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Characterization of shiga toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC) in raw yak (*Poephagus grunniens*) milk and milk products

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ABSTRACT

Thirty-one shiga toxin-producing (STEC) and 6 enteropathogenic *Escherichia coli* (EPEC) were isolated from 87 raw yak milk and 63 'churpi' samples. Of 18 stx_1 positive isolates (48.6%), 14 carried stx_{1c} (77.7%). Subtyping of 28 stx_2 positive isolates (75.7%) revealed the presence of stx_{2c} (9, 32.1%), stx_{2d} (3, 10.7%), stx_{2e} (1, 3.57%) and stx_{2f} (3, 10.7%) variants. Furthermore, intimin (*eaeA*), enterohaemolysin (*ehxA*), autoagglutinating adhesin (*saa*), *iha* (adherence conferring protein), *efa1* (EHEC factor for adherence), bundle forming pilli (*bfpA*) and *toxB* (type III secreted protein encoded on LEE Island, similar to toxin B of *Clostridium difficile*) genes were detected in 14, 16, 12, 4, 3, 2 and 2 isolates, respectively. Univariate and multivariate analysis depicted that both stx_1 and stx_2 or their variants were more likely to occur in isolates from Arunachal Pradesh ($p < 0.04$) rather than Sikkim. Dendogram constructed on the basis of RAPD and ERIC PCR profile distributed the STEC and EPEC isolates in separate clusters irrespective of their sources and serotypes. The STEC and EPEC isolates exhibited resistance against erythromycin, amikacin, azithromycin, amoxicillin, ampicillin + cloxacillin, cephalothin, furazolidone, gentamicin, kanamycin, streptomycin and tetracycline. This is the first ever report on occurrence and characterization of STEC and EPEC isolated from yak milk and milk products.

1. Introduction

Shiga-toxin producing (STEC) and enteropathogenic *Escherichia coli* (EPEC) are serologically diverse, emerging food borne pathogens and leading cause for a spectrum of human illness ranging from haemorrhagic diarrhea to even fatal consequences such as hemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpurea (TTP) and haemorrhagic colitis (HC) (Croxen and Finlay, 2010; Gyles and Fairbrother, 2010). Although, the production of shiga toxins and intimin constitutes a touchstone in virulence of STEC and EPEC they may carry additional plasmid-borne colonization factors – *iha* (adherence-conferring protein that is similar to Vibrio cholera *IrgA*), *saa* (an autoaglutinating adhesin), *bfpA* (bundle forming pilli) and *toxB* (type III secreted protein encoded on LEE Island, similar to toxin B of *Clostridium difficile*) mediating their strong adherence to the intestinal mucosa and prevent their elimination by peristalsis (Islam et al., 2008; Wani et al., 2009; Bosilevac and Koohmaraie, 2011). Furthermore, they may carry virulence factors like *ehxA* (enterohemolysin), *etpD* (a novel gene cluster) and *katP* (bifunctional catalase peroxidase). Food producing animals are the major reservoirs of STEC and EPEC. Recurrent outbreaks of life-threatening human infections were attributed to STEC/EPEC contaminated milk and milk products (Pradel et al., 2008; Dadie et al., 2010; Martin and Beutin, 2011). Recently, yak (*Poephagus grunniens*) was determined as a natural source of STEC (Bandyopadhyay et al., 2009). The tribal highlanders and nomadic yak herdsmen (brokpas) often consume undercooked or raw meat, milk and milk products like 'churpi' (dried and smoked hard cheese of yak milk). In India, the occurrence of STEC and EPEC from animal products is poorly investigated. Taken together, the present study was undertaken to find out the occurrence, virulent gene(s) profile, molecular characterization and drug resistance pattern of shiga-toxin producing (STEC) and enteropathogenic (EPEC) *E. coli* in milk and 'churpi' of yak.

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2. Materials and methods

2.1. Collection of samples and isolation of E. coli

The investigation was carried out in four major yak inhabited districts of Arunachal Pradesh (West Kameg, Tawang) and Sikkim (East and North district), India, where about 19,000 yaks are reared under transhumance system of migration by the brokpas of these regions. A total of 87 raw milk (20 ml each) and 63 'churpi' samples (50 g each) were collected for the present study. Single animal milk samples were collected from brokpas and 'churpi' samples from the local markets. Collected samples were stored in sterile vials containing transport medium and carried to the laboratory in ice cold condition. Samples were incubated overnight at 37 °C in EC broth (HiMedia, Mumbai, India) and subjected to multiplex polymerase chain reaction (m-PCR) for putative virulence markers characteristic for STEC or EPEC like shiga-toxin producing gene(s) (*stx*$_1$, *stx*$_2$), intimin (*eaeA*), enterohaemolysin (*ehxA*) and STEC autoagglutinating adhesin (*saa*) (Bandyopadhyay et al., 2011). Details regarding PCR condition and primers used are described in Table 1. Broth cultures positive for at least one virulence gene were further processed for isolation of *E. coli* as previously described (Bandyopadhyay et al., 2008, 2011) and subjected to standard morphological and bio-chemical tests (Holt et al., 1994).

2.2. Detection of different virulence gene(s) and colonization factors using PCR

Again, m-PCR was carried out for all the individual isolates as stated above. All the *stx*$_1$ and *stx*$_2$ positive isolates were screened for the presence of their respective variants (*stx*$_{1c}$: *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$ and *stx*$_{2f}$) (García-Aljaro et al., 2005; Vu-Khac and Cornick, 2008). The presence of plasmid mediated additional virulent factors like *toxB*, *iha*, *bfpA, efa1, katp* and *etpD* genes was investigated by PCR (Table 1) as earlier reported (García-Aljaro et al., 2005; Islam et al., 2008; Pradel et al., 2008; Wani et al., 2009). A human O157:H7 strain (STEC3/VTEC3) harboring the above-mentioned genes and a K12 *E. coli* strain were used as positive and negative controls, respectively. PCR products were analyzed by gel electrophoresis in 1–2% agarose containing ethidium bromide (0.5 μg ml^{-1}) as per the standard protocol.

2.3. Serogrouping

'O' antigen of the isolates harboring characteristic virulence gene(s) for STEC or EPEC was determined in National Salmonella and Escherichia Centre, Central Research Institute, Kausali, Himachal Pradesh, India.

2.4. Antimicrobial susceptibility test

Drug susceptibility of these isolates was carried out by disc diffusion method following the recommendation of Clinical and Laboratory Standards Institute guidelines (CLSI, 2008) using the commercial discs (Hi Media, Mumbai, India). The antibiotics used were chloramphenicol (25 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), gentamicin (10 μg), neomycin (30 μg), norfloxacin (10 μg), streptomycin (30 μg), oxytetracycline (30 μg), cephalothin (30 μg), amikacin (30 μg), ceftazidime (30 μg), kanamycin (30 μg), ceftriaxone (30 μg), levofloxacin (5 μg), amoxicillin + clavulanic acid (20 + 10 μg), cefaclor (30 μg), cefuroxime (30 μg), azithromycin (30 μg), piperacillin + tazobactam (100 + 10 μg), cefepime + tazobactam (30 + 10 μg), ampicillin + cloxacillin (10 μg), enrofloxacin (10 μg), amoxicillin (25 μg), erythromycin (10 μg), furazolidone (50 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), tetracycline (30 μg), cephataxime (30 μg), doxycycline hydrochloride (300 μg) and pefloxacin (5 μg).

2.5. Molecular characterization of the virulent isolates

Molecular typing of all the isolates carrying characteristic virulence markers was performed by RAPD (Randomly Amplified Polymorphic DNA) and ERIC (Enterobacterial Repetitive Intergenic Consensus) PCR (Barman et al., 2008; Prabhu et al., 2010). DNA fingerprinting thus obtained from RAPD and ERIC PCR was analyzed visually in a gel documentation system. Furthermore, the banding information of both the RAPD and ERIC PCR was coded as 1 (band present) and 0 (band absent). The binary data obtained from both the tools were merged together for statistical analysis by Squared Euclidean Distance (SED) using the software SPSS 16.0 to plot a dendogram displaying clonal relationship among the isolates.

2.6. Statistical analyses

All statistical analyses were performed in Epi Info 2010, SPSS 16.0 and Win Episcope 2.0. The frequency of enterovirulent isolates in two geographical areas (AP and Sikkim) and sources (milk and churpi) of sampling was determined with exact 95% confidence interval (CI) and compared by Fisher's exact test. Accordingly, odds ratio (OD) and relative risk (RR) were determined at 95% CI. Univariate and multivariate logistic regression analyses were performed to find association between common virulent genes (*stx*$_1$, *stx*$_2$ and their variants, *eaeA*, *ehxA*, *saa* as dichotomous dependent variable) and sampling areas as well as source (as independent variables). Other genes like *bfpA, toxB* and *iha* were excluded from analysis because of their low frequency. Odds ratios (ORs) with corresponding 95% CIs were derived from the models to determine the strength of association between the variables. Level of significance was determined by likelihood ratio test. A $p < 0.05$ was considered statistically significant for the study.

3. Results

Of 87 milk and 63 'churpi' samples tested, 23 milk (22.2%, 95% CI: 12.2, 34.5) and 14 churpi (25.3%, 95% CI: 16.5, 35.7) samples were positive for at least one of the virulent gene(s) targeted in the initial m-PCR (OR: 1.25, 95% CI: 0.6, 2.6; RR: 1.19 95% CI: 0.6, 2.1). Area wise comparison revealed that 28 samples from Arunachal Pradesh (28.5%, 95% CI: 20, 39) and 9 from Sikkim (17.3%, 95% CI: 8.2, 30) yielded positive result in the m-PCR (OR: 1.9, 95% CI: 0.8, 4.6; RR: 1.65, 95% CI: 0.8, 3.23). A total of 37 samples positive for *E. coli* were isolated carrying the virulence attributes in different combination investigated in the present study. Of them 31 isolates were STEC and remaining six were EPEC. Thirty-one STEC isolates belonged to 22 divergent serotypes namely, O2, O5, O8, O11, O17, O22, O28, O43, O44, O54, O60, O61, O91, O92, O104, O113, O138, O141, O146, O147, O159, O172 and one was untypable. Remaining six EPEC isolates were distributed within five different serotypes – O13, O97, O104, O113 and O146 while one was untypable. However, no isolate belonged to the serogroup O157. The virulence gene(s) profile of all the 37 isolates characterized in the study is illustrated in Table 2. Three isolates possessed *stx*$_1$ gene but not *stx*$_2$ and 13 carried *stx*$_2$ but not *stx*$_1$. Fifteen isolates produced positive amplicon for both *stx*$_1$ and *stx*$_2$. In total, *stx*$_1$ was present in 18 and *stx*$_2$ in 28 isolates. The *stx* variants like *stx*$_{1c}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$ and *stx*$_{2f}$ were present in 14, 9, 3, 1 and 3 isolates, respectively. Additional plasmid mediated putative virulence factors – *eaeA*, *ehxA*, *saa, iha, toxB* and *efa1* genes were detected in 14, 16, 12, 4, 2 and 3 isolates, respectively. Two EPEC isolates were