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Isolation, Characterization and Association of Shiga Toxin-Producing *Escherichia coli* from Bovines and their Handlers in Jammu, India.

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Shiga toxin-producing *Escherichia coli* (STEC) are one of the emerging zoonotic pathogens with cattle being considered as the major reservoirs of infection. Outbreaks of human disease are often traced to contacts with cattle or their products. The present study was, thus, undertaken to isolate and characterize STEC from bovines and their handlers. Faecal samples from bovines (n=103) and their handlers' stool and fingertip rinses (n=70) were collected over an eight month from August, 2011 to March, 2012. Multiplex polymerase chain reaction (mPCR) for *stx*$_1$, *stx*$_2$, *eaeA* and *hlyA* genes detected STEC in 15 (11.90%) of the 126 *E. coli* isolates with the most frequent virulence gene combination as *stx*$_1$, *stx*$_2$ and *hlyA*. Both *stx*$_1$ and *stx*$_2$ genes in combination were found to be more frequent (66.6%) than only *stx*$_{2-}$ (26.6%) and *stx*$_1$ (6.66%) genes among the STEC isolates. The intimin and enterohaemolysin encoding genes, *eaeA* and *hlyA* were detected in 6 (40%) and 11 (73.3%) of the STEC isolates. The overall prevalence of STEC in cattle, buffalo and bovine handlers was 15%, 6.7% and 4.28% respectively. Though there were no similar serogroups of STEC isolated from bovines and their handlers, their virulence gene profile was similar in one farm. A positive correlation (r=0.958) of STEC prevalence of bovines and their handlers existed in different localities. The study indicates that bovines in the region harbour STEC, and are the probable source of STEC transmission to humans especially occupationally exposed groups.

Key words: Association, bovines, bovine handlers, prevalence, STEC.

Shiga toxin-producing *Escherichia coli* (STEC) have emerged as a group of highly pathogenic *Escherichia coli* strains characterized by the production of potent cytotoxins that inhibit protein synthesis within eukaryotic cells.[1,2] These toxins are either termed verocytotoxins (VT), because of their activity on Vero cells, or Shiga toxins (Stx), because of their similarity with the toxin produced by *Shigella dysenteriae*.[3] In humans the diseases they cause range from asymptomatic carriage, watery diarrhoea to haemorrhagic colitis and or haemolytic uremic syndrome.[4,5] Haemolytic uraemic syndrome, a life-threatening complication, is characterized by thrombocytopenia, microangiopathic haemolytic anaemia, and acute renal failure. The latter syndrome occurs especially among children, the elderly and the immuno-compromised.[6] STEC represent the pathogenic group of *E. coli* with a definite zoonotic

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origin; cattle being recognised as the major reservoir for human infections.[7] Most human cases have been linked to direct or indirect contact with cattle though some have been reported with other species.

In India cattle farming is mostly practised on a house hold scale with routine contacts with cattle. Thus, a large human population could be at risk of STEC transmission from bovine reservoirs. Despite this, little is known about the epidemiology and burden of STEC infections in bovine handlers. Though there are reports of STEC isolation from both animals[8] and humans[9] in various parts of India, there is paucity of information on this aspect in Jammu region. Relatively less information exists about STEC in cattle and buffalo handlers of the region. The present study was, therefore, undertaken to isolate and characterize STEC and to find an association, if any, between STEC from bovines and their handlers of the area.

MATERIALS AND METHODS

Sample collection

Bovines

One hundred three faecal samples were collected from 60 cattle and 43 buffaloes per rectally from two cattle farms viz., Belicharana and F.V.Sc, R.S. Pura, and from the household farms of Sidher, Khanachak, and Kotli villages of R.S. Pura area of Jammu during the period from August, 2011 to March, 2012. Calf samples were obtained by rectal swabs.

Bovine handlers

Stool samples and fingertip rinses were obtained from the persons who handled the cattle and buffaloes at these places. A total of twenty eight stool samples and forty two fingertip rinses were collected.

Isolation and identification of *E. coli*

Enrichment was done using Mac Conkey broth followed by selective plating.[10] Two to three lactose fermenting colonies from each Mac Conkey agar plate were streaked on Eosin Methylene Blue agar for appearance of characteristic metallic sheen and then subjected to biochemical identification.[11,12] The purified cultures were maintained in 0.75% nutrient agar media slants in triplicate.

Serogrouping

Serogrouping of the *E. coli* isolates was got done from National Salmonella and Escherichia Centre, Central Research Institute, Kasauli-173204 (H.P, India) on the basis of their 'O' antigen.

Extraction of bacterial DNA

The *E. coli* isolates were first revived in Mac Conkey agar to obtain fresh isolates. Hundred microlitre of nuclease free water was taken in a separate micro centrifuge tube and a loopful of each isolate was mixed with it thoroughly. The suspended isolates in the micro centrifuge tubes were then subjected to heat lysis by keeping in boiling water for 10 minutes and quickly placed in ice for 10 minutes followed by centrifugation at 10,000 rpm for 10 minutes. Two microlitres of the supernatant were taken as template DNA.

Multiplex polymerase chain reaction (mPCR) of the *E. coli* isolates

Target gene specific multiplex polymerase chain reaction was performed for the molecular detection of virulence genes of *E. coli* isolates. Primers used in the study are listed in Table.1. All the *E. coli* isolates were screened for the detection of Shiga toxin-producing (*stx*) genes by multiplex polymerase chain reaction.[13] The mPCR was carried out in a final reaction volume of 25µl using 0.2 ml thin wall sterile and nuclease free PCR tubes (Eppendorf, Germany). The PCR mixture contained a final concentration of 2 mM $MgCl_2$, 0.6 mM concentrations of each 2'-deoxynucleoside 5'-triphosphate (dNTPs), 5 µl of 5X assay buffer, 0.5µl of forward and reverse primers, 2.0µl template DNA and 1.0 U of GoTaq DNA Polymerase (Promega Corporation, Madison, U.S.A). PCR was performed in a Thermocycler (Applied Biosystems Gene Amp PCR System 2400) with heated lid using the steps and cycle conditions as: initial denaturation at 95°C for 2 minutes followed by 15 cycles; each cycle consisting of denaturation at 95°C for 1 minute, annealing at 65°C for 2 minutes and extension for 1.5 minutes at 72°C. A second phase of 20 cycles was followed with each cycle consisting of denaturation for 1 minute at 95°C, annealing at 60°C for 2 minutes and extension for 2 minutes at 72°C. A final extension was done at 72°C for 5 minutes. The PCR product was analysed by agarose gel electrophoresis for the amplicon sizes of 180 base pairs (bp), 255 bp, 384 bp and 534 bp.

RESULTS

The serogroups and virulence gene profile of STEC isolated from bovines and their handlers during the present study have been shown in Table 2. Of the total of 126 *E. coli* isolates screened, fifteen (11.9%) *E. coli* isolates revealed the presence of Shiga toxin producing genes. The most frequent gene combination was stx_1, stx_2 and *hlyA* (Fig. 1; Table 2). From the sixty cattle, a total of fifty one *E. coli* isolates were obtained with thirteen (25.49%) possessing at least one virulence gene of which nine isolates (17.6%) possessed stx_1 and or stx_2 genes; four from adults and five from calves. The overall prevalence of STEC was 15% in the cattle; higher in calves (19.2%) compared to adults (11.76%). In cattle, *eaeA* genes were present in 44.4% of the STEC isolates. Of the thirty five *E. coli* isolates obtained from buffaloes; six isolates (14.28%) possessed at least one virulence gene. Among the three STEC isolates, two were from adults and one from buffalo calf. The overall prevalence of STEC in buffaloes was 6.97%. From the bovine handler samples, forty *E. coli* isolates were screened for the presence *stx* genes of which three revealed the presence of stx_1 and or stx_2 genes; two from fingertip rinses and one from stool sample (Table.2). The overall prevalence of STEC in bovine handlers was 4.28%.

No STEC were isolated from the cattle, buffalo or their handlers' samples taken from the households of Sidher, Kotli, and Khanachak villages. However, they were isolated both from bovines as well as their handlers' samples of F.V.Sc and Belicharana cattle farms (Table 3). A positive correlation (r= 0.958) between the prevalence of

Table 1. List of primers (5' -3') used in the multiplex PCR reaction

Primer	Sequence (5' –3')	Amplicon size (bp)	Reference
stx_1-F	ATAAATCGCCATTCGTTGACTAC	180	Paton and Paton (1998)
stx_1-R	AGAACGCCCACTGAGATCATC		
stx_2-F	GGCACTGTCTGAAACTGCTCC	255	
stx_2-R	TCGCCAGTTATCTGACATTCTG		
eaeA-F	GACCCGGCACAAGCATAAGC	384	
eaeA-R	CCACCTGCAGCAACAAGAGG		
hlyA-F	GCATCATCAAGCGTACGTTCC	534	
hlyA-R	AATGAGCCAAGCTGGTTAAGCT		

Table 2. Description of STEC isolates carrying one or more virulence genes

S. No.	Isolate no.	Serogroup	Species	Virulence gene profile
1	F-21	UT	Cattle	stx_1, stx_2, *hlyA*
2	F-23	UT	Cattle	stx_1, stx_2, *hlyA*
3	F-26	UT	Cattle	stx_1, stx_2, *hlyA*
4	F-76	UT	Cattle	stx_1
5	F-85	O-76	Cattle	stx_1, stx_2, *hlyA*
6	F-93	R	Cattle	stx_1, stx_2, *eaeA*, *hlyA*
7	F-99	R	Cattle	stx_2, *eaeA*, *hlyA*
8	F-100	UT	Cattle	stx_1, stx_2, *eaeA*, *hlyA*
9	F-105	UT	Cattle	stx_2, *eaeA*, *hlyA*
10	F-63	R	Buffalo	stx_1, stx_2, *hlyA*
11	F-87	UT	Buffalo	stx_1, stx_2
12	F-107	UT	Buffalo	stx_1, stx_2, *eaeA*, *hlyA*
13	A.H-23	UT	Fingertip rinse	stx_1, stx_2, *hlyA*
14	A.H-26	UT	Fingertip rinse	stx_2
15	A.H-39	O-106	Stool sample	stx_2, *eaeA*

UT=Untypeable, R = Rough

Table 3. Prevalence of STEC in different localities

S. No.	Place of collection	Bovines		Bovine handlers	
		No of samples taken	No. of samples positive for STEC	No of samples taken	No of samples positive for STEC
1	Sidher	10	0 (0)	16	0
2	Kotli	4	0 (0)	8	0
3	Khanachak	5	0 (0)	8	0
4	Cattle farm, F.V.Sc	26	3 (11.5)	16	1 (6.25)
5	Cattle farm, Belicharana	58	9 (15.5)	22	2 (9.09)

Figures in parenthesis indicate percentage out of number of samples.

Table 4. Comparison of virulence gene profile of STEC in bovines and their handlers

Locality	Bovines			Bovine handlers		
	Isolate no.	Source	Virulence gene profile	Isolate no.	Source	Virulence gene profile
Cattle farm, F.V.Sc	F-21	Cattle	*stx1, stx2, hlyA*	A.H-23	Fingertip rinse	*stx1, stx2, hlyA*
	F-23	Cattle	*stx1, stx2, hlyA*			
	F-26	Cattle	*stx1, stx2, hlyA*			
Cattle farm, Belicharana	F-63	Buffalo	*stx1, stx2, hlyA*	A.H-26	Fingertip rinse	*stx2*
	F-76	Cattle	*stx1*			
	F-85	Cattle	*stx1, stx2, hlyA*			
	F-87	Buffalo	*stx1, stx2*			
	F-93	Cattle	*stx1, stx2, eaeA, hlyA*	A.H-39	Stool	*stx2, eaeA*
	F-99	Cattle	*stx2, eaeA, hlyA*			
	F-100	Cattle	*stx1, stx2, eaeA, hlyA*			
	F-105	Cattle	*stx2, eaeA, hlyA*			
	F-107	Buffalo	*stx1, stx2, eaeA, hlyA*			

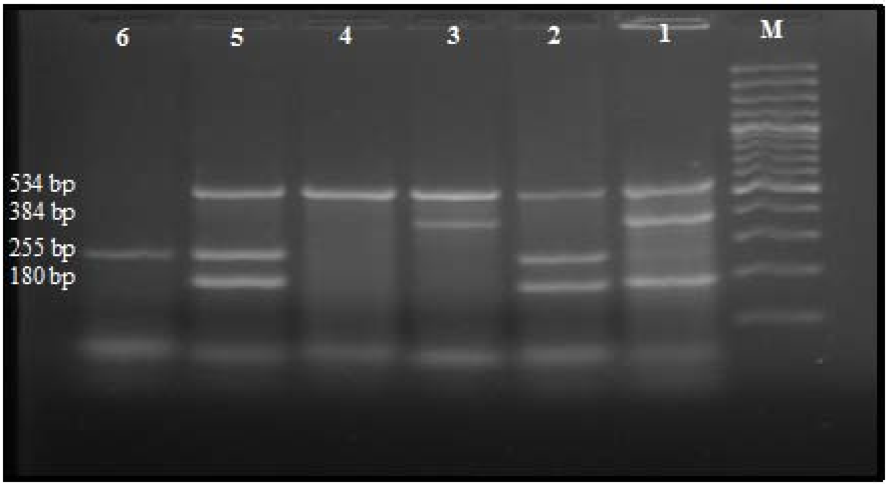

Fig. 1. Agarose gel showing multiplex PCR amplification products of *stx*$_1$, *stx*$_2$, *eaeA* and *hlyA* genes in *E. coli* isolates. Lane M: 100 bp Molecular weight marker, Lane 1: Positive control, Lanes 2 and 5: Amplified products of *stx*$_1$, *stx*$_2$ and *hlyA* genes, Lane 3: Amplified products of *eaeA* and *hlyA* genes, Lane 4: Amplified product of *hlyA* gene, Lane 6: Amplified product of *stx*$_2$ gene

STEC in bovines and their handlers was observed in F.V.Sc and Belicharana cattle farms. The virulence gene profile of STEC isolates obtained from bovines and their handlers was same at Cattle farm, F.V.Sc (Table 4).

DISCUSSION

The present findings reveal a greater prevalence of stx_1 and stx_2 genes in combination than stx_2 and stx_1 genes singly in the STEC isolates. Besides, there was dominance of stx_2 gene compared stx_1 gene in STEC isolates especially from cattle. Though such findings have earlier been reported[14,15], the high incidence of stx_2 genes observed in this study is a matter of some concern, as the carriage of stx_2 genes has been linked to more-severe *E. coli* infection.[16] Our findings also revealed a high prevalence of *eaeA* gene (40%) and of *hlyA* gene (73.3%) in the STEC isolates. A high incidence of *hlyA* positive STEC isolates from cattle was also reported in Silesia, Italy[17] and Hessia, Germany[18] and of *eaeA* positive STEC isolates from Egypt[19]; however a lesser prevalence (17%) was found among Swiss cattle.[20] An interesting finding was that all the STEC isolates from cattle carried *hlyA* gene. Such a finding is especially important in view of the fact that STEC strains from patients suffering from haemorrhagic colitis or haemolytic uraemic syndrome are frequently stx_2 and *eaeA* positive and many also carry the *hlyA* gene.[21]

Though an association of STEC from bovines and their handlers based on similar serogroups could not be established as most of them were rough or untypeable, the presence of STEC in handlers only at those farms (F.V.Sc and Belicharana) where bovines were positive probably indicates the transmission of STEC from bovines to their handlers ($r = 0.958$). The similar virulence gene profile of STEC isolates from bovines and their handlers of Cattle farm, F.V.Sc could also suggest a relationship as STEC isolates from cattle have been found to have high homology with human isolates in areas where stockbreeding is prolific.[22] Moreover, several cases of STEC infections in humans have been associated with STEC strains without the *eaeA* gene, but with stx_2 and other factors for adhesion.[23,24]

CONCLUSION

This study indicates that in Jammu region, cattle and buffaloes are important reservoirs of STEC and their routine handling may lead to human infections. A high proportion of stx_2, *hlyA* and *eaeA* genes in bovine strains warrant the high pathogenic potential of STEC in this region. Screening of a larger bovine and human population in rural areas should be carried out to further elucidate the information on epidemiology and zoonotic potential of STEC as well as their role in human infections.

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