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RESEARCH ARTICLE
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Isolation of pathogenic *Escherichia coli* from stool samples of diarrhoeal patients with history of raw milk consumption

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Abstract

Aim: To detect the occurrence of pathogenic *Escherichia coli* from stool samples of diarrhoeal patients with history of raw milk consumption and to determine the public health significance of isolates, especially their role in causing human diseases.

Materials and Methods: A total of 100 stool samples from diarrhoeal patients, with history of raw milk consumption were collected from primary health centres in and around Anand city, under aseptic conditions and a total of 50 raw milk samples were collected from milk vendors, retail shops located in Anand city in sterilized sample bottles. MacConkey broth was used for the enrichment of all the samples and inoculation was done on MacConkey agar and EMB agar was used as the selective media. This was followed by the confirmation of isolates using biochemical tests. For the serotyping, *E. coli* isolates were sent to the National Salmonella and Escherichia Centre, Central Research Institute (CRI), Kasauli, Himachal Pradesh. Detection of virulence genes was performed using PCR technique.

Results: During the present investigation, 26 (52%) *E. coli* isolates from 50 milk samples and 59 (59%) *E. coli* isolates from 100 stool samples were recovered. Out of 85 *E. coli* isolates sent for serotyping, 74 isolates could be typed which were further distributed into 13 different serogroups O2, O4, O8, O17, O22, O25, O29, O36, O45, O60, O90, O116 and O172, whereas 8 isolates were found untypable and 3 isolates were reported rough isolates. Of the 59 *E. coli* isolates from stool samples of diarrhoeal patients tested, 15 isolates (25.42%) were reported to be positive for *stx* genes, among that 6 (10.16%) were positive for *stx1* gene, 9 (15.25%) isolates were positive for *stx2* gene, while 3 isolates (5.08%) were positive for *eaeA* gene. In this study, 21 *E. coli* isolates were found to be Shiga toxin producing *E. coli* (STEC) while none of the isolates were positive for the serotype O157.

Conclusions: Our present findings indicate that raw milk may act as a source of pathogenic *E. coli* and it may be responsible for the occurrence of diarrhoea and various other health-related complications in humans. We therefore recommend proper managemental practices and effective control measures for improved hygiene and sanitation.

Keywords: diarrhoea, *E. coli*, milk, serotype, shiga toxin producing *E. coli* , stools

Introduction

Escherichia coli is a facultative anaerobe, Gram negative and belongs to the family Enterobacteriaceae, and is usually a commensal organism [1]. Entero-pathogenic *E. coli* which affect humans are categorised into six groups: Shiga toxin producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC); entero toxigenic *E. coli* (ETEC); entero invasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) [2].

STEC is a group of food borne pathogenic microorganisms linked to a broad range of human diseases, ranging from diarrhoea to hemorrhagic colitis (HC), thrombocytopenia, hemolytic uremic syndrome (HUS), and can also lead to human mortality [3]. STEC represents the only pathogenic group of *E. coli* having a zoonotic significance [4]. Shiga toxins are the major virulence factors contributing to pathogenicity of the organism, and they consist of two types of toxins; stx_1 and stx_2. STEC may be responsible for various disease outbreaks across the world. In Italy, an outbreak of *E. coli* O26 was reported during 2005, which occurred upon consumption of buffalo milk products [5]. The outbreak of haemolytic uremic syndrome was caused by O145:H28 and O26:H11 which was associated with consumption of ice-cream prepared from pasteurised milk [6].

In view of the above facts, the current study was undertaken to detect the occurrence of pathogenic *E. coli* from stool samples of diarrhoeal patients with history of raw milk consumption but not from those who consumed boiled milk, collected from primary health centres in and around Anand city.

Materials and Methods

Sample collection: From September 2012 to February 2013, a total of 50 raw milk samples (50 ml) were collected from milk vendors, retail shops located in Anand city in sterilized sample bottles and a total of 100 stool samples from diarrhoeal patients with history

Table-1. Details of primers used for PCR reaction

Sr. No.	Target genes	Primer sequence (5'→3')	Product Size (bp)	Reference
1.	*stx1*	F:ACACTGGATGATCTCAGTGG		
		R:CGTAATCCCCCTCCATTATG	614	[9]
2.	*stx2*	F:CCATGACAACGGACAGCAGTT		
		R:CCTGTCAACTGAGCAGCACTTTG	779	[9]
3.	*eaeA*	F:GACCCGGCAACAAGCATAAGC		
		R:CCACCTGCAGCAACAAGAGG	384	[9]
4.	*rfbO157*	F:AAGATTGCGCTGAAGCCTTTG		
		R:CATTGGCATCGTGTGGAC	497	[10]

(F= Forward primer; R = Reverse primer)

of raw milk or milk product consumption were collected from primary health centres in and around Anand city. Samples from patients were collected preferably before the initiation of antimicrobial therapy using multipurpose sample collecting bottles and were brought to the Post Graduate Research Laboratory of the Veterinary Public Health department in an ice box for further processing and microbiological analysis.

Isolation and identification of *E. coli:* Samples were processed to isolate *E. coli* as described by the Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration (USFDA)[7]. The samples were inoculated into MacConkey broth for enrichment at 37°C for 24 hrs, the enrichments were streaked on MacConkey agar and incubated for 24 hrs at 37ºC. Pink coloured colonies were sub cultured on Eosin Methelene Blue (EMB) agar. Colonies producing greenish metallic sheen on EMB agar were considered as having *E. coli.* In addition, various biochemical tests were done for the confirmation of *E. coli* as proposed by Edward and Ewing [8].

Serotyping of *E. coli* isolates: *E. coli* isolates recovered from milk and stool samples were serotyped based on their somatic (O) antigens at the National Salmonella and Escherchia Centre (NSEC), Central Research Institute (CRI), Kasauli, H. P., India.

DNA isolation: DNA was isolated from *E. coli* by using boiling method [9]. Approximately a loopful of culture was mixed with 100 µl of sterilized DNAse and RNAse free water in a micro centrifuge tube. This was followed by denaturation at 95ºC for 10 min using the thermal cycler (Applied Biosystems, Sweden). Finally, cellular debris was removed by centrifugation (10000 rpm for 5 min) and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture.

Polymerase chain reaction (PCR): Screening of all the *E. coli* isolates was done to detect the presence of virulence associated genes using the PCR technique. The PCR was standardized for the detection of stx_1, stx_2, *eaeA* and *rfb*O157 following the methods described by Osman et al. [10] for detection of stx_1,stx_2 *and eaeA* genes and methods described by Dhanashee and Mallaya [11] for detection of *rfbO157* with suitable modifications (Table-1). Standardization of PCR was done using standard reference strain of *E. coli* O157:H7 and EPEC. The reactions were performed in a thermal cycler (Applied Biosystems, Sweden) with pre-heated lid (Lid temp. 105ºC). For the confirmation of targeted PCR amplification, 1 µl of 6X gel loading buffer along with 5 µl of the PCR product was electrophoresed along with DNA molecular weight marker (Gene Ruler, MBI Fermentas). Agarose gel (2%) along with ethidium bromide (at the rate of 0.5 µg/ml) was used. Electrophoresis was performed in 0.5X Tris Borate EDTA buffer at 5V/cm for 60 min. Visualization of amplified product was done under ultraviolet light and was recorded using gel documentation system (SynGene, Gene Genius BioImaging System, UK).

Results

Prevalence of *E. coli*: During the present investigation, 26 (52%) *E. coli* isolates from 50 milk samples and 59 (59%) *E. coli* isolates from 100 stool samples from diarrhoeal patients with history of milk consumption were recovered.

Serotyping of *E. coli* isolates: Out of 85 *E. coli* isolates sent for serotyping, 74 isolates could be typed which were distributed into 13 different serogroups, whereas 8 isolates were found untypable and 3 isolates were reported to be rough (Table-2). Among the 42 isolates, the different serogroups detected in the descending order were 16 isolates (18.82%) of O90; 12 isolates (14.11%) each of O60 and O4; 9 isolates (10.58%) each of O2 and O116; 7 isolates (8.23%) of O8; 3 isolates (3.52%) of O25; 1 isolate (1.17%) each of O17, O22, O29, O36, O45 and O172.

Detection of virulence genes: In present study, out of 85 *E. coli* isolates from milk and stool samples tested, 21 isolates (24.70%) were reported to be positive for *stx* genes, among them 8 (9.41%) were positive for stx_1 gene, 13 (15.29%) isolates were positive for stx_2 gene, while 4 isolates (4.70%) were positive for *eaeA* gene. In this study, 21 *E. coli* isolates belong to STEC, while all the isolates were negative for serotype O157 (Table- 3).

Discussion

Raw milk is a good source of various pathogenic microorganisms. Various pathogenic bacteria can grow and survive in the raw milk. A multitude of factors are responsible for the occurrence of *E. coli* in milk. Contamination of milk with *E. coli* may occur due to unhygienic conditions, poor management, improper storage conditions and improper processing of milk.

Table-2. Serotype of *E. coli* isolates

Sr. No.	'O' Serogroup	No. of *E. coli* isolated from		Total
		Milk samples	Stool samples	
1	O2	3	6	9
2	O4	4	8	12
3	O8	3	4	7
4	O17	0	1	1
5	O22	1	0	1
6	O25	1	2	3
7	O29	0	1	1
8	O36	0	1	1
9	O45	1	0	1
10	O60	5	7	12
11	O90	6	10	16
12	O116	1	8	9
13	O172	0	1	1
14	UT	1	7	8
15	R	0	3	3
Total		26	59	85

Table-3. PCR amplification of virulence genes

Sr. No.	Virulence genes	*E. coli* isolates positive from	
		Milk samples	Stool samples
1	*stx1*	2 (7.69%)	6 (10.16%)
2	*stx2*	4 (15.38%)	9 (15.25%)
3	*eaeA*	1 (3.84%)	3 (5.08%)
4	*rfbO157*	0 (0.00%)	0 (0.00%)

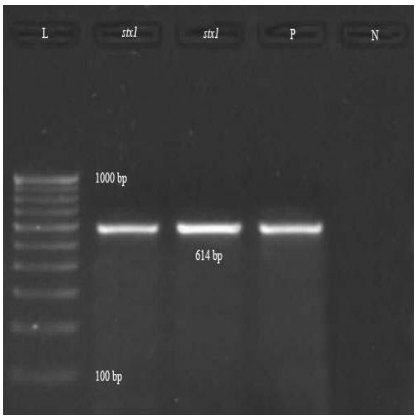

Figure-1. Agarose gel showing PCR amplified product (614 bp) for *stx1* gene in *E. coli* isolates. P: Positive control, N: Negative control, L: DNA Ladder, *stx1:* Positive samples

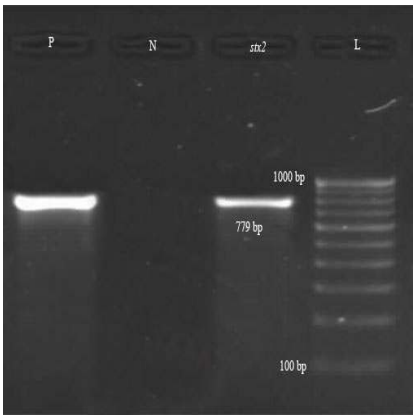

Figure-2. Agarose gel showing PCR amplified product (779 bp) for *stx2* gene in *E. coli* isolates. P: Positive control, N: Negative control, L: DNA Ladder, *stx2:* Positive sample

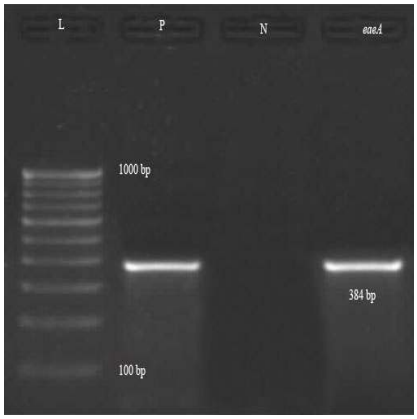

Figure-3. Agarose gel showing PCR amplified product (384 bp) for *eaeA* gene in *E. coli* isolates. P: Positive control, N: Negative control, L: DNA Ladder, *eaeA:* Positive sample

Unhygienic conditions during storage and supply of milk for human consumption may be responsible for the occurrence of pathogenic *E. coli* in milk. Consumption of contaminated milk by humans may produce pathogenic conditions like diarrhoea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) etc.

In present study, of the 59 *E. coli* isolates from stool samples of diarrhoeal patients tested, 15 isolates (25.42%) were positive for *stx* genes, among them 6 (10.16%) were positive for stx_1 gene, 9 (15.25%) isolates were positive for stx_2 gene, while 3 isolates (5.08%) were positive for *eaeA* gene. During the present investigation, the stool samples were collected from diarrhoeal patients with history of raw milk consumption, and the occurrence of *E. coli* in stool samples may be due to the consumption of raw milk contaminated with *E. coli*. In the present study, the virulence genes reported from raw milk were also recovered from stool samples which indicate that raw milk may be responsible for the occurrence of virulence genes of *E. coli* in humans. The report of virulence genes suggest that prevalence of STEC in raw milk may be involved in outbreaks of human HC and HUS and is of significant importance from the viewpoint of public health and hygiene.

Among bacterial infections *E. coli* has a significant role in causing severe food poisoning in man. Many diseases have appeared due to changes in domestic animal managemental practices, food industry and changes in feeding habits of humans.

The specific serogroup of *E. coli* can be linked to the occurrence of certain clinical conditions. Hygienic condition, geographical area and prevalence of *E. coli* in humans and animals in a particular region may be responsible for the distribution of different serotypes of

E. coli. Serotype O8 isolated in the present investigation was associated with juvenile diarrhoea and gastroenteritis in adult humans as reported by Beutin *et al.* [12]. Serotype O8 was also associated with bloody diarrhoea in children in Argentina as reported by Rivas et al. [13]. Serotypes O2, O8, O25 and O60 were responsible for the occurrence of urinary tract infections as per the report of Kausar et al. [14]. Serotype O2 has been considered common in urinary tract infections in humans and was also linked with other clinical conditions such as HUS and HC [15].

As in the present study, serotypes O8 and O25 have also been reported by Nishikawa et al. [16] from human diarrhoeal illness in Japan. Arthur *et al.* [17] reported that serogroups O8, O non typable and rough autoagglutinable strains are linked to human diseases. Serotype O45 recovered in our investigation was previously linked to human illness as reported by Brooks et al. [18].

Careful dairy practices can only minimize, but not completely eliminate, the risk for raw milk contamination [19]. The existence of animal faeces at the milking area and the existence of several risk points throughout the milking and handling processes may result in faecal contamination of milk. Contamination with as few as 10 *E. coli* O157 bacteria might be sufficient to cause human infection [20]. Hence, for the prevention of microbial risks associated with milk, pasteurization of milk is necessary, which not only considerably reduces microorganisms in milk but also prevents transmission of diseases very efficiently [21].

Gastrointestinal diseases, food poisoning, and even death in some cases make these microbes a dangerous threat for human health [22]. Studies showed that raw animal milk samples and different dairy products may act as major reservoirs of STEC [23]. The occurrence of STEC outbreaks were related to the consumption of raw seafood products, traditional dairy products, unpasteurized milk, contaminated food with pollution sources such as faeces, contaminated water, contaminated equipment, fast-food, contaminated plant products, and finally raw or even undercooked foods [24].

Conclusion

Different serotypes (13 different O serogroups) like O2, O4, O8, O17, O22, O25, O29, O36, O45, O60, O90, O116 and O172 were recovered during the present investigation. Majority of these serotypes were reported in various human disease conditions as well as animals, thus revealing the significance of our findings and the zoonotic significance of these serotypes. 21 *E. coli* isolates were found to be STEC in the present investigation, which have significant role in causing HC and HUS in humans. A number of untypable isolates were also found in our study. We presume that these isolates might belong to some rare or different serotypes which are not yet identified from milk and milk products. They certainly deserve special attention in future studies.

Authors' contributions

PKV, JBN, MNB and HC: Conceived and designed the experiment. PKV collected the samples, analysed the samples and interpreted the results. All authors drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

References

1. Tchaptchet, S. and Hansen, J. (2011) The Yin and Yang of host-commensal mutualism. *Gut Microbes*.2: 347–352.
2. Farrokh, C., Kieran, J., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., Thevenot, D., Condron, R., De Reu, K., Govaris, A., Heggum, K., Heyndrickx, M., Hummerjohann, J., Lindsay, D., Miszczycha, S., Moussiegt, S., Verstraete, K. and Cerf, O. (2012) Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. *Int. J. Food Microbiol.* http://dx.doi.org/10.1016/j.ijfoodmicro.2012.08.008,10/04/2013.
3. Karmali, M. A., Gannon, V. and Sargeant, J. M. (2010) Verocytotoxinproducing*Escherichia coli* (VTEC). *Vet. Microbiol.* 140: 360–370.
4. Caprioli, A., Morbito, S., Brugere, H. and Oswald, E. (2005) Enterohaemorrhagic*Escherichia coli*: emerging issue on virulence and mode of transmission. *Vet. Res.* 36: 289–311.
5. Lorusso, V., Dambrosio, A., Quaglia, N. C., Parisi, A., La Salandra, G., Lucifora, G., Mula, G., Virgilio, S., Carosielli, L., Rella, A., Dario, M. and Normanno, G. (2009) Verocytotoxin-producing *Escherichia coli* O26 in raw water buffalo (Bubalusbubalis) milk products in Italy. *J. Food Prot.*72(8): 1705-1708.
6. Buvens, G., Posse, B., De Schrijver, K., De Zutter, L., Lauwers, S. and Pierard, D. (2011) Virulence profiling and quantification of verocytotoxin-producing *Escherichia coli* O145:H28 and O26:H11 isolated during an ice cream-related haemolytic uremic syndrome outbreak. *Foodborne Pathogens and Disease.* 8: 421–426.
7. Kumar, R., Surendran, P. K. and Thampuran, N. (2008) Evaluation of culture, ELISA and PCR assays for the detection of *Salmonella* in seafood. *Letters in Applied Microbiol*. 46(2): 221-226.
8. Edwards, P. R. and Ewing, W. H. (1972)Identification of *Enterobacteriaceae. Emerg. Infect. Dis.* 12:154–159.
9. Beverley, C., Millar, X. J., John, E. M. and John A. P. E. (2000) A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. *Journal of Microbiological Methods.* 42: 139-147.
10. Osman, K. M., Mustafa, A. M., Elhariri, M. and AbdElhamed, G. S. (2013) The Distribution of *Escherichia coli* serovars, virulence genes, gene association and combinations and virulence genes encoding serotypes in pathogenic *E. coli* recovered from diarrhoeic calves, sheep and goat. *Transbound Emerg. Dis.* 60(1):69-78.
11. Dhanashree, B. and Mallya, P. S. (2008) Detection of shiga-toxigenic *Escherichia coli* (STEC) in diarrhoeagenic stool & meat samples in Mangalore, India. *Indian J. Med. Res*. 128: 271-277.

12. Beutin, L., Montenegro, M. A., Orskov, I., Orskov, F., Prada, J., Zimmermann, S. and Stephan, R. (1989)Close association of verotoxin (shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. *J. Clin. Microbiol.* 27: 2559–2564.
13. Rivas, M., Miliwebsky, E., Chinen, I., Roldan, C. D., Balbi, L., Garcia, B., Fiorilli, G., Sosa-Estani, S., Kincaid, J., Rangel, J. and Griffin, P. M. (2006) Characterization and epidemiologic subtyping of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina. *Foodborne Pathog. Dis.* 3: 88-96.
14. Kausar, Y., Chunchanur, S. K., Nadagir, S. D., Halesh, L. H. and Chandrasekhar, M. R. (2009) Virulence factors, Serotypes and Antimicrobial Suspectibility Pattern of *Escherichia coli* in Urinary Tract Infections. *Al Ameen J. Med. Sci.* 2(1):47-51.
15. Karama, M., Johnson, R. P., Holtslander, R., McEwen, S. A. and Gyles, C. L. (2008) Prevalence and characterization of verotoxin-producing *Escherichia coli* (VTEC) in cattle from an Ontario abattoir. *The Canadian J. Vet. Res.* 72: 298-302.
16. Nishikawa, Y., Zhou, Z., Hase, A., Ogasawara, J., Kitase, T., Abe, N., Nakamura, H., Wada, T.,Ishii, E. and Haruki, K. (2002) Diarrheagenic *Escherichia coli* isolated from stools of sporadic cases of diarrheal illness in Osaka city, Japan between 1997 and 2000: Prevalence of Enteroaggregative *E. coli* heat stable enterotoxin 1 gene- possessing *E. coli*. *Jpn. J. Infect. Dis.* 55:182-190.
17. Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M., Hoekstra, R. M. and Strockbine, N. A. (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J. Infect. Dis.* 192: 1422-1429.
18. Arthur, T. M., Barkocy-Gallagher, G. A., Rivera-Betancourt, M. and Koohmaraie, M. (2002) Prevalence and characterization of non-O157 shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. *App. Environ. Microbiol.* 68(10): 4847-4852.
19. Leedom, J. M. (2006) Milk of nonhuman origin and infectious diseases in humans. *Clin. Infect. Dis.* 43:610–615.
20. Guh, A., Phan, Q., Randall, N., Purviance, K., Milardo, E., Kinney, S., Mshar, P., Kasacek, W. and Cartter, M. (2010) Outbreak of Escherichia coli O157 Associated with Raw Milk, Connecticut, 2008. *Clin. Infect. Dis.* 51(12): 1411-1417.
21. LeJeune J. T. andRajala-Schultz P. J. (2009) Food safety: unpasteurized milk: a continued public health threat. *Clin Infect Dis.* 48:93–100.
22. Solomakos, N., Govaris, A., Apostolos, S., Spyros, A., Angeliki, P., Burriel, R., Spyridon, K., Demetrios, K. and Papageorgiou, K. (2009) Occurrence, virulence genes and antibiotic resistance of *Escherichia coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. *Food Microbiol*. 26(8): 865–871.
23. Stephan, R., Schumacher, S., Corti, S., Krause, G., Danuser, J. and 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(7): 2561-2565.
24. Madic, J.,Vingadassalon, N., de Garam,C. P., Marault, M.,Scheutz, F., Brugere, H. Jamet, E. and Auvray, F. (2011) Detection of Shiga toxin-producing Escherichia coli serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 in raw-milk cheeses by using multiplex real-time PCR. *Applied and Environmental Microbiology* 77(6): 2035–2041.

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