extensive and indiscriminate use of antimicrobial agents for therapy, prophylaxis and growth promotion in dairy animals (Fahrenfeld *et al*. 2013). The emergence of resistant strains has posed an increasing threat to successful treatment of *E. coli* related diarrheic diseases (Tadesse *et al*. 2012). The rising drug resistance in bacteria is mainly due to genetic elements that can be readily spread through bacterial populations (Kumarasamy *et al*. 2010, Martins *et al*. 2016). This can raise an alarming situation due to ease of transfer to non-pathogenic strains. The present study was, therefore, aimed to detect and characterize *E. coli* isolates from calves of below 3 months of age and their molecular characterization based on virulence genes.

MATERIALS AND METHODS

Sample collection: Faecal samples were collected during March 2013 to April 2015 from 350 untreated diarrheic bovine calves below 3 months of age. The affected calves showed watery faeces, signs of dehydration and weakness. Out of 350 samples, 210 were from Holstein-Friesian calves reared on organized dairy farms and remaining 140 samples were collected from Holstein-Friesian and Jersey crossbred calves of local livestock owners from different regions of Jammu. The samples were collected in specimen collection

Table 1. List of primer sequences and predicted amplicon length

Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
Stx1 F	TTCGCTCTGCAATAGGTA	*stx1*	555	Paton *et al*. (1995)
Stx1 R	TTCCCCAGTTCAATGTAAGAT			
Stx2 F	GTGCCTGTTACTGGGTTTTTCTTC	*stx2*	118	Paton *et al.* (1993)
Stx2 R	AGGGGTCGATATCTCTGTCC			
Sta F	GCTAATGTTGGCAATTTTTATTTCTGTA	*sta*	190	Sekizaki *et al*. (1985)
Sta R	AGGATTACAACAAAGTTCACAGCAGTAA			
eae F	ATATCCGTTTTAATGGCTATCT	*eae*	425	Yu and Kaper (1992)
eae R	AATCTTCTGCTGCGTACTGTGTTCA			
F41 F	GCATCAGCGGCAGTATCT	*f41*	380	Fidock *et al*. (1989)
F41 R	GTCCCTAGCTCAGTATTATCACCT			
F5 F	TATTATCTTAGGTGGTATGG	*f5*	314	Roosendaal *et al.* (1984)
F5 R	GGTATCCTTTAGCAGCAGTATTTC			
Lt F	ATTTACGGCGTTACTACTCTC	*lt*	281	Van Boost *et al*. (2001)
Lt R	TTTTGGTCTCGGTCAGATATG			

vials (Himedia, India), transported to the laboratory on ice and processed immediately.

Isolation and biochemical characterization: For isolation and identification of *E.coli*, faecal samples were streaked on MaConkey's lactose agar and incubated at 37°C for 48 h. Well isolated lactose fermenting 2–3 colonies were selected from each plate and streaked on EMB Agar and incubated for 48 h at 37°C. Isolated colonies with green metallic sheen on EMB agar plate were stabbed and streaked on nutrient agar slants for further processing. All isolates from slants were characterized on the basis of biochemical tests with Himotility *E.coli* test kit (Himedia, India) incorporating tests for motility, indole, citrate utilization, nitrate reduction, ONPG, lysine utilization, lactose, glucose, sucrose and sorbitol. Results were recorded by comparing the colour change in the wells as per manufacturer's instructions. In addition to this, catalase and oxidase tests were also performed separately.

Serogrouping: All the isolates identified as *E. coli* were sent to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (Himachal Pradesh) for serogrouping of *E.coli* 'O' antigen.

Molecular characterization by PCR: For extraction of bacterial DNA, a loopful of confluent bacterial growth was taken from Nutrient agar slant and suspended in 300 µl sterile distilled water in a 2 ml microcentrifuge tube. The DNA was then extracted by Snap chill method which was used as a template for PCR detection of various virulence factors.

E. coli virulence genes *sta, stx1, stx2, eae, f41* and *f5* were amplified in a multiplex reaction by the method described by Franck *et al.* (1998) while *lt* was amplified as described by Blanco *et al.* (2004). Oligonucleotide primers were procured from Integrated DNA Technologies, Bengaluru, India. List of primer sequences and predicted lengths of amplified products are presented in Table 1. PCR reaction was performed in a thermal cycler (Eppendorf, Mastercycler Gradient, Germany). The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) for 45 min at 90 volts.The gel was then analyzed with UV illumination and imaged with gel documentation system (Biometra, Germany).

Assay for antimicrobial resistance: PCR positive *E. coli* isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method of Kirby and Bauer. Isolates were tested against commonly used antibiotics (Himedia, India), viz. amoxicillin (Ax, 10 µg), cephalexin (CN, 30 µg), enrofloxacin (Ex, 10 µg), ciprofloxacin (Cf, 10 µg), cloxacillin (COX, 5 µg), gentamicin (G, 30 µg), oxytetracycline (O, 30 µg), amikacin (A, 30 µg). Diameters of zones of inhibition of different antibiotics were measured with Hiantibiotic Zone scale (Himedia, India). The zones of inhibition were interpreted as per reference given in charts provided with antibiotic discs from Himedia, India.

RESULTS AND DISCUSSION

Colonies (800) showing cultural characteristics of *E. coli* (green metallic sheen on EMB and lactose fermenting colonies on MLA) were randomly selected for further processing. Out of these isolates, 700 showed all the biochemical characters suggestive of *E. coli*. Out of these 700 isolates, 395 were from HF cows (organized farms) and 305 were from crossbreed cows (unorganized sector).

These 700 *E. coli* isolates were screened for the presence of genes encoding for different virulence factors. Out of 700, only 65 isolates (9.2%) were virulent, i.e. they were positive for at least one virulence gene. Among these 65 virulent *E. coli,* 37 (5.2%) belonged to crossbreeds of unorganized sector and 28 (4.0%) belonged to HF of

Table 2. Farm wise distribution of *E. coli* virotypes

Virotype	Organized sector (HF)	Unorganized sector (Crossbreds)
ETEC (total 20)	9	11
STEC (total 27)	12	15
EPEC (total 15)	6	9
Unclassified (total 3)	1	2
Total isolates	28 (4%)	37 (5.2%)

HF, Holstein Friesian

Table 3. Virulence gene profile and antibiotic sensitivity pattern of *E. coli* isolates

Virotype	Virulence profile		Antibiotic sensitivity (No. of resistant isolates)								Serogroups
			Ax	A	Cf	Ex	G	COX	O	CN	
ETEC	*Sta*	13	13	0	10	3	3	5	3	3	O1, O2, O9 and O15
Total isolates –	*f41 and sta*	1	1	0	0	1	0	0	1	0	
20 (2.4%)	*f41*	4	4	0	3	1	0	1	1	0	
	Sta and lt	1	1	0	1	1	1	0	0	1	
	f5	1	1	0	1	1	0	1	0	0	
STEC	*stx1*	20	15	0	3	5	2	5	18	2	O8, O9, O12, O60 and O160
Total isolates –	*stx2*	4	3	0	1	1	0	1	3	3	
27 (3.8%)	*stx1 and eae*	3	2	0	1	1	1	0	2	1	
EPEC	*eae*	15	3	0	6	3	9	9	12	6	O15, O68 and O82
Total isolates – 15 (2.1%)											
Unclassified virotype	*f5 and stx2*	3	2	0	1	2	2	1	1	1	O9 and O101
Total		65	45	0	27	19	18	23	41	17	

organized farm. Farm wise distribution of virulent *E.coli* isolates is shown in Table 2. Distribution of virulence genes among virulent *E. coli* strains is presented in Table 3 and images of amplified PCR products of various virulence genes on agarose gel is shown in Figs 1,2.

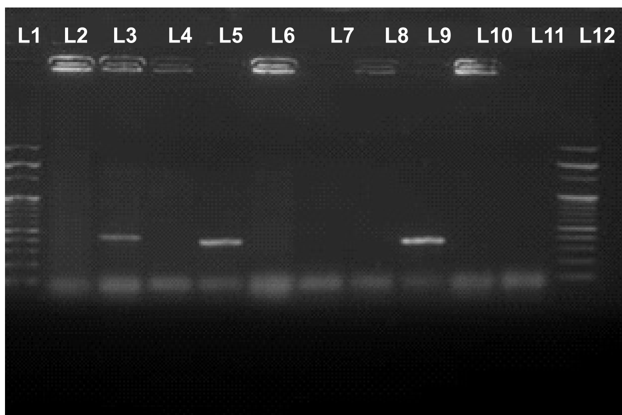

Fig. 1. Multiplex PCR showing amplified product of *eae* gene at 425 bp, *f5* at 315 bp and *f41* gene at 380 bp. Lanes L1 and L12, molecular weight marker of 1500 bp; lane L2, negative control; lane L3, *eae* gene at 425 bp; lane L5, *f41* gene at 380 bp; lane L9, *f5* gene at 315 bp; lanes L4, L6, L7, L8, L10, L11, negative samples.

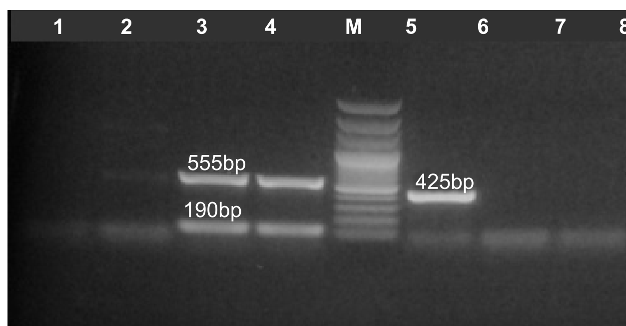

Fig. 2. Amplified PCR product of *eae, stx1* and *sta* gene. Lane M, Molecular weight marker of 100 bp (Himedia, India); lanes 1, 2, 6, 7 and 8, virulence gene negative isolate; lanes 3 and 4, positive for *stx1* and *sta* gene; lane 5, positive for *eae* gene.

In this study, *E. coli* strains harbouring enterotoxins genes (*sta* and/or *lt*) or fimbrial genes (*f41* or *f5*) either alone or with enterotoxins (*st* and *lt*) were classified as ETEC, the strains possessing eae^{+} genotype were classified as EPEC while the strains with or without the eae^{+} genotype that harboured Shiga toxin genes (*stx1* and/or *stx2*) were classified as STEC. Thus, out of 65 PCR positive isolates, 27 (3.8%) were STEC, 20 (2.8%) were ETEC, 15 (2.1%) were EPEC whereas remaining 3 (0.4%) could not be grouped as any recognized virotype as they possesed a new combination.

This study detected 2.8% prevalence of ETEC in calves in Jammu. Similar prevalence rate of ETEC was observed in diarrheic mithun calves (2.8%) and buffalo calves (4.95%) of India (Roosendal *et al*. 1984, Rajkhowa *et al*. 2009). Similarly, EPEC showed a prevalence rate of 2.1% in calves which was comparable to the study of Mainil *et al*. (1993), who reported EPEC in 3.2% of calves below 3 months of age. A higher prevalence of EPEC strains (9.3%) was reported from Kashmir, India (Wani *et al*. 2003) which may be due to difference in climatic condition and animal husbandry practices in both regions. Also, prevalence of STEC (3.8%) was in accordance with reports from Spain (5%) and India (6.02%) (Orden *et al*. 1998, Blanco *et al*. 2004).

Interestingly, 3 *E. coli* isolates positive for *f5* gene harboured *stx2* gene also, showing emergence of new phenotype causing diarrhea in calves. This may be due to the fact that Shiga toxin gene is encoded by bacteriophage which is possibly being transferred in *E. coli* population. Such study was also reported from Vietnam (Tan *et al*. 2010).

On serogrouping of the 700 isolates, only 598 (85.4%) could be typed for 'O' antigen, 27 (3.8%) were untypeable and 75 (10.7%) were rough isolates. These 598 typeable isolates belonged to 25 different serogroups. Among them,

most predominant were O2 (13.9%) and O6 (13.9%). The presence of these serogroups, i.e. O2, O6, O8, O26, O55, O60, O82, O86, O101, O121, O123, O170 was in agreement with previous reports from India (Sharma *et al*. 2004) and Japan (Nishikawa 2002). The distribution of different serogroups varies with geographical regions, climatic conditions and age of animals. This wide diversity in *E. coli* serogroups from calf diarrhea has been reported in several studies.

The ETEC strains in the present study belonged to serogroups O1, O2, O9 and O15. The isolation of ETEC serogroup O1 was in agreement with other reports from India, in which this serogroup was isolated from diarrhoeic calves in Kashmir (Wani *et al*. 2013). *E. coli* serogroups O9, O20 and O141 were found to be enterotoxigenic in calves throughout the world (Shahrani *et al*. 2014). In this study also, isolates with the serogroup O9 were found positive for *sta* and hence were grouped as ETEC. Serogroup O20 was negative for both the enterotoxin genes and O141 was not detected in our study. This may be due the fact that these genes are encoded by plasmid which may get lost on sub culturing. STEC isolates from present study belonged to serogroups O8, O9, O12, O60 and O160. Previously also, O8, O9, O26, O111, O113, O126 and O145 were found associated with diarrhea and enteritis in calves due to STEC (Hussain *et al*. 2003). The serogroups O15, O68 and O82 in present study were classified as EPEC as they were positive for *eae* gene. Previously also, the O15 and O68 EPEC serogroups were isolated from children of Kashmir with diarrhea (Wani *et al*. 2006). The serogroups isolated in the present study, viz. O6, O20, O22, O26, O121, O129 and O169 were negative for all the virulence characters tested. One possible explanation for this finding could be that these strains were non-pathogenic *E. coli* and the diarrhea was probably caused by some other infectious agent.

Only virulent *E.coli* isolates positive for any of the virulence genes tested were selected for antimicrobial testing. ETEC isolates were found resistant to amoxicillin (100%), oxytetracycline (80%), cloxacillin (40%), enrofloxacin (20%) and gentamicin (20%). STEC isolates were resistant to oxytetracycline (91.66%), amoxycillin (75%), cloxacillin (25%), enrofloxacin (25%), cephalexin (16.66%) and gentamicin (8.3%). EPEC isolates were resistant to oxytetracycline (80%), cloxacillin (40%), amoxicillin (60%), enrofloxacin (20%) and gentamicin (60%). Whereas, all *E. coli* isolates were sensitive for amikacin. This was in agreement with results of antimicrobial sensitivity of *E. coli* isolated from calves in India (Khan *et al.* 2002).

Multidrug resistance (resistance to more than 3 drugs) was also observed in 84.6% of tested *E. coli* strains; 32.14% of the isolates were resistant to 3 antibiotics, 42.85% to 4 antibiotics and 7.14% were resistant to 5 antibiotics. Similar multiple resistances have often been reported among the *E. coli* isolated from calves (Marin *et al*. 2007).

Diarrhea due to *E.coli* was prevalent in calves from both organized and unorganized farms of Jammu. Most commonly encountered serogroup in this area were O2 and O6, which are known to be associated with pathogenic strains. Multiplex PCR was successfully employed for virotyping of *E.coli* which will facilitate in the diagnosis and epidemiological studies of *E. coli* diarrhea. Also, multiple resistances are increasing in diarrhegenic *E.coli* strains, which is a matter of concern for animals as well as community.

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