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The prevalence and drug resistance profile of Shiga-toxin producing (STEC), enteropathogenic (EPEC) and enterotoxigenic (ETEC) *Escherichia coli* in free ranging diarrheic and non-diarrheic yaks of West Kameng, Arunachal Pradesh, India

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ABSTRACT

Food producing animals are the major reservoirs of Shiga-toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC). The yak (*Poephagus grunniens*) is a unique multipurpose bovid, reared by highlanders in the Himalayan region. A total of 67 STEC, 5 EPEC and 22 ETEC strains were isolated from 256 rectal swabs of free ranging yaks from the West Kameng district of Arunachal Pradesh, India. Among the STEC isolates, shiga toxin producing genes *stx2* was predominant, followed by *stx1*. Of all the *stx* variants, *stx1c*, *stx2d, stx2c*, *stx2e* and *stx2f* were detected in 23, 11, 2 and 1 isolates, respectively. Further, genes such as *eaeA*, *ehxA, saa*, *iha* and *toxB* were detected in 16, 35, 28, 10 and 2 isolates, respectively. One of the EPEC isolates possessed a *bfpA* gene and was categorized as typical EPEC. Among the ETEC isolates, genes such as *LT*, *STa*, *STb*, *F41* and *K99* were detected in 14, 5, 13, 2 and 2 isolates, respectively. The majority of the STEC, EPEC and ETEC isolates exhibited multi-drug resistance. The study revealed the presence of multi-drug resistant diarrhoea genic *E. coli* in free ranging yaks from the West Kameng district of Arunachal Pradesh. Moreover, the presence of STEC/EPEC can be a potential public health risk for tribal highlanders residing in close proximity of the reservoir yaks.

Key words: enteropathogenic *E. coli*, enterotoxigenic *E. coli*, Shiga-toxin producing *E. coli*, yak, India

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Introduction

Food producing animals are major reservoirs of Shiga-toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC). Due to the carriage of these virulent pathogens, there is a strong possibility of contamination in animal borne food products, leading to fatal human ailments. On the other hand, enterotoxigenic *E. coli* (ETEC) are also responsible for significant economic loss of neonatal calves, lambs, and suckling piglets, with enormous morbidity and mortality.

The yak (*Poephagus grunniens*) is a unique multipurpose bovid reared by highlanders in the Himalayan terrain, such as in the Arunachal Pradesh and Sikim states of India, China, Nepal, Bhutan, Mongolia and Russia. Domestic yaks are economically important animals to nomadic pastoralists in high-altitude environments. In the recent past there have been a few reports of the occurrence of enterovirulent *E. coli* from livestock resources of North Eastern India, including Arunachal Pradesh (BARMAN et al., 2008; BANDYOPADHYAY et al., 2009; BANDYOPADHYAY et al., 2011; BANDYOPADHYAY et al., 2012; DUTTA et al., 2011; RAJKHOWA et al., 2010; RAJKHOWA and SARMA, 2014). However, such reports are meager considering the huge population of yaks in these regions. The occurrence of Shiga-toxin producing and Enteropathogenic *E. coli* in food products of yak origin is more worrisome, as tribal people often like to consume uncooked or undercooked food products (BANDYOPADHYAY et al., 2012a).

Considering the paucity of literature regarding the enteric pathogens in North-Eastern regions of India, the present study was undertaken to determine the virulence gene and drug resistance profile of enterovirulent *E. coli* isolated from free-ranging yaks in the West Kameng district of Arunachal Pradesh, India.

Materials and methods

The present study was carried out in the West Kameng district (91° 30' to 92° 40' East longitude and 26° 54' to 28° 01' North latitude) of Arunachal Pradesh, India. The district has a long international border with Bhutan to the west and Tibet to the north. A total of 256 rectal swabs were aseptically collected from the major yak tracts of Nyukmadung (138), Chander (58) and Mandlaphudung (60) and processed for the detection of diarrhea genic or enterovirulent *E. coli*. Out of 256 samples, 52 were collected from yaks with diarrhoea and 204 samples were collected from apparently healthy yaks.

Faecal samples were aseptically collected from the rectum by sterile cotton swabs (HiMedia, India) and were carried in transport medium to the laboratory on ice. Following incubation in *E. coli* broth (Hi Media, Mumbai) at 37 ^{0}C overnight, the supernatant was subjected to multiplex PCR for putative virulence markers characteristic for STEC, EPEC and enterotoxigenic *E. coli* (ETEC), such as Shiga-toxin gene(s) (*stx1*, *stx2*), intimin

(*eaeA*), enterohaemolysin (*ehxA*), STEC autoagglutinating adhesin (*saa)*, and heat labile enterotoxin (*LT*), heat stable enterotoxin (*STa* and *STb*).

Broth cultures positive for at least one virulence gene were streaked on MacConkey's agar (HiMedia, India) and incubated at 37 °C overnight. On the next day rose pink colonies were randomly picked and transferred to EMB agar (HiMedia, India) and again incubated overnight at 37 °C. The following day single colonies were streaked on nutrient agar (HiMedia, India) slant and subjected to biochemical confirmation.

For PCR-based detection of diarrhoea genic *E. coli* isolates, DNA was extracted by the previously described method (BANDYOPADHYAY et al., 2011).

Characterization of different virulence attributes of *E. coli* like shiga toxin-producing gene(s) (*stx1*, *stx2*), respective variants (*stx1c, stx2c*, *stx2d*, *stx2e* and *stx2f*) of *stx* genes, intimin (*eaeA*), enterohemolysin (*ehxA*), STEC autoagglutinating adhesion (*saa*), enteroaggregative *E. coli* heat-stable enterotoxin 1 (*EAST1*), type III secreted protein encoded on LEE island (*toxB*), adherence conferring protein (*iha*), bundle forming pilli (*bfpA*), EHEC factor for adherence (*efa1*), catalase-peroxidase (*katP*), type II secretion protein (*etpD*) and specific virulence factors of ETEC such as heat stable enterotoxins (*STa*, *STb*) and heat labile enterotoxin (*LT*), colonization factors (*F5* and *F41*) were carried out as described earlier (BANDYOPADHYAY et al., 2009; BANDYOPADHYAY et al., 2012; BANDYOPADHYAY et al., 2011).

'O' antigen of all the positive isolates was determined at the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kausali, Himachal Pradesh, India.

Drug susceptibility of these isolates was tested by disk diffusion method, following the recommendations of the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008) using the commercially available disks (Himedia, India).

Results

Out of 256 samples collected, 68 and 18 samples were positive for characteristic virulence genes for STEC/EPEC and ETEC, respectively, in the initial screening. Finally, 67 STEC (67/256, 26.1 %), 5 EPEC (5/256, 1.9 %) and 22 ETEC (22/256, 8.5 %) strains were obtained (Table 1). Individual screening of STEC/EPEC samples revealed that the *stx2* gene was the most prevalent gene, being present in 60 isolates (60/67, 89.5 %) followed by *stx1* in 54 isolates (54/67, 80.5 %) only. The *stx* variants such as *stx1c, stx2c*, *stx2d*, *stx2e* and *stx2f* were detected in 30 (30/67, 44.7 %), 11 (11/67, 16.4 %), 23 (23/67, 34.3 %), 2 (2/67, 2.9 %), 1 (1/67, 1.4 %) isolates, respectively. The gene intimin (*eaeA*) was detected in 16 isolates, of which 5 were enteropathogenic strains (EPEC) possessing the *eaeA* gene alone. One enteropathogenic strain was typical, being positive for the *bfpA* gene. Other gene(s) such as *ehxA* and *saa* were quite predominant, detected in 35 and 28 isolates respectively. The adhesin gene(s), such as *iha*, *toxB,* were detected in 10 and

2 isolates, respectively. EAST1 was detected in 9 isolates only (Table 1). None of the isolates was positive for *efa1*, *etpD* and *katP*.

Table 1. Virulence characteristics of the STEC, EPEC and ETEC strains isolated from yaks of West Kameng district, Arunachal Pradesh

Sl. No.	Virulence gene profile	Total positive isolate(s)	Serotype(s)
Shiga-toxin producing *E. coli* (STEC)			
1.	*stx_1 stx_{2d} ehxA*	3	O10, O22, O76,
2.	*stx_{1c} stx_2 ehxA*	1	O140
3.	*stx_{1c} stx_{2c} ehxA, EAST1*	1	O60
4.	*stx_{1c} stx_{2d} ehxA*	2	O2, O59
5.	*stx_{1c} stx_{2d} ehxA, EAST1*	2	O110, OUT
6.	*stx_{1c} stx_{2d} ehxA, iha*	2	O87, O91
7.	*stx_1 stx_2 ehxA saa, EAST1*	1	O163
8.	*stx_1 stx_{2c} ehxA saa*	2	O2, O60
9.	*stx_{1c} stx_2 ehxA saa, iha*	2	O60, O44
10.	*stx_{1c} stx_{2d} ehxA saa*	4	O21, O105, O158, OR
11.	*stx_1 stx_2*	1	O61,
12.	*stx_1 stx_{2d}*	3	O22, O85, O105
13.	*stx_{1c} stx_2*	2	O25, O69
14.	*stx_{1c} stx_{2d}*	4	O4, O110, O120, OUT
15.	*stx_1 stx_2 saa EAST1*	2	O85(2)
16.	*stx_{1c} stx_2 saa*	2	O21, O132,
17.	*stx_{1c} stx_{2c} saa*	3	O118, O120(2)
18.	*stx_{1c} stx_{2e} saa, EAST1*	2	O110, OR
19.	*stx_{1c} stx_{2f} saa*	1	O22
20.	*stx_1*	2	O110, O84
21.	*stx_2*	1	O13
22.	*stx_{2c}*	2	O85, O166,
23.	*stx_2 saa*	1	O97
24.	*stx_{2c} saa*	2	O85, O110
25.	*stx_{2c} ehx_A saa*	1	OUT
26.	*stx_2 ehx_A*	2	O158, O79
27.	*stx_1 ehxA saa EAST1*	1	O85
28.	*stx_2 ehxA saa*	1	O76
29.	*stx_{1c} ehxA saa, iha*	2	O91, O113
30.	*stx_2 ehxA saa iha*	1	O44
31.	*stx_1 eae*	2	O158, OUT

Table 1. Virulence characteristics of the STEC, EPEC and ETEC strains isolated from yaks of West Kameng district, Arunachal Pradesh (continued)

32.	*stx_2 eaeA*	1	OUT
33.	*stx_1 stx_2 eaeA*	1	O20
34.	*stx_1 stx_{2d} eaeA ehxA*	3	OUT, O158(2)
35.	*stx_2 eaeA, toxB, iha*	1	O111
36.	*stx_1 stx_2 eaeA ehxA*	2	O76, O110
37.	*stx_1 stx_2 eaeA, toxB, iha*	1	O26
Enteropathogenic *E. coli* (EPEC)			
1.	*eaeA*	2	O25, O88
2.	*eaeA, bfpA*	1	OUT
3.	*eaeA, ehxA, iha*	2	O6, OUT
Enterotoxigenic *E. coli* (ETEC)			
1.	*LT, STb*	3	OUT, O115, O149
2.	*STb, K99, F41*	1	O141
3.	*LT*	3	O20, O64, OUT
4.	*LT, EAST1*	2	O119, O147
5.	*STa*	1	O101
6.	*STa, EAST1*	2	O47, O107
7.	*STb*	2	O18, O116
8.	*LT, STa*	1	OUT
9.	*LT, ST_b, EAST1, F41, K99*	3	O8, O138, O147
10.	*LT, STb, EAST1*	1	OUT
11.	*STb, EAST1*	2	O93, O98
12.	*LT, STa, STb*	1	O9

The STEC and EPEC isolates were distributed among 33 sero-groups and rough (R) and untypeable (UT). The serogroups are O2, O4, O6, O10, O13, O21, O22, O25, O26, O44, O59, O60, O61, O69, O76, O79, O84, O85, O87, O88, O91, O97, O105, O110, O111, O113, O118, O120, O132, O140, O158, O163, and O166. Whereas, the ETEC isolates belonged to 14 sero-groups such as O8, O18, O20, O47, O64, O101, O107, O115, O116, O119, O138, O141, O147, O149 and three were untypeable.

Among the various enterotoxin gene(s) investigated, *LT*, *STa* and *STb* were detected in 14 (14/22, 63.6 %), 5 (5/22, 22.7 %) and 13 (13/22, 59 %) of the ETEC strains recovered from yaks of the West Kameng district. Other fimbrial adhesin gene(s), *F41* and *K99* were detected in 4 and 3 isolates, respectively. However, the *EAST1* gene was detected in 10 ETEC isolates (Table 1).

The majority of the STEC, EPEC and ETEC isolates exhibited resistance to the antibiotics furazolidone, nalidixic acid, nitrofurantoin, chloramphenicol, gentamicin, erythromycin, and streptomycin. Some of the isolates were also resistant to clavulanic acid potentiated amoxicillin, fluoroquinolones derivatives, third or fourth generation cephalosporin, and macrolides.

Discussion

There is a paucity of literature regarding the occurrence of enterovirulent *E. coli* in yaks from the North Eastern Indian states. The present finding strongly demonstrates again the fact that yaks are significant reservoirs of STEC and EPEC. The study reflected that pathogenic *E. coli* are distributed in wide sero-groups, many of which were previously reported in yaks (BANDYOPADHYAY et al., 2009; BANDYOPADHYAY et al., 2012, BANDYOPADHYAY et al., 2012a) and sheep (BANDYOPADHYAY et al., 2011) in India. Some of the serogroups identified in the present study were also observed in yaks in neighbouring countries such as China (BAI et al., 2013). Moreover, the isolated serogroups O2, O9, O103, O104, O113, O117 O146 and O172 in the present study are associated with fatal human diseases such as haemorrhagic diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (PRADEL et al., 2008; ISLAM et al., 2010).

In the present study, 26.1 % and 1.9 % of the rectal swab samples collected from yaks were positive for STEC and EPEC, respectively. Similarly, an earlier study also detected 28.76 % STEC in captive yak maintained at an institutional farm in the West Kameng district of Arunachal Pardesh, India (BANDYOPADHYAY et al., 2009). However, in another study conducted among free range yaks in the Tawang district of Arunachal Pradesh, India, the occurrence of STEC and EPEC was 15.39 % and 2.2 %, respectively (BANDYOPADHYAY et al., 2012), indicating the existence of variations in occurrence rates between the districts. In total, *stx2* was detected in a higher number of isolates than *stx1*, which is in support of the previous observations in yaks in India (BANDYOPADHYAY et al., 2009; BANDYOPADHYAY et al., 2012) and China (BAI et al., 2013). The higher frequency of *stx2* positive isolates may be a possible public health concern due to its correlation with HUS in humans (ISLAM et al., 2010).

Further characterization of the *stx1* positive isolates produced 55.56 % *stx1c* positive isolates, which is also in corroboration with the earlier study (BANDYOPADHYAY et al., 2012). Of all the *stx2* variants, *stx2d* was predominant, being detected in 23 isolates, followed by *stx2c*, *stx2e* and *stx2f* in 11, 2 and 1 isolates, respectively, which is also consistent with an earlier study of yaks (BANDYOPADHYAY et al., 2012).

The intimin gene (*eaeA*) was detected in 16 (22.22 %) isolates, and five of them harboured *eaeA* genes alone without the *stx* gene(s), hence they are considered as EPEC. The *eaeA* gene was reported crucial for the full virulence of EPEC as it confers upon

bacterial strains the ability to adhere to the intestinal epithelia, producing characteristic attaching and effacing (A/E) lesions. One of the EPEC isolates possessed the *bfpA* gene and was categorized as typical EPEC; the remaining four were atypical EPEC (aEPEC). Previously, typical and atypical EPEC have been reported from sheep (WANI et al., 2009), calves (WANI et al., 2007), and yaks (BANDYOPADHYAY et al., 2012) in India. Typical EPEC strains, the leading cause of infantile diarrhoea in developing countries, possess EAF (EPEC adherence factor) plasmid, which produces bundle forming pillus (encoded by *bfpA* gene) to mediate localized adherence on a cultured cell line, like human enterocytes.

Other associated virulence factors, such as *ehxA, saa*, *iha* and *toxB,* were detected in 35, 28, 10 and 2 isolates, respectively. None of the *saa* positive isolates carried the *eaeA* gene, but many of them were positive for the *ehxA* gene. The observation confirmed our previous findings in yak and sheep that the gene *saa* was present exclusively in LEE negative STEC strains (BANDYOPADHYAY et al., 2012; BANDYOPADHYAY et al., 2011). The precise role of *ehxA* and *saa* in human diseases is not known so far. However, several STEC diseases such as HUS have been recorded in *eaeA* negative and *ehxA* positive isolates, or *saa* positive isolates (JENKINS et al., 2003). The gene *iha* was found to be quite predominant, being detected in 11 isolates and with a strong correlation with the enterohemolysin (*ehxA*) gene (in 9 isolates). This putative adhesion marker was widely reported among the STEC strains isolated from yaks (BANDYOPADHYAY et al., 2012) and from beef samples in the United States (BOSILEVAC and KOOHMARAIE, 2011). The gene *toxB* was detected in two EPEC isolates only. This putative virulence factor was first described on the pO157 plasmid to accentuate the production of type III secreted protein encoded on LEE Island. Thus it was generally presumed to present only in LEE positive isolates and be expressed concomitantly with the *eaeA* gene.

In the present study 8.5 % of the rectal swab samples collected from yaks were positive for ETEC, whereas a slightly higher prevalence (9.8 %) of ETEC was observed in our previous study in free ranging yaks from the nearby Tawang district of Arunachal Pradesh, India (BANDYOPADHYAY et al., 2012). A similar form of serogroup distribution in ETEC isolates was also previously observed in earlier studies in yak and sheep (BANDYOPADHYAY et al., 2012; BANDYOPADHYAY et al., 2011). Most of the ETEC isolates recovered from yaks of the West Kameng district were *LT* and *STb* producers. This was in contrast to earlier observations in yaks (Tawang district) and water buffalo in India, where most of the ETEC isolates were found *STa* positive (BANDYOPADHYAY et al., 2012; MAHANTI et al., 2014). Fimbrial adhesins such as *K99* and *F41* were detected in 18.18 % and 13.64 % ETEC isolates, respectively. A higher prevalence of these fimbrial adhesins was reported in our previous studies in yak and water buffalo (BANDYOPADHYAY et al., 2012; MAHANTI et al., 2014). These are the proteinaceous appendages that helped the bacteria to adhere to the microvilli of the small intestinal epithelial cells (SAMANTA et al., 2013).

The *EAST1* gene was detected in seven ETEC (31.82 %) and nine STEC (13.43 %) isolates. However, the earlier study in free ranging yaks from the Tawang district detected a higher occurrence of the *EAST1* gene (44.4 %) in ETEC and a lower occurrence of the *EAST1* gene (7.14 %) in STEC isolates (BANDYOPADHYAY et al., 2012).

The antimicrobial resistance feature as reflected by these isolates is in corroboration with the previous observations in yaks from the Tawang district (BANDYOPADHYAY et al., 2012), which suggests that multi-drug resistance has become common and dominant among the ETEC isolates from yaks of the Arunachal Pradesh, India.

The present study reflected that yaks from the West Kameng district, Arunachal Pradesh, India are a potential reservoir of enterovirulent *E. coli* such as STEC, EPEC and ETEC. The presence of STEC and EPEC is a critical public health concern for the nomadic tribal highlanders, who share the same premises with the yaks for their daily life. Again, the presence of ETEC may be an important but undeniable factor in the recurrent occurrence of fatal hemorrhagic diarrhea in yaks, with high morbidity and mortality. Most of the isolates were multi-drug resistant, which is again crucial as the resistance feature may be transferred to other flora through e mobile genetic transfer elements.

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BANDYOPADHYAY, S., A. MAHANTI, C. LODH, I. SAMANTA, T. K. BISWAS, T. K. DUTTA, K. K. BARUAH, D. BHATTACHARYA: Prevalencija i otpornost na lijekove šiga-toksigenih (STEC), enteropatogenih (EPEC) i enterotoksigenih (ETEC) izolata bakterije *Escherichia coli* u slobodno držanih jakova s proljevom i bez proljeva u pokrajini West Kameng, Arunachal Pradesh, Indija. Vet. arhiv 85, 501-510, 2015.

SAŽETAK

Životinje koje služe za proizvodnju hrane mogu biti rezervoar šiga-toksigenih (STEC) i enteropatogenih (EPEC) sojeva bakterije *Escherichia coli*. Jak (*Poephagus grunniens*) je jedinstveni multipari bovid kojeg uzgajaju gorštaci na Himalaji. Ukupno je bilo izdvojeno 67 STEC sojeva, 5 EPEC sojeva i 22 ETEC soja iz 256 rektalnih obrisaka slobodno držanih jakova na području West Kameng, Arunachal Pradesh, Indija. Među izolatima STEC prevladavao je gen *stx2* za prizvodnju šiga-toksina, a slijedio ga je gen *stx1*. Od svih varijanata gena *stx*, *stx1c* bio je dokazan u 23 izolata, *stx2d* u 11 izolata, *stx2c* u dva izolata, a *stx2e* i *stx2f* u jednog izolata. Nadalje gen *eaeA* dokazan je u 16 izolata, gen *ehxA* u 35, gen *saa* u 28, gen *iha* u 10 te gen *toxBu* dva izolata. Jedan od izolata EPEC posjedovao je gen *bfpA* i svrstan je u tipične EPEC izolate. Gen *LT* dokazan je u 14 ETEC izolata, gen *Sta* u pet, gen *STb* u 13, gen *F41* u dva i *K99* također u dva izolata. Većina izolata STEC, EPEC i ETEC pokazivala je otpornost na više lijekova. Istraživanje je pokazalo da postoji višestruka otpornost bakterije *E. coli* izdvojene iz slobodno držanih jakova s proljevom na području West Kameng, Arunachal Pradesh. Povrh toga, prisutnost STEC/EPEC može predstavljati rizik za javno zdravstvo za gorštačka plemena koja dolaze u bliski dodir s jakovima.

Ključne riječi: enteropatogena *E. coli*, enterotoksigena *E. coli*, šiga-toksin, Indija, jak