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Virulence Gene Profile and Serogroups of STEC in Diarrhoeic Buffalo Calves

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A B S T R A C T

A study was undertaken to investigate virulence gene profiles of shiga toxin-producing *E. coli* serogroups in diarrhoeic buffalo calves of Andhra Pradesh (AP) and Telangana States (TS). A total of 375 faecal samples from diarrhoeic buffalo calves of 1 to 90 days were collected from various districts in AP and TS, of which 302 *E. coli* (80.53%) were isolated. The virulence genes of STEC were detected using multiplex PCR and serogrouping was carried at National *Salmonella* and *Escherichia* centre, Central research institute, Kasauli, Himachal Pradesh. Among the virulence genes of STEC the *stx1, stx2& hlyA* genes carrying isolates constitutes about 4.63% of which 36% were identified as O157, distributed in Krishna and West Godavari districts of Andhra Pradesh. The serogrops of STEC associated with calf diarrhoeia includes O8, O9, O10, O26, O29, O49, O84, O86, O120, O121, O125, O126, O128, O141 and O157. The present study concluded that diarrhoeic buffalo calves are the source of about 15 STEC serogroups including O157 and O26 which are having public health significance.

Introduction

Escherichia coli is an important enteric pathogen of buffalo calves causing neonatal diarrhoea and economic loss to the dairy producers. Epidemiological studies of cattle and water buffalo calves have revealed that *E. coli* was the major cause of neonatal diarrhoea

(Fagiolo *et al*., 2005 and Foster and Smith, 2009). Shiga toxin-producing *Escherichia coli* (STEC) are one of six different categories of diarrhoeagenic *E. coli* recognized so far. STEC has been implicated as an etiological factor of calf diarrhea, (Sandhu and Gyles, 2002) and cattle are regarded as the main reservoirs of STEC which produce Shiga toxins (*Stx1* and *Stx2*) encoded by *stx1* and *stx2* genes (Boerlin *et al*., 2005). The other virulence factors of STEC are intimin and enterohaemolysin encoded by *eae* and *ehxA* variants of *stx1* and more than 20 variants of *stx2* have genes respectively (Blanco *et al*., 2004). Different serotypes of STEC that were associated with diarrhoea in calves (manly O5, O26, and O118) have been recognized (Orden et al., 2003) Therefore, studying the prevalence of various serogroups of STEC in buffalo calves in this geographic area is important to monitor the epidemiology of infection due to *E. coli* associated calf diarrhoea.

Materials and Methods

A total of 375 faecal samples from diarrhoeic buffalo calves of 1 to 7, 8-30, 31-60 and 61-90 day age groups were collected randomly from organized dairy farms and individual farmers of Vizianagaram, Vishakapatnam, East Godavari, West Godavari, Krishna, Guntur, Prakasam, Districts of Andhra Pradesh State and Ranga Reddy and Khammam Districts of Telangana State. Geographical distribution and age of diarrhoeic calves were recorded during sampling. Fecal samples were collected using sterile rectal swabs. After collection, the swabs were immediately transported to the department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram in ice-cooled containers for *E. coli* isolation. All the samples were inoculated on to Maconkey agar and incubated at 37^{o}c for 24 hours. The pink colonies obtained were again inoculated in EMB agar and the colonies showing green metallic sheen were selected and confirmed as *E. coli* by standard biochemical tests (Cruickshank 1970). Bacterial DNA was obtained by boiling the cells at 100^{0} C for 15 min and then pelleting the cells by centrifugation. The supernatant was then used in the PCR reaction.

Molecular Characterization of *E. coli* isolates

The biochemical results were confirmed by PCR amplification using *E. coli* 16s rRNA specific primers quoted by Sun Dong-bo *et al.*(2011) (E16S-F: ATCAACCGAGATTCCCCCAGT E16S-R: TCACTATCGGTCAGTCAGGAG) with 231bp amplified product.

PCR conditions for detection *E.coli 16SrRNA*

PCR reactions were carried out in an Eppendorf thermal cycler. The amplification conditions were 5 min of denaturation at 95°C, followed by 35 cycles of 95°C for 1 min, 50°C for 50 s, and 72°C for 1 min, and a final extension step of 72°C for 10 min. DNA amplified by PCR was subjected to 2% agarose gel electrophoresis as described by sambrook and Russel (2001).

The primers used in the present study for the detection of shiga toxin producing *E. coli* were as described by Paton and Paton (1998) (Table 1)

Standardization of multiplex PCR protocol for detection of *stx*1, *stx*2, *eae*A, *hly*A virulence genes

PCR for amplification of the *stx*1, *stx*2, *eae*A, *hly*A genes was set up in 25µl reaction. Following initial trails with varying concentration of components the reaction mixture was optimized as below

Primer F (20 p mol)	0.12 µl × 4
Primer R (20 p mol)	0.12 µl × 4
Master mix	12.5 µl
Template	5.00 µl
NFW water	6.54 µl
Total	**25 µl**

PCR tube containing the reaction mixture was flash spun in a microcentrifuge to settle the reactants at the bottom. PCR assay was performed in Eppendorf thermocycler with heated lid. Samples were subjected to 35 cycles as per the procedure of paton and paton (1998). The cycle consisting of one min of denaturation at 95^{o} C, two min of annealing at 65^{o}C and 1.5 min of elongation at 72^{o}C for the first 10 cycles, decrementing annealing temperature to 60^{o}C by cycle 15, one min of denaturation at 95^{o}C, two min of annealing at 60^{o}C and 1.5 min of elongation at 72^{o}C, incrementing to 2.5 min from cycles 26 to 35. On completion of the reaction, tubes with PCR products were held at 4^{o}C. DNA amplified by PCR was subjected to 2% agarose gel electrophoresis as described by sambrook and Russel (2001).

Serogrouping of *E. coli* isolates

The isolates of *E. coli* were sent to National *Salmonella* and *Escherichia* centre, Central research institute, Kasauli, Himachal Pradesh on nutrient agar slants for serogrouping.

Results and Discussion

Out of 375 faecal samples collected from diarrhoeic buffalo calves, 302 (80.53%) samples were found positive after biochemical and molecular characterization for *E. coli*. Among different age groups, highest rate (85.04%) of *E. coli* isolated from faecal samples of 1-7 day old buffalo calves, followed by 83.96, 63.27 and 50.00 % isolated from 8-30, 31-60 and 61-90 day old buffalo calves, respectively. Similar to present findings, higher prevalence (70%) was observed in buffalo calves of less than 4 weeks age (Borriello *et al.,* (2012). Compared to the prevalence rate observed in this study, lower prevalence rate of *E. coli* was reported in diarrhoeic buffalo calves by Deepti Naag *et al.,* (2015) in Jabalpur, India (59.37%), Abdulgayeid *et al.,* (2015) and Helal *et al.,* (2014) in Egypt (66 and 72%), Anwarullah *et al.,* (2014) in Pakistan(14.6%), Paul *et al.,* (2010) and Islam *et al.,* (2008) in Bangladesh (45 and 37.9%) and in healthy buffalo calves (Helal *et al.,* 2014) in Egypt. On the other hand, higher prevalence (95.6%) of *E. coli* in buffaloes was also reported by Raghavendra *et al.,* (2016) in Uttar Pradesh, India. The differences of the prevalence rates of *E. coli* may be attributed to the geographical locations and management practices as well as hygienic measures which influence the susceptibility of calves to *E. coli* infection (Cho and Yoon, 2014 and Içen *et al.*, 2013).

In Andhra Pradesh, highest prevalence of *E. coli* in diarrhoeic buffalo calves was observed in Krishna district (95.58) and lowest (62.5%) was found in Vizianagaram district while in Telangana state prevalence was high in Khammam (76%) compared to Ranga Reddy district (62.85%).

The Association between virulence genes and serogroups of STEC in diarrhoeic buffalo calves is presented in Table 2.

Among the *E. coli* isolates from diarrhoeic buffalo calves, 35.01% isolates were STEC and the isolates carrying virulence genes of *eaeA*& *hlyA* combination were highest (45%). This was in accordance with the findings of Islam *et al.,* (2008) who detected 37.9 % STEC mostly *eae* positive in faecal samples of buffaloes in Bangladesh (Table 2).

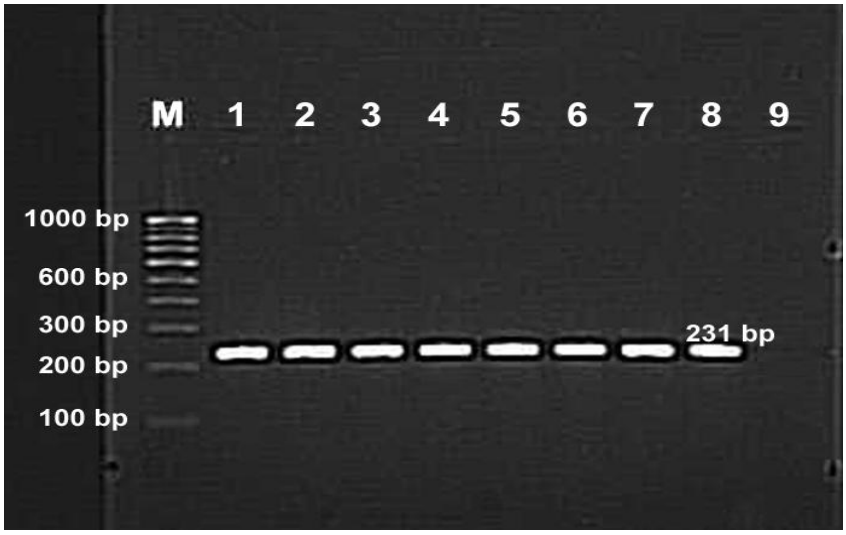
Table.1 Details of the primers used for the detection of *stx*1, *stx*2, *eae*A and *hly*A genes

S.NO	Primer	Sequence (5'---- 3')	Target gene	Expected Amplicon size (bp)
1	*Stx*1F	ATAAATCGCCATTCGTTGACTAC	*stx*1	180
	*Stx*2 R	AGAACGCCCACTGAGATCATC		
2	*Stx*2 F	GGCACTGTCTGAAACTGCTCC	*stx*2	254
	*Stx*2 R	TCGCCAGTTATCTGACATTCTG		
3	*eae*A F	GACCCGGCACAAGCATAAGC	*eae*A	384
	eae R	CCACCTGCAGCAACAAGAGG		
4	*hly*A F	GCATCATCAAGCGTACGTTCC	*hly*A	534
	*hly*A R	AATGAGCCAAGCTGGTTAAGCT		

Table.2 Association between virulence genes and serogroups of STEC in diarrhoeic buffalo calves

Virulence gene	n=302	%	Serogroups
stx1	17	5.62	O8 (4), O121(4), O9 (2), O26 (2) and UT (5)
stx2	13	4.30	O29 (4), O125 (3), O10(2), O8 (1) and UT (3)
stx1,stx2 & hlyA	14	4.63	O128 (5), O157 (5) and O9 (4)
stx1,eaeA & hlyA	10	3.31	O26 (5) and O120 (5)
stx1& hlyA	7	2.31	O125 (4) and O86 (3)
eaeA& hlyA	45	14.90	O84 (13), O128 (10), O126 (6), O141(5), O49 (2), O121(2) and UT(7)

Fig.1 Amplified product of 16S r RNA



Lane M: 100 bp DNA ladder
Lane 1: *E. coli* 16S r RNA positive control
Lane 2 to 8 : *E. coli* isolates carrying 16S r RNA gene
Lane 9: Negative control.

Fig.2 Multiplex PCR for detecting Shiga toxin genes. *Stx*1 (180bp), *Stx*2 (254bp) *eae*A (384bp), *hly*A (534bp) genes

Lane M: 100 bp DNA ladder
Lane 1: *E. coli* positive control carrying all four genes
Lane 2 to 7: *E. coli* isolates carrying shiga toxin genes
Lane 8: Negative control.

The serogroups of STEC associated with calf diarrhoeia includes O8, O9, O10, O26, O29, O49, O84, O86, O120, O121, O125, O126, O128, O141 and O157. Among these serogroups, the frequency of O128, O8, O121, O9, O126 and O125 were high compared to other serogroups identified (Table 2). The *stx1*, *stx2*& *hlyA* genes carrying isolates constitutes about 4.63% of which 5 (36%) were identified as O157 which is known for its zoonootic significance. The World Health Organization also recognizes O26 as having the potential to cause severe disease in humans (WHO, 1995). The present study also observed that 50 % of *stx*, *eaeA* & *hlyA* and 12% of *stx*1 genes carrying isolates detected as O26.

Parallel to the present findings 42.5% prevalence of O157 in buffalo calves recorded in Italy (Sandra *et al*., 2010). In a similar study the incidence of 14.4% of the serotype O157 was reported from the STEC isolates carrying *eae* positive genes in buffaloes of Bangladesh (Islam *et al*., 2008) while in Italy adult water buffalo has been reported as a natural reservoir of the serotype O157 (Galiero *et al*., 2005). Consistent with present findings, O9 serogroup in diarrhoeic mithun calves in Nagaland (Rajkhowa *et al*., 2009) and O86 serogroup in diarrhoeic calves in Brazil (Rigobelo *et al*., 2006) reported. Toni *et al*., (2006) also recognized O8 and O9 serogroups in diarrhoeic pre-weaned piglets. Contrary to the present findings, Vagh and Jani (2010) detected serotype O56 was highest (14.28 %) in diarrhoeic buffalo calves in Gujarat (Fig. 1 and 2).

The present study detected the prevalence of STEC serogroups that are having public health significance like O157 in Krishna and West Godavari districts and O26 in Vishakhapatnam, East Godavari, West Godavari, Guntur districts of Andhra Pradesh and Khammam district of Telangana state. Therefore, the diarrhoec buffalo calves may be the potential source of these serogroups

which are having zoonootic importance in this geographis area.

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