Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. (July–Sept 2013) 83(3):423–429
DOI 10.1007/s40011-012-0145-7

RESEARCH ARTICLE

Prevalence, Serogroups, Shiga-toxin Genes and Pulsed Field Gel Electrophoresis Analyses of *Escherichia coli* Isolated from Bovine Milk

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Received: 12 September 2012 / Revised: 1 November 2012 / Accepted: 6 December 2012 / Published online: 3 January 2013

Abstract Shiga toxin-producing *Escherichia coli* (STEC) including non-O157 strains have been linked to outbreaks and sporadic cases of illness worldwide. A total of 647 milk samples were collected at different levels of collection and processing (udder, milking utensils, milk collection centres and receiving dock) within West Coast region of India. The milk samples were screened for the presence of *E. coli* and further tested for the Shiga-toxin (*stx*) genes by PCR. The isolates were characterized for their serogroups and *Xba*I digestion patterns of total DNA separated by pulsed-field gel electrophoresis (PFGE). A total of 77 (11.90 %) isolates were confirmed as having *E. coli*. The serogroups reported were O4, O60, O112, O56, O159, O120, O2, O83, O88, O95, O141, O21, O25, O80, O140, O97, O24, O166, O146, O51, O169, O147, O103, O18, O100, O15, O69, O43, O7, O3, O45, O124, O110, O84, and O114. Out of the 77 *E. coli* isolates, 25 (32.46 %) could be classified as Shiga-toxigenic based on PCR results. Of these 11, 3 and 11 isolates were positive for *stx*1, *stx*2, and both *stx*1 and *stx*2, respectively. PFGE profiles indicated genetic diversity of *E. coli* strains. Much variation was observed among isolates recovered at different levels of collection. Further research is needed to uncover unique characteristics and resistance of non-O157 STEC strains.

Keywords Shiga toxin-producing *Escherichia coli* · Milk · Shiga-toxin gene · Pulsed-field gel electrophoresis · Serogroups

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Introduction

Milk and milk products may be a pool of a variety of microorganisms and can be important source of foodborne pathogens. Foodborne pathogens can gain entrance in milk due to direct contact with contaminated sources in the dairy farm environment and through an infected udder [1]. Milk also gets contaminated at various stages of production and processing including the animal, milker, extraneous dirt or unclean process water [2, 3]. Therefore, contamination of raw milk with foodborne pathogens either directly or indirectly poses the risk of ingestion and transmission of foodborne pathogens.

The presence of the pathogenic bacteria in milk is of considerable public health concern, particularly for individuals drinking raw milk [4]. The true incidence of milkborne disease remains obscure. A number of microorganisms including *Escherichia coli* can contaminate milk and milk products. *E. coli* inhabits the intestinal tract of animals, humans and birds. Though most *E. coli* are harmless, some are known to be pathogenic, causing both severe intestinal and extra-intestinal diseases in human beings [5, 6]. Pathogenic *E. coli* have been classified into

different pathotypes that cause a common disease using common and assortments of virulence factors [6]. The Shiga toxin-producing *E. coli* (STEC) causes severe clinical syndromes in humans such as haemorrhagic colitis and haemolytic uremic syndrome [7]. The virulence factors of STEC are the cytotoxins, Shiga-toxins 1 and 2 (encoded by the *stx*1 and *stx*2 genes, respectively) and the protein intimin (encoded by the chromosomal gene *eae*) responsible for the intimate attachment of the bacteria to intestinal epithelial cells and the enterohaemolysin, encoded by the *ehx*A gene [7]. In 2006, 19 outbreaks in the European Union have been linked to STEC, in which 111 persons with 42 hospitalizations were involved [8]. Earlier studies also documented the presence of some *E. coli* from raw milk and products possessing virulence markers [9–11]. Recent outbreak of *E. coli* O104:H4 in more than 14 European countries has caused 42 deaths and illness of more than 4,000 people [12]. Schaffzin et al. [13] summarized two outbreaks caused by non-O157 STEC in USA. These reports highlight the ability of non-O157 STEC to cause outbreaks and call for concerted efforts for their effective control.

Guaranteeing a greater food safety level for consumer products warrants an integrated approach to control food safety throughout the food chain [14, 15]. A number of regulations have been developed and introduced to assure food safety at different stages of the food production chain. Given many potential and emerging hazards along the chain, it is of practical importance to prioritize attributes. Though dairy products are deemed to be one of the safest classes of food, there is considerable concern about the possibility of hazards from dairy products to affect a large number of consumers [15].

Livestock production including dairy plays a multipurpose role in the agriculture systems of India. Dairy plays a dynamic role in India's agro-based economy. Today, India ranks the first in the world in terms of milk production. In India, limited information is available regarding the STEC in animals including cattle [16], sheep [17], fish [18], beef [19] and human faeces [19]. The objective of the study was to screen milk samples for the presence of *E. coli* and to further test for Shiga-toxin (*stx*) genes by PCR. The isolates were also characterized for their serogroups and *Xba*I digestion patterns of total DNA separated by pulsed-field gel electrophoresis (PFGE).

Material and Methods

Samples

A total of 647 milk samples from dairy cows were collected at different levels of collection and processing (udder, milking utensils, milk collection centres and receiving dock) within West Coast region, India during 2006–2010. All the samples were collected aseptically, transported to the laboratory under chilled conditions and processed for microbiological analysis within 24 h of collection.

Isolation and Identification

The samples were plated on MacConkey's agar (HiMedia, Mumbai, India) plates and incubated at 37 °C for 18–24 h. Pure and a single population of bacterial colonies were recorded. Five randomly selected colonies from MacConkey's agar were picked up and subcultured on eosin methylene blue (EMB) agar (HiMedia, Mumbai, India) plates to observe the characteristic metallic sheen of *E. coli*. The well separated pure colonies were picked up on nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests [20].

Detection of Virulence Genes by PCR

The singleplex PCR was carried out using two sets of oligonucleotide primers for the *stx*1 and *stx*2 genes. The PCR primers for *stx*1 and *stx*2 genes were previously described by Cebula et al. [21]. The primers used to amplify the *stx*1 gene 5′-CAGTTAATGTGGTGGCGAAG G-3′ and 5′-CACCAGACAATGTAACCGCTG-3′and the *stx*2: 5′-ATCCTATTCCCGGGAGTTTACG-3′ and 5′-GC GTCATCGTATACACAGGAGE-3′ were synthesized by Sigma Aldrich, USA. The expected size for PCR-amplified products was 348 bp for the *stx*1 and 584 bp for the *stx*2. In brief, the genomic DNA of all the isolates was extracted using bacterial DNA extraction kit (Chromous Biotech, Bangalore, India). The PCR mixture of 25.0 µl contained 10× PCR buffer, 1.5 mM of $MgCl_2$, each primer within the two primer sets at a concentration of 40 nM, 200 µM each of dNTPs, 1.0 U of *Taq* DNA polymerase and 2.0 µl of template DNA. The PCR reaction was performed in a thermal cycler (Eppendorf, Germany). The samples were initially denatured at 94 °C for 3 min and then subjected to 35 cycles, each consisting denaturing for 1 min at 94 °C, annealing at 64 °C for 1.5 min and extension at 72 °C for 2 min. A final extension step was done for 10 min at 72 °C. The fragments were separated on 1.2 % agarose gel followed by ethidium bromide staining. Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system (Alpha Imager, USA). Both positive and negative controls were used in PCR experiments. *E. coli* MTCC 723 obtained from Institute of Microbial Technology, Chandigarh, India was used as control which was positive for the *stx*1 and *stx*2 genes.

Serotyping

Escherichia coli cultures were referred to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India, for sero-grouping. The isolates were serogrouped for 'O' antigen.

Pulsed-Field Gel Electrophoresis

Genomic DNA fingerprints of the STEC isolates were further determined using PFGE according to a standard protocol developed by PulseNet for *E. coli* O157:H7 [22]. Briefly, agarose-embedded DNA was digested with 50 U of *Xba*I for 3 h in a water bath at 37 °C. DNA fragments were separated by electrophoresis in 0.5× Tris–borate–EDTA buffer at 14 °C for 18 h on a CHEF-II Mapper electrophoresis system (Bio-Rad Laboratories), with a pulse time of 2.2–54.2 s at constant voltage of 6 V/cm. A PFGE Marker I (New England Biolabs) was included in each agarose run. The generated PFGE patterns were analyzed using the Gel Compare II (Applied Maths) software. The pattern clustering was performed by the unweighted-pair group algorithm and the Dice correlation coefficient with a tolerance of 1 %. The results of the clustering analysis were confirmed by visual comparison of the PFGE profiles. A similarity coefficient of 80 % was selected to define the pulsed-field type clusters.

Results and Discussion

Milk may get contaminated with *E. coli* during different stages of production and processing. Therefore, the present work was intended to study the occurrence of *E. coli* in the milk. The bacteriological examination of milk samples ($n = 647$) revealed the presence of *E. coli* in 77 (11.90 %) samples. Biochemically, the isolates were identified as *E. coli*. Maximum isolates were detected at market level followed by the receiving dock. In earlier studies [23], *E. coli* was isolated from 33 (66 %) of the 50 raw milk and product samples tested. A study in Pakistan also reported heavy contamination (57 %) of the raw milk and products by *E. coli* [24].

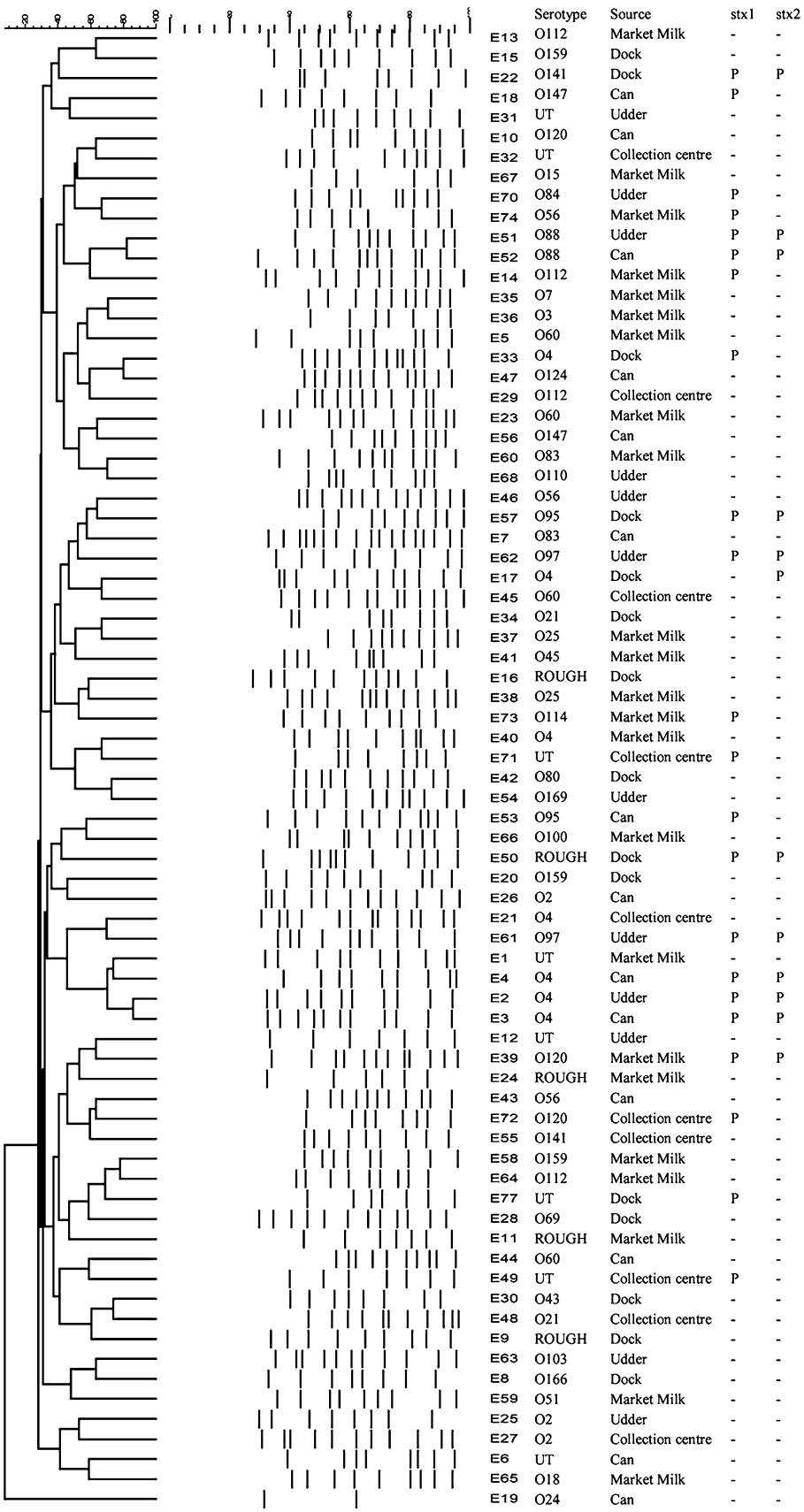
Of the 77 *E. coli* isolates, 64 were typeable and belonged to 35 different O-serogroups (O2, O3, O4, O7, O15, O18, O21, O24, O25, O43, O45, O51, O56, O60, O69, O80, O83, O84, O88, O95, O97, O100, O103, O110, O112, O114, O120, O124, O140, O141, O146, O147, O159, O166, O169). Eight isolates were considered as O untypeable (UT) and five as rough strains. In the present study, a total of thirty-five different serogroups of *E. coli* were isolated, of which O4 was the predominant. Previous studies in India [25] showed the predominance of O9, O8, O60 and O25 in animal cases. Mishra et al. [26] found O2, O19, O20 and O78 as predominant serogroups amongst the *E. coli* isolates from 250 clinical specimens from poultry in India. Sanath Kumar et al. [18] reported the presence of STEC in seafood of serogroups other than that of O157. These findings indicate the variable distribution of different serogroups of *E. coli* in different geographical regions in India. Studies in USA reported that *E. coli* O26, O45, O103, O111, O113, O121, O145, and O157 have been the most commonly identified O-serogroups associated with STEC implicated in outbreaks of human illness all over the world [27]. Zweifel et al. [28] detected a notable proportion of non-O157 STEC serogroups associated with human infections in semi-hard and hard raw milk cheese. In another study, about 77.4 % of the isolates form mastitic milk belonged to four different O-serogroups (O26, O86, O111, and O127) [29]. In the context of lack of data on the occurrence of STEC in milk from India, the study is of significance. Studies in India have confirmed that cattle are the principal reservoir of non-O157 serogroups [19].

In this study, PCR assay yielded amplified products of ~384 and ~584 bp specific for the *stx*1 and *stx*2 genes, respectively. Of 77 *E. coli* strains recovered, 25 (32.46 %) had one or more of the genes responsible for virulence of *E. coli* (Table 1). PCR assay revealed that 11/77 (14.29 %) of the isolates carried the *stx*1 gene alone, 3/77 (3.89 %) possessed the *stx*2 alone and 11/77 (14.29 %) carried both the *stx*1 and *stx*2 genes.

In this study, the *stx*1 gene was the predominant *stx* type (28.57 %), in contrast to previous studies in cattle and humans [30, 31] and dairy cattle [22, 32]. It has been demonstrated that the *stx*2 gene is associated with high virulence in humans [22, 33]. Characterization of STEC isolated from Swiss raw milk cheese revealed thirteen of the 24 strains typeable with O antisera belonging to the serogroups O2, O22, and O91 and the *stx*2 (86 %) was more prevalent than the *stx*1 gene (46 %) [34]. On the contrary, Carneiro et al. [35] could not detect *stx*1 and *stx*2 genes from strains isolated from *E. coli* isolated from

Table 1 Virulence profiles for STEC isolates recovered from milk samples

STEC strains origin	Isolates	Genes		
		*stx*1 No. (%)	*stx*2 No. (%)	*stx*1 + *stx*2 No. (%)
Udder	13	1 (7.69)	1 (7.69)	4 (30.76)
Can	14	2 (14.28)	–	3 (21.42)
Milk collection centre	11	2 (18.18)	1 (9.09)	–
Receiving dock	16	3 (18.75)	1 (6.25)	3 (18.75)
Market milk	23	3 (13.04)	–	1 (4.34)
Total	77	11 (14.28)	3 (3.89)	11 (14.28)



	Serotype	Source	stx1	stx2
E13	O112	Market Milk	-	-
E15	O159	Dock	-	-
E22	O141	Dock	P	P
E18	O147	Can	P	-
E31	UT	Udder	-	-
E10	O120	Can	-	-
E32	UT	Collection centre	-	-
E67	O15	Market Milk	-	-
E70	O84	Udder	P	-
E74	O56	Market Milk	P	-
E51	O88	Udder	P	P
E52	O88	Can	P	P
E14	O112	Market Milk	P	-
E35	O7	Market Milk	-	-
E36	O3	Market Milk	-	-
E5	O60	Market Milk	-	-
E33	O4	Dock	P	-
E47	O124	Can	-	-
E29	O112	Collection centre	-	-
E23	O60	Market Milk	-	-
E56	O147	Can	-	-
E60	O83	Market Milk	-	-
E68	O110	Udder	-	-
E46	O56	Udder	-	-
E57	O95	Dock	P	P
E7	O83	Can	-	-
E62	O97	Udder	P	P
E17	O4	Dock	-	P
E45	O60	Collection centre	-	-
E34	O21	Dock	-	-
E37	O25	Market Milk	-	-
E41	O45	Market Milk	-	-
E16	ROUGH	Dock	-	-
E38	O25	Market Milk	-	-
E73	O114	Market Milk	P	-
E40	O4	Market Milk	-	-
E71	UT	Collection centre	P	-
E42	O80	Dock	-	-
E54	O169	Udder	-	-
E53	O95	Can	P	-
E66	O100	Market Milk	-	-
E50	ROUGH	Dock	P	P
E20	O159	Dock	-	-
E26	O2	Can	-	-
E21	O4	Collection centre	-	-
E61	O97	Udder	P	P
E1	UT	Market Milk	-	-
E4	O4	Can	P	P
E2	O4	Udder	P	P
E3	O4	Can	P	P
E12	UT	Udder	-	-
E39	O120	Market Milk	P	P
E24	ROUGH	Market Milk	-	-
E43	O56	Can	-	-
E72	O120	Collection centre	P	-
E55	O141	Collection centre	-	-
E58	O159	Market Milk	-	-
E64	O112	Market Milk	-	-
E77	UT	Dock	P	-
E28	O69	Dock	-	-
E11	ROUGH	Market Milk	-	-
E44	O60	Can	-	-
E49	UT	Collection centre	P	-
E30	O43	Dock	-	-
E48	O21	Collection centre	-	-
E9	ROUGH	Dock	-	-
E63	O103	Udder	-	-
E8	O166	Dock	-	-
E59	O51	Market Milk	-	-
E25	O2	Udder	-	-
E27	O2	Collection centre	-	-
E6	UT	Can	-	-
E65	O18	Market Milk	-	-
E19	O24	Can	-	-

Fig. 1 Dendrogram of the 74 *E. coli* strains based on PFGE patterns after digestion with enzyme *Xba*I

pasteurized milk. The *stx*1 and *stx*2 genes were detected in 3 and 6.1 %, respectively of *E. coli* strains isolated from raw milk samples in Saudi Arabia [23]. Montenegro et al. [36] reported that most of the STEC isolates of bovine origin encoded for *stx*1 gene. In another study, of the 16 strains detected from raw milk cheese samples, 11 were typed into 7 *E. coli* O groups (O2, O15, O22, O91, O109, O113, O174), whereas 5 strains were nontypeable and the *stx*1 and *stx*2 variants were detected in 1 and 15 strains, respectively [13]. Osman et al. [29] investigated non-O157 isolates for the *stx* genes, however, none was *stx* positive. STEC O83 has been reported to be associated with human illness [37] raising the possibility that O83 might be transmitted to humans via milk products. Since not all STEC strains are equally pathogenic to humans, evaluation of virulence-associated factors is necessary to assess the potential of individual isolate to cause human illness.

Large foodborne outbreaks have been linked to STEC and some of which have been linked to dairy products [22]. Many factors, such as geographical location and sampling, isolation, and testing methods, make comparisons of different studies difficult [34]. There is a paucity of data on contamination by STEC in raw milk and there is growing concern over the emergence of highly virulent non-O157 STEC serogroups that are globally distributed, several of which are associated with outbreaks and/or severe human illness, such as HUS and HC [37, 38].

Pulsed-field gel electrophoresis of *Xba*I-digested DNA fragments isolated from the 74 *E. coli* isolates generated 69 unrelated *Xba*I-PFGE subtypes at 80 % similarity (Fig. 1). Pulsed field electrophoresis with *Xba*I endonuclease is currently considered as being a highly discriminatory method for molecular typing of STEC. The isolates belonging to the same serogroups, differed in PFGE profiles and *stx* genotypes. Isolates of the same serogroups tended to cluster together, however, polymorphism among the isolates of the same serogroup was also observed, according to different PFGE patterns. All other isolates had their own specific PFGE profiles, with similarity indices ranging from 40 to 90 %. In a study [22] PFGE of *Xba*I-digested DNA fragments isolated from the 118 STEC isolates generated 74 unrelated *Xba*I-PFGE subtypes. PFGE demonstrated a wide diversity of STEC strains isolated at different levels of collection. This diversity may be linked to the different factors such the handling, utensils used, new bovines and environmental contamination.

The non-O157 STEC serogroups described in this study are associated worldwide with diseases in human beings and represent a risk for the public health [27]. For this, any microbiological control in dairy farms should be targeted not only to the search of O157:H7 serotype. Consumption of contaminated food has been considered as the main route of STEC infections in humans [39]. STEC strains have been isolated from a large variety of different foods including raw-milk cheeses [7, 40, 41].

The morbidity and mortality associated with several large outbreaks of gastrointestinal diseases caused by Shiga toxin-producing *Escherichia coli* (STEC) indicate the threat of these organisms to public health [42]. These are commonly recovered from food animals and were found responsible for severe gastrointestinal and systemic diseases such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) leading to diarrhoea, especially among the infants in the developing countries [42, 43]. Although, in India, reports are available on isolation, identification and characterization of STEC in human and animals [17–20, 25, 44, 45], there appears no information on association of STEC in raw milk. Detection of STEC in raw milk in the present study is probably the first report in India.

In conclusion, the findings provide the information about the involvement of STEC in raw milk in India. In India, a much larger segment of the population, particularly the rural population, consumes raw unpasteurized milk directly and indirectly. The fact that the serogroups reported from raw milk samples were common to that reported from animal populations suggested that the source of contamination of milk might be from animals. Once entered into the dairy food processing plants, the foodborne pathogens can lead to persistence through formation of biofilms, subsequently contaminating the processed milk products and exposure of consumers to pathogenic bacteria [1]. Furthermore, recontamination of dairy products may occur with pathogens such as *E. coli* which survive and thrive in post-pasteurization processing. Under these circumstances, unpasteurized dairy products as well as dairy products that become re-contaminated after pasteurization with foodborne pathogens pose a risk to the consumer.

The isolation and diversity of STEC serogroups found in this study have confirmed that raw milk can be an important reservoir of STEC. The serogroups carrying genes related to human diseases suggest a risk to the population. This should be taken into account in the control and preventive measures to minimize the risk of STEC foodborne infection in humans.

Acknowledgments The authors are thankful to the Director, ICAR Research Complex for Goa, Old Goa for providing necessary facilities. DD is working under Faculty Improvement Programme of University Grants Commission, New Delhi.

References

1. Oliver SP, Jayarao BM, Almeida RA (2005) Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathog Dis 2:115–129
2. Bonfoh B, Wasem A, Traore AN, Fane A, Spillmann H, Simb CF, Alfaroukh IO, Nicolet J, Farah Z, Zinsstag J (2003)

Microbiological quality of cow's milk taken at different intervals from the udder to the selling point in Bamako (Mali). Food Control 14:495–500
3. Hayes MC, Ralyea RD, Murphy SC, Carey NR, Scarlett JM, Boor KJ (2001) Identification and characterization of elevated microbial counts in bulk tank raw milk. J Dairy Sci 84:292–298
4. Ryser ET (1998) Public health concerns. In: Marth EH, Steele JL (eds) Applied dairy microbiology. Marcel Dekker Inc., New York, pp 263–403
5. Fairbrother JM, Nadeau E (2006) *Escherichia coli*: on-farm contamination of animals. Rev Sci Tech 25:555–569
6. Kaper JB, Nataro JP, Mobley HLT (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140
7. Solomakos N, Govaris A, Angelidis AS, Pournaras S, Burriel AR, Kritas SK, Papageorgiou DK (2009) Occurrence, virulence genes and antibiotic resistance of *Escherichia coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiol 26:865–871
8. European Food Safety Authority (EFSA) (2007) The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2006. EFSA J 130:1–352
9. Holko I, Bisova T, Holkova Z, Kmet V (2006) Virulence markers of *Escherichia coli* strains isolated from traditional cheeses made from unpasteurised sheep milk in Slovakia. Food Control 17: 393–396
10. Jayarao BM, Henning DR (2001) Prevalence of foodborne pathogens in bulk tank milk. J Dairy Sci 84:2157–2162
11. Paneto BR, Schocken-Iturrino RP, Macedo C, Santo E, Marin JM (2007) Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil. Arquivo Brasileiro de Medicina Vet Zootec 59:508–512
12. WHO (2011) The enterohaemorrhagic *Escherichia coli* (EHEC) outbreak. http://www.euro.who.int/en/what-we-do/health-topics/emergencies/international-health-regulations/ehec-outbreak-in-germany. Accessed 10 June 2012
13. Schaffzin JK, Coronado F, Dumas NB, Root TP, Halse TA, Schoonmaker-Bopp DJ, Lurie MM, Nicholas D, Gerzonich B, Johnson GS, Wallace BJ, Musser KA (2011) Public health approach to detection of non-O157 Shiga toxin-producing *Escherichia coli*: summary of two outbreaks and laboratory procedures. Epidemiol Infect 140:283–289
14. Stefan G (1997) Food safety issues affecting the dairy beef industry. J Dairy Sci 80:3458–3462
15. Valeeva NI, Meuwissen MP, Lansink AG, Huirne RB (2005) Improving food safety within the dairy chain: an application of conjoint analysis. J Dairy Sci 88:1601–1612
16. Pal A, Ghosh S, Ramamurthy T, Yamasaki S, Tsukamoto T, Bhattacharya SK, Nair GB, Takeda Y (1999) Shiga-toxin producing *Escherichia coli* from healthy cattle in a semi urban community in Calcutta, India. Indian J Med Res 110:83–85
17. Bhat MA, Nishikawa Y, Wani SA (2008) Prevalence and virulence gene profiles of Shiga toxin—producing *Escherichia coli* and enteropathogenic *Escherichia coli* from diarrhoeic and healthy lambs in India. Small Rumin Res 75:65–70
18. Sanath Kumar H, Otta SK, Karunasagar I, Karunasagar I (2001) Detection of Shiga-toxigenic *Escherichia coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR. Lett Appl Microbiol 33:334–338
19. Khan A, Yamasaki S, Sato T, Ramamurthy T, Pal A, Datta S, Chowdhury NR, Das SC, Sikdar A, Tsukamoto T, Bhattacharya SK, Takeda Y, Nair GB (2002) Prevalence and genetic profiling of virulence determinants of non-O157 Shiga-toxin producing *Escherichia coli* isolated from cattle, beef and humans, Calcutta, India. Emerg Infect Dis 8:54–62
20. Chattopadhayay UK, Dutta S, Deb A, Pal D (2001) Verotoxin producing *Escherichia coli*—an environment induced emerging zoonosis in and around Calcutta. Int J Environ Health Res 1:107–112
21. Cebula TA, Payne WL, Feng P (1995) Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. J Clin Microbiol 33:248–250
22. Fremaux B, Raynaud S, Beutin L, Rozand CV (2006) Dissemination and persistence of Shiga toxin-producing *Escherichia coli* (STEC) strains on French dairy farms. Vet Microbiol 117: 180–191
23. Altalhi AD, Hassan SA (2009) Bacterial quality of raw milk investigated by *Escherichia coli* and isolates analysis for specific virulence-gene markers. Food Control 20:913–917
24. Soomro AH, Arain MA, Khaskheli M, Bhutto B (2002) Isolation of *Escherichia coli* from milk and milk products in relation to public health sold under market conditions at Tandojam. Pak J Nutr 1:151–152
25. Wani SA, Samanta I, Bhat MA, Nishikawa Y (2004) Investigation of Shiga toxin-producing *Escherichia coli* in avian species in India. Lett Appl Microbiol 39:389–394
26. Mishra A, Sharda R, Chhabra D, Moghe MN (2002) *Escherichia coli* isolates from domestic poultry. Indian J Anim Sci 72: 727–729
27. DebRoy C, Roberts E, Valadez AM, Dudley EG, Cutter CN (2011) Detection of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the *wzx* gene of the O-antigen gene cluster. Foodborne Pathog Dis 8:651–652
28. Zweifel C, Giezendanner N, Corti S, Krause G, Beutin L, Danuser J, Stephan R (2010) Characteristics of Shiga toxin-producing *Escherichia coli* isolated from Swiss raw milk cheese within a 3-year monitoring program. J Food Prot 73:88–91
29. Osman KM, Mustafa AM, Aly MA, AbdElhamed GS (2012) Serotypes, virulence genes, and intimin types of Shiga toxin-producing *Escherichia coli* and enteropathogenic *Escherichia coli* isolated from mastitic milk relevant to human health in Egypt. Vector Borne Zoonotic Dis 12:297–305
30. Padola NL, Sanz ME, Blanco JE, Blanco M, Blanco J, Etcheverria AI, Arroyo GH, Usera MA, Parma AE (2004) Serotypes and virulence genes of bovine Shiga-toxigenic *Escherichia coli* (STEC) isolated from a feedlot in Argentina. Vet Microbiol 100:3–9
31. Rivas M, Miliwebsky E, Chinen I, Roldán CD, Balbi L, García B, Fiorilli G, Sosa-Estani S, Kincaid J, Rangel J, Griffin PM, Case-Control Study Group (2006) The case-control study group 2006 characterization and epidemiologic subtyping of Shiga toxing producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina. Foodborne Pathog Dis 3:88–96
32. Irino K, Kato MA, Vaz TM, Ramos II, Souza MA, Cruz AS, Gomes TA, Vieira MA, Guth BE (2005) Serotypes and virulence markers of Shiga toxin-producing *Escherichia coli* (STEC) isolated from dairy cattle in Sao Paulo State. Braz Vet Microbiol 105:29–36
33. Rasooly R, Do PM (2010) Shiga toxin *Stx*2 is heat stable and not inactivated by pasteurization. Int J Food Microbiol 136:290–294
34. Xia X, Meng J, McDermott PF, Ayers S, Blickenstaff K, Tran TT, Abbott J, Zheng J, Zhao S (2010) Presence and characterization of Shiga toxin-producing *Escherichia coli* and other potentially diarrheagenic *E. coli* strains in retail meats. Appl Environ Microbiol 76:1709–1717
35. Carneiro LA, Lins MC, Garcia FR, Silva AP, Mauller PM, Alves GB, Rosa AC, Andrade JR, Freitas-Almeida AC, Queiroz ML (2006) Phenotypic and genotypic characterization of *Escherichia coli* strains serogrouped as enteropathogenic *E. coli* (EPEC) isolated from pasteurised milk. Int J Food Microbiol 108:15–21

36. Montenegro MA, Ite MB, Trumpf T, Aleksic S, Reuter G, Bulling E, Helmuth R (1990) Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. J Clin Microbiol 28:1417–1421
37. Bettelheim KA (2007) The non-O157 Shiga-toxigenic (verocytotoxigenic) *Escherichia coli*: under-rated pathogens. Crit Rev Microbiol 33:67–87
38. Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. J Infect Dis 192:1422–1429
39. Meng J, Doyle MP, Zhao T, Zhao S (2007) Enterohemorrhagic *Escherichia coli*. In: Doyle MP, Beuchat LR (eds) Food microbiology: fundamentals and frontiers, 3rd edn. ASM Press, Washington, DC
40. Caro I, Garcia-Armesto MR (2007) Occurrence of Shiga toxin producing *Escherichia coli* in a Spanish raw ewe's milk cheese. Int J Food Microbiol 116:410–413
41. Stephan R, Schumacher S, Corti S, Krause G, Danuser J, Beutin L (2008) Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* in Swiss raw milk cheeses collected at producer level. J Dairy Sci 91:2561–2565
42. Paton AW, Paton JC (1998) Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohaemorrhagic *E. coli hlyA*, *rfbO111* and *rfbO157*. J Clin Microbiol 36:598–602
43. Narato JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201
44. Farooq S, Hussain I, Mir MA, Bhat MA, Wani SA (2009) Isolation of atypical enteropathogenic *Escherichia coli* and Shiga toxin1 and 2f-producing *Escherichia coli* from avian species in India. Lett Appl Microbiol 48:692–697
45. Dutta TK, Roychoudhury P, Bandyopadhyay S, Wani SA, Hussain I (2011) Detection and characterization of Shiga toxin producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) in poultry birds with diarrhoea. Indian J Med Res 133:541–545