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Subtype Analysis of Shiga Toxin-Producing *Escherichia coli* and Enteropathogenic *Escherichia coli* Isolated from Cattle and Sheep

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ABSTRACT

The present study was conducted to determine *stx* and *eae* virulent genes in *E .coli* and their subtyping during 2021-2022. For this, One hundred fifty six (156) rectal swab samples from healthy cattle and sheep were investigated for the presence of stx_1, stx_2, *eae, ehxA* and *bfp* virulence genes using polymerase chain reaction (PCR). In all, 133 *Escherichia coli* (*E. coli*) isolates possessed at least one virulence gene targeted. Eighty nine Shiga toxin-producing *Escherichia coli* (STEC) isolates (sixty two from cattle and twenty seven from sheep) were obtained. STEC were more prevalent in cattle (63.11%) than in sheep (45.76%). Enteropathogenic *Escherichia coli* (EPEC) were detected from 10 (10.30%) cattle and 1 (1.69%) sheep rectal swab samples. All the EPEC isolates detected were atypical. Subtype analysis of *stx* genes in cattle exhibited 18.39% as *stx1*, 34.48% as *stx2* and 18.93 as both *stx1 & 2*. Similarly, *stx* analysis in sheep revealed stx_1 in 45.65%, *stx2* in 6.15% and both *stx1 & 2* in 6.15% isolates. Among 20 (15.03%) *eae* positive isolates of *E. coli* recovered from Cattle and Sheep, *eaeA* gene was found associated with 16 (18.39%) and 4(8.69%) rectal swab of Cattle and Sheep respectively. Upon intimin subtyping, *int-β* was detected in 03 (75%) and 04 (25%) while as *Int-γ* was detected in 01 (25%) and 10 (62.50%) of sheep and cattle rectal swabs respectively. Two *E. coli* isolate positive for *eae* gene form rectal swab of cattle could not be subtyped. The present investigation indicates that stx_1, stx_2, *stx1&2*, *eae- β* and *Int-γ* could be common subtypes prevalent in STEC and EPEC strains of healthy cattle and sheep in India. Statistically, the difference between prevalence of STEC and EPEC in Cattle and Sheep was found significant ($p = 0.05$).

HIGHLIGHTS

- This article describes the subtyping of *stx* and *eae* virulent genes in *E.coli*.
- The high occurrence of *E. coli* highlights that the importance of animals as being super-shedders.
- High diversity of STEC, EPEC strains circulating in the animals represents a risk for Public Health.

Keywords: PCR, Shiga toxin, STEC, subtype, typical EPEC

Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) represent two of the at least six different categories of diarrhoeagenic *E. coli* recognized so far (Kaper *et al*., 2004). STEC is an important causative agent of haemorrhagic colitis (HC) and diarrhoea-associated haemolytic uremic syndrome (HUS) with or without neurological complications (Islam *et al.,* 2007). Domestic animals mainly cattle and sheep have been implicated as the principal reservoirs

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of STEC (Cornick *et al*., 2007). The pathogenicity of STEC is mediated mainly through Shiga toxins 1 and 2 encoded by stx_1 and stx_2 genes, respectively. Another virulence-associated factor expressed by STEC is a protein called intimin (encoded by *eae* gene), which is responsible for intimate attachment of the STEC to the enterocytes, causing attaching and effacing (A/E) lesions in the intestinal mucosa (Mora *et al*., 2005). One more factor that may affect the virulence of STEC is called as enterohaemolysin (Ehly) or enterohaemorrhagic *E. coli* haemolysin (EHEC-HlyA) which is encoded by the *ehxA* gene (Blanco *et al*., 2003).

EPEC strains are defined as intimin-containing diarrhoeagenic *E. coli* that possess the ability to form A/E lesions on intestinal cells but do not possess *stx* genes (Kaper, 1996). EPEC and STEC can also cause disease in animals using many of the same virulence factors that are present in human strains (Kaper *et al*., 2004). EPEC strains are further classified as typical and atypical. Typical EPEC possess EPEC adherence factor (EAF) plasmid which carry genes for bundle forming pili (*bfpA*) that mediates localized adherence (LA) on cultured epithelial cells (Blanco *et al*., 2005). Atypical EPEC strains are negative for both *EAF* plasmid and *bfpA* gene but show localized-like adherence patterns (Trabulsi *et al*., 2002). Atypical EPEC have been isolated frequently from humans and animals, however, typical EPEC have been rarely reported from animals (Blanco *et al*., 2005; Krause *et al*., 2005). Atleast, 18 types and nine subtypes of intimin (α, α2, β1 to 3, γ1, γ2, δ, ε, ε2 to 4, ζ, η, η2, θ, ι, ι2, κ, λ, μ, ν, ξ, ο, π, ρ, and σ) gene variants have been reported (Ito *et al*., 2007). But there seems little information available about the variants of *eae* gene from India (Wani *et al*., 2006).

The purpose of the present work was to analyse *stx* and *eae* virulence genes for their different variants as well as to ascertain the typical and atypical nature of the EPEC from cattle and sheep.

MATERIALS AND METHODS

Sampling and isolation of *E. coli*

A total of 156 faecal samples (97 from cattle and 59 from sheep) were collected from different organized Cattle and Sheep Breeding farms (Mountain livestock Research Institute, Mansbal; Sheep Research Station, Faculty of Veterinary Science & Animal Husbandry, SKUAST-K, Srinagar; Sheep Breeding Farm Daksum, Sheep Husbandry Department, Govt. of Jammu and Kashmir) and from private Cattle and Sheep farms of the Kashmir valley from March 2021 to November 2022. All the samples were from healthy animals without diarrhoea. The samples were collected by rectal swabbing and immediately carried to laboratory on ice.

Faecal samples were immediately processed as per Cookson *et al*. (2007). Briefly, the samples collected were enriched in buffered peptone water (20 ml) and incubated at 37°C for 5-6 hours. A loopful of inoculum from the enriched broth was streaked onto Sorbitol MacConkey agar plate supplemented with cefixime (50 µg/ml) and tellurite (2.5 mg/ml) and Tryptone bile X-glucuronide agar plate. The streaked plates were incubated at 37°C for 18-24 hours. Multiple colonies of pink sorbitol-fermenting (SF) colony from Sorbitol MacConkey agar plate and well separated blue / colourless colonies from the tryptone bile X-glucuronide agar plate were picked up and further subjected to molecular testing. After overnight incubation at 37ºC bacterial colonies from each culture was processed for extraction of DNA for detection of stx_1, stx_2, *eae, bfp* and *ehxA* virulence genes by multiplex-PCR. The isolates were subjected to standard morphological, biochemical tests as described by Holt *et al*. (1994) to ascertain their identity as *E. coli.*

Extraction of bacterial DNA

The isolated bacterial colonies from Sorbitol MacConkey agar plate and tryptone bile X-glucuronide agar plate were suspended in 150µl of sterile distilled water in 1.5 ml microcentrifuge tubes and mixed by gentle vortexing. The bacteria were lysed by boiling for 10 mins in a water bath and then cooled on ice for 20 min. The lysate was centrifuged at 10,000×g for 5 min. in a table-top cooling microcentrifuge and the supernatant was directly used as template for multiplex polymerase chain reaction (m-PCR).

Polymerase chain reaction (PCR)

All the *E. coli* isolates were screened by PCR for the presence of *eae, stx1, stx2,* bundle forming pilli (*bfp*)

and entero-hemolysin (*ehx*). The PCR assay was carried out in 25 µl reaction volume containing 2.0 µl template DNA, 2.5 µl 10X buffer, 0.2 µl 100 mM dNTP mix, 1U of Taq DNA polymerase (Bangalore Genei, India) and sterile distilled water. The concentration of $MgCl_2$ was 2.5 mM. The details of primer sequences used in this study are given in table 1. The m-PCR assays were performed in Gene Amp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were procured from M/S Sigma GENOSYS or M/S Integrated DNA Technologies, Inc.

Detection of stx_1, stx_2, eae, bfp and ehxA genes

All *E coli* isolates were subjected to multiplex PCR for detection of stx_1, stx_2, *eae* and *bfp* genes. PCR cyclic conditions and primer sequences were essentially the same as described by (cookson *et al*., 2002). Amplified PCR products were analysed by gel electrophoresis in 2% agarose containing 0.5 µg/ml of ethidium bromide (Sambrook and Russell, 2001).

Subtyping of *eae* genes

Subtyping of *eae* gene for detection of commonly reported subtypes (*eae-α*, *eae-β*, *eae-g*, *eae*-δ) was carried out as per the standard protocol described by (Cookson *et al*., 2002). The cyclic conditions for PCR were initial denaturation for 2 minutes at 94º C followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 55º C for 1minute and extension at 72 º C for 2 minutes. The final extension was carried out at 72ºC for 10 minutes.

Classification of EPEC

To ascertain the typical or atypical nature, all EPEC isolates were examined for presence or absence of *ehxA* and *bfpA* genes. For the detection of *ehxA* by PCR, procedure of Wang *et al*. (2002) was followed with minor modifications. The samples were denatured at 95°C for 8 min, followed by 30 cycles of amplification with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 7 min. The primer concentration used was 0.2 mM of each primer (*ehx F* 1:5′ GCTATGGGCCTGTTCTCCTCTG3′ and *ehx R*: 5′ TGTCTTGCGTCATATCCATTCTCA′). These primers amplify a 1779 bp product in positive samples. Detection of *bfpA* gene was carried out by PCR as described earlier by cookson *et al*. (2002) except the *bfpF* (5′AAATCCTCCATGAAGCCAGT 3′) and *bfpR* primers (5′TTCTTTCCGACTGGGCGCAA3′) were used at a concentration of 0.1 mM each which amplifies a product of 1088 bp as a positive result.

Analysis of PCR Products

Amplified PCR products were analysed by gel electrophoresis in 0.5-2% agarose gel containing 0.5 µg/ml of ethidium bromide (Sambrook and Russel, 2001). The products were visualized with ultra violet illumination and photographed by Gel Documentation System (Ultracam Digital Imaging, Ultra Lum. Inc, Claremont, CA).

Statistical analysis

The data on prevalence of STEC and EPEC strains in Cattle and Sheep was analysed by χ^2 test.

RESULTS AND DISCUSSION

Prevalence of STEC and EPEC in cattle and sheep

Among 156 faecal samples screened, 133 were positive for *E. coli* with an overall occurrence of 85.25% percent. Among the rectal swab samples collected from cattle and sheep, the occurrence of *E. coli* was highest in cattle 87 (89.69%) than the rectal swab samples of sheep (76.96%). All the *E. coli* isolates from 133 faecal samples showed the presence of at least one of the four virulence genes. Eighty Nine of these isolates possessed *stx* gene(s) and thus were designated as STEC. Five (3.75%) STEC isolates possessed *ehx* gene also. Nine (6.76%) STEC isolates possessed also *eae* gene as well (Fig. 1). Eleven isolates harboured *eae* gene alone so were denoted as EPEC. Out of these, none of the isolates carried *bfpA* gene and all the eleven isolates were termed as atypical EPEC.

Occurrence of *eae gene* was highest 16 (18.39%) in cattle rectal swab than sheep rectal swab 4 (8.69%) and the difference was statistically significant. In this study, *ehx* gene was found to be associated more with cattle rectal swab 4(4.59%) than sheep rectal swab and 01 (2.17%) respectively.

Overall occurrence of typical STEC was observed to be 05(5.61%), associated more with cattle rectal swab 04 (6.45%) than rectal swab from sheep 01(3.70%). However, the overall occurrence of atypical STEC was recorded as 84 (94.38%), highest occurrence was noted in sheep rectal swab 26 (96.29%) than cattle rectal swab 58(93.54%). Atypical STEC observed were more 84 (94.38%) as compared to typical STEC (5.61%).

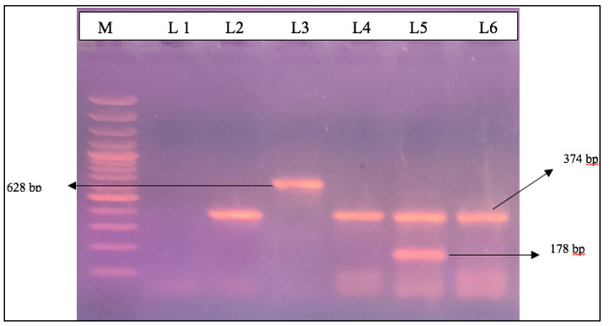

Fig. 1: Detection of *eae, stx*$_1$ and *stx*$_2$, *bfp* genes by Polymerase chain reaction

Lane M, 100 bp DNA ladder; **Lane 1,** Negative Control, **Lane 2,** positive sample for *stx2*,; **Lane 3,** positive sample for *eae*; **Lane 4,** positive sample for *stx2*; **Lane 5,** positive sample for *stx*$_1$ and *stx2*; Lane 6, positive for *stx*$_2$

The virulence gene profiling of 133 *E. coli* isolates from the rectal swab samples collected from cattle and sheep, revealed that 20 (15.03%) isolates positive for *eae* gene, 37 (27.81%) isolates for *Stx1*, 33 (24.81%) isolate for S*tx2* gene, 19(14.28%) isolates for both S*tx1* and S*tx2* genes and 5 (3.75%) isolates for *ehx* gene. However, *bfp* gene was not noted from any of the isolates.

Occurrence of *eae gene* was noted highest 16 (18.39%) in cattle rectal swab than sheep rectal swab 4 (8.69%) and the difference noted was statistically significant. *Stx1* gene was found more prevalent 21 (45.65%) in sheep rectal swab than the cattle rectal swab samples 16 (18.39%). The occurrence of *Stx2* was found more in cattle rectal swab 30 (34.48%) than sheep rectal swab 3 (6.15%). *ehx* gene was found to be associated more with cattle rectal swab 4(4.59%) than sheep rectal swab 01 (2.17%).

In the present study, EPEC was noted in 11 (7.05%), associated more with rectal swab of cattle 10 (10.30%) compared to sheep rectal swabs 01(1.69%). All the EPEC isolates were identified as atypical.

Among 20 (15.03%) *eae* positive isolates of *E.coli* recovered from animals, eae gene was found associated with 16 (18.39%) rectal swab of cattle and 4(8.69%) rectal swab of sheep. Upon intimin subtyping, *int-β* was detected in 03 (75%) and 04 (25%) while as *Int-γ* was detected in 01 (25%) and 10 (62.50%) of sheep and cattle rectal swabs respectively. Two *E. coli* isolate positive for *eae* gene form rectal swab of cattle could not be subtyped using the available set of primers.

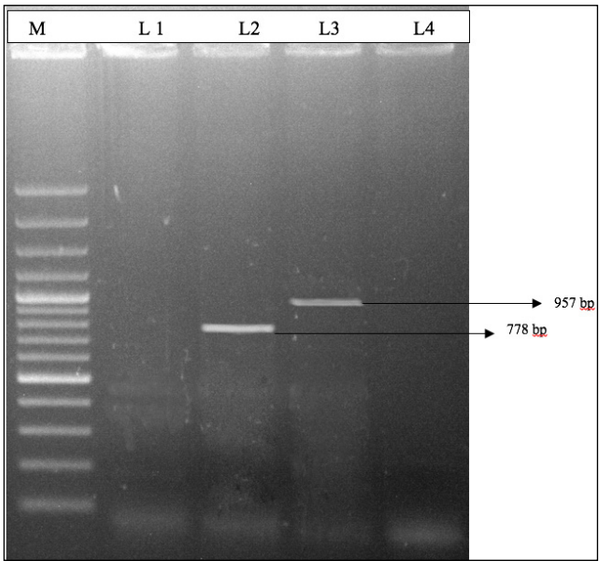

Fig. 2: Detection of int-β and Int-γ gene by Polymerase chain reaction. **Lane M,** 100 bp DNA ladder; **Lane 1,** Negative Control; **Lane 2,** positive sample for int-β; **Lane 3,** positive sample for Int-γ

The high occurrence of *E. coli* highlights that the importance of animals as being super-shedders which may be challenging, due to the fact that shedding may be highly variable even in the same animal.

Occurrence of STEC was found more 62 (63.91%) from cattle rectal swab samples as compared to 27 (45.76%) from sheep rectal swab. The finding is in agreement with Cerqueira *et al*. (2003), Joaquim *et al*. (2004), who reported the prevalence of STEC in healthy cattle in Brazil as high as 69% and Vettorato *et al*., 2003 who reported STEC in sheep in the range from 17.2% to 52.1%. Other studies with cattle reported an occurrence ranging from 16. 5% to 78.3% (Moreira *et al*., 2003; Farah *et al*., 2007; Pigatto *et al*., 2008; Salvadori *et al*., 2003; Ferreira *et al*., 2014,

Table 1: Primer equences used

Primer Name	Target gene	Primer sequence (5'-3')	Product Size
eaeAF(mid)	*eae*	TGCGGCACAACAGGCGA	628 bp
eaeAR(mid		CGGTCGCCGCACCAGGATTC	
stx1F	*Stx1*	GCCAGATGGAAGAGTCCGTGGGATTACGC	178 bp
stx1R		CACAATCAGGCGTCGCCAGCGCACTTGCT	
stx2F	*Stx2*	CCACATCGGTGTCTGTTATTAACCACACC	374 bp
stx2R		GCAGAACTGCTCTGGATGCATCTCTGGTC	

2018). However, our finding are contrary to Wani *et al.* (2003), Bhat *et al.* (2008) who recorded lower occurrence of STEC i.e. 9.73% in cattle and 17.5% from lambs.

The result of our study indicates that STEC colonization is widespread among cattle and sheep in Kashmir Valley. High occurrence of STEC in non diarrhoeic ruminants in present study clearly points out towards animals as being principal reservoirs of STEC which corroborates with the findings of Gyles, 2007; La Ragione *et al*., 2009.

Among ruminants, cattle (especially ruminating post-weaning calves and heifers) are considered to be the most important STEC reservoirs without symptomatic colonization (Caprioli *et al*., 2005; Gyles, 2007).

EPEC was found associated more with rectal swab of cattle 10 (10.30%) compared to sheep rectal swabs 01(1.69%). Several other researchers have reported EPEC prevalence close to these values. 5.8% and 5.9% in cattle feces from Netherland (Bolton *et al*., 2014) and from spain (Orden *et al*., 2003), respectively.

Low occurrence of EPEC from sheep (1.25%) was noted as comapred to cattle in the present study. Therefore, cattle might be possible reservoirs of EPEC. Isolation of atypical EPEC in the present work corroborated the findings of other workers (wani *et al*., 2007; Trabulsi *et al*., 2002; Nakazato *et al.*, 2004). This is in also in agreement with the study of Chandran and Mazumder, 2014 that showed humans are the only living reservoir of tEPEC.

Higher prevalence of *stx2* gene (34.48%) than *stx1* gene (18.39%) in cattle, in the present study, corroborates with the findings of Blanco *et al*. (2004) who reported predominance of *stx2* over *stx1* in bovines.

Similarly, *stx1* gene (45.65%) was predominant as compared *stx2* in sheep which corroborates with the findings of Martins *et al*. (2015). Detection of *stx1* in higher proportion in sheep in the present study may not be of any grave concern for the animal handlers as *stx1* was reported to be less intricately associated with dreadful human diseases like HUS (Bandyopadhyay *et al*., 2011).

Present study clearly shows that different STEC serotypes preferentially colonize healthy cattle and sheep, and that only a small percentage (approximately 12%) of these isolates possesses intimin. Although intimin probably plays an important role in tissue tropism, little is known about the effects of intimin sequence variation on cell adherence and host range. This finding corroborates with the observations of Beutin *et al*. (1993), Brett *et al.* (2003), Djordjevic *et al*. (2001).

This study indicates that the concurrent carriage of multiple *eae*-positive bacterial strains is not an unusual occurrence in cattle and sheep. However, the possible tissue tropism and specificity of adherence for the majority of *eae* subtypes remains to be established and their frequency of distribution may relate to the range of sites at which adherence may occur.

Our results show that the high diversity of STEC, EPEC strains circulating in the apparently healthy animals in the Kashmir valley harbors several virulence determinants related to moderate to severe illness in humans, and therefore, represents a risk for Public Health. Thus, preventive and control strategies should not be focused on detecting serogroups or serotypes, but instead, on detecting the molecular determinants of virulence.

CONCLUSION

In conclusion, this study revealed that though STEC and EPEC are prevalent in Cattle and Sheep. STEC with stx_1, stx_2 subtypes are prevalent in animals. Typical EPEC are prevalent in lambs and could be a source of infection to

human. The *int-β* and Int-γ could be common subtype of *eae* gene prevalent in Cattle and Sheep in India.

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