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RESEARCH ARTICLE

MICROBIOLOGY

ISOLATION OF SHIGATOXIGENIC *ESCHERICHIA COLI* FROM FAECES AND FARM WATER SAMPLES OF SHEEP USING PCR

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ABSTRACT

Apart from cattle, sheep and goat are important natural reservoirs of Shigatoxigenic *Escherichia coli*. Faecal contamination during dressing of carcass accounts for the presence of STEC in raw meat. A total of 201 samples (104 sheep faecal and 97 sheep farm water samples) collected from various sources were subjected to PCR analysis for the presence of Shigatoxigenic *Escherichia coli* using primers specific to STEC virulent genes, shiga toxins (*stx*1 and *stx*2) and enterohaemolysin (*hly*A) with amplification size of 614bp, 779bp and 361bp respectively. Out of 201 samples, 93 showed presence of STEC (36 faecal and 27 water samples). Of the 93 STEC positive isolates, 63 (67.74%) showed presence of *stx*1, 32 (34.4%) showed *stx*2, 47 (50.53%) showed *hly*A gene and 16 (17.2%) showed both *stx*1 and *stx*2. Among 58 STEC positive isolates, 7.52% (4 faecal and 3 water samples) isolates possessed all the 3 virulent genes. Enrichment with modified Tryptic Soy Broth (mTSB) containing novobiocin gave good results compared to modified *Escherichia coli* (mEC) broth with novobiocin.

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KEY WORDS

STEC, PCR, Sheep faeces, Sheep farm water, Shiga toxins, mTSB and mEC.

INTRODUCTION

Shiga-toxin producing *E. coli* (STEC), also called verotoxin producing *E. coli* (VTEC) is the most important recently emerged group of foodborne pathogens [3, 4]. Shiga toxin genes have been found in over 200 serotypes of *E. coli* including both O157 and non-O157:H7 STEC[21]. The serotype O157:H7 remains the prototype of STEC and accounts for 70-80% of recognized clinical cases of disease[14]. STEC produce two types of toxins, *stx*-1 and *stx*-2 which are responsible for several diseases such as haemorrhagic colitis (HC), haemorrhagic uraemic syndrome (HUS) and thrombocytopaenic purpurea (TTP) in man[13]. *Stx*-2 producing strains appear to be more commonly responsible for serious complications such as HUS than those only *stx*1 producing[60]. *E. coli* strains carrying *stx*2 gene along with enterohaemolysin (*hly*A) gene are potentially dangerous to human health[22]. The reservoirs and main source of infection for humans are ruminants, particularly faeces of cattle and sheep[5,32] and contamination of animal foods occurs through improper handling of meat and meat products by workers.

Conventional techniques for toxin detection require either bioassays[9] or cell culture assays[17] which are time consuming and unsuitable for routine diagnosis. Immunological methods for the detection of toxins by latex agglutination and ELISA tests are available commercially and in routine use[7]. However, a rapid, single method which can be used for the detection of all *E. coli* toxins is desirable. The PCR involves the enzymatic amplification of a DNA sequence initiated from a pair of short DNA fragments (primers) which bind either side of the chosen target sequence[26]. The amplified DNA product of this reaction can be easily detected by gel electrophoresis followed by ethidium bromide staining and UV transillumination. Sophisticated developments of the PCR have been used to simultaneously detect several virulence genes within a single reaction[12]. So, in the detection of *E. coli* O157:H7 and STEC, PCR is mainly employed to detect the genes that are responsible for the production of SLTs or VTs. This method has proved to be more rapid, sensitive and specific to detect *stx* genes as compared to DNA colony hybridization tests[6,15]. With this procedure DNA is amplified to increase the level of target DNA when STEC are present in very low number.

The present study was undertaken to detect presence of STEC in faeces and farm water samples of sheep (needs some correction) by using PCR.

MATERIALS AND METHODS

A total of 201 samples (104 sheep faecal samples and 97 sheep farm water samples) were collected from different sheep farms and lairages in and around Hyderabad, India. Faecal and water samples (10gm/ml each) were enriched in 90 ml of modified *Escherichia coli* (mEC) broth and modified Tryptic Soy broth (mTSB) both supplemented with novobiocin at 37^0C for 18 hours. An *Escherichia coli* O157:H7 strain, obtained from National Institute of Cholera and Enteric diseases, Kolkata was used as known positive strain in PCR analysis. All the enriched samples were subjected to PCR analysis for the presence of STEC by using oligonucleotide primers against three virulence genes i.e. *stx*1, *stx*2[20] and *hly*A[30] with specific amplification size of 614, 779 and 361bp respectively (fig-1).

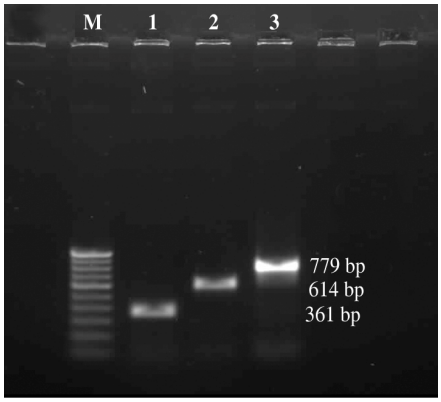

Fig. 1
Results of the PCR assay showing hlyA, stx1 and stx2 bands
Lane M: 100-bp DNA marker
Lane 1: amplifying 361-bp segment of hly A
Lane 2: amplifying 614-bp segment of stx 1
Lane 3: amplifying 779-bp segment of stx 2

1.5 ml of enriched broths were taken into centrifuge tubes and bacteria were pelleted by centrifuging at 6000rpm for 5 min and supernatant was discarded. To the pellet 50µl of molecular grade water was added and kept in a water bath at 65^0C for 15 min and snap chilled. Then centrifuge at 13000rpm for 5 min and the supernatants were used as DNA templates for PCR analysis.

Bacterial DNA amplification was done in 20µl reaction mixture containing 2µl of 10X Taq DNA polymerase buffer (containing 100mM Tris with P^H 9.0, 500mM KCl, 15mM $MgCl_2$ and 1% Triton X-100), 2µl of 10mM of dNTP mix, 0.9U of Taq DNA polymerase (Genei, Bengaluru), 2µl each of 4 p.moles/µl of forward and reverse primers (Genei, Bengaluru) and 5µl of crude bacterial cell lysate. Make this mixture to 20µl using molecular grade water. Amplification was done in thermal cycler (Eppendorf) following standardized conditions (Table.1).

Table.1
Cycling conditions used for three sets of primers

S.No.	Step	*hly* A	*stx*1 and *stx*2
1.	Initial denaturation	94^0C/5min	94^0C/5min
2.	Final denaturation	94^0C/1min	94^0C/1min
3.	Annealing	52^0C/1min	60^0C/1min
4.	Initial extension	74^0C/2min	72^0C/2min
5.	Final extension	74^0C/10min	72^0C/10min

The amplified DNA fragments were resolved by agarose (1%) gel electrophoresis, stained with ethidium bromide (0.5µg/ml) and visualized with an UV transilluminator (Fig.1 & 2).

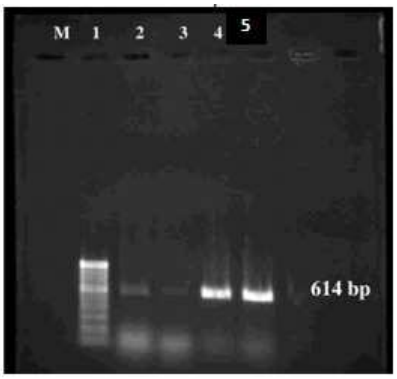

Fig.2
Evaluation of PCR compatibility of enrichment broths for detection of (stx1)
Lane 1: 100-bp DNA ladder
Lane 2 &3: amplifying 614-bp segment of stx 1 enriched in mEC broth
Lane 4 & 5: amplifying 614-bp segment of stx 1 enriched in mTSB broth

RESULTS

In this study, the growth of STEC was good in mTSB compared to mEC as shown in fig-2. Results for the presence of STEC in sheep faecal and farm water samples are presented in Table 2.

Table.2
Occurrence of STEC virulent genes in sheep faecal and farm water samples

S.No.	Sample	No. of samples	STEC	*stx*1	*stx*2	*hly*A	Both *stx*1& *stx*2	All the 3 genes
1.	Sheep faeces	104	57	36	19	26	9	4
2.	Farm water	97	36	27	13	21	7	3
	Total	201	93	63	32	47	16	7

In this study, the incidence of STEC in sheep faecal samples was found to be 54.80% (57 out of 104). Also STEC were detected in 37.11% (36 out of 97) of the farm water samples. Out of total 93 STEC positive isolates (both faeces and water), 67.74% (63 out of 93) samples showed presence of *stx*1, 34.40% (32 out of 93) samples showed presence of *stx*2, 17.20% (16 out of 93) were positive for both *stx*1 and *stx*2.

When tested for presence of '*hly*A' by PCR, 50.53% (47 out of 93) of the total STEC positive samples showed presence of *hly*A. The *hly*A gene was detected in 45.6% (26 out of 57) of STEC positive sheep faecal isolates and 58.33% (21 out of 36) positive sheep farm water isolates. Among 93 STEC positive isolates, 7.52% (4 faecal and 3 water samples) isolates possessed all the 3 virulent genes.

DISCUSSION

The intestinal tract of ruminants, in particular sheep and cattle, has been shown to be a major reservoir of STEC[2,18]. During the dressing of carcass, faecal contamination of the carcasses occurs either by direct or indirect contact with the animal's fleece, legs and/or hooves or via the workers or from dirty equipment[1].

Traditional methods for detecting and identifying STEC are labour intensive and time consuming. Commercially available ELISA kits and molecular methods like PCR have reduced the time and labour involved in the analysis of food products and stool samples. Non-selective and/or selective enrichment combined with PCR have been applied to the detection of many bacteria pathogens[27] to improve sensitivity and to dilute the PCR-inhibitory substances usually present in faeces[10]. In contrast to culture based methods, PCR methods may also detect cells that are non-culturable[11].

The PCR technique works primarily on the detection by amplification of specific nucleotide sequences with the help of different sets of oligonucleotide primers and restriction endonucleases (RE) generally upto the size of genes. This amplified DNA/nucleic acid end product is detected by gel electrophoresis. PCR amplification of *stx* gene sequences present in faecal samples has been accepted as the most sensitive and specific means for STEC screening[23].

In the present study, the growth of STEC was good in mTSB compared to mEC**.** Some of the published studies revealed that modified Tryptic Soy Broth (mTSB) took less time for doubling of STEC strains (i.e. 28 min) than mEC (58 min)[24]. In this study, sheep faecal samples yielded high percentage of STEC compared to sheep farm water samples. Many studies have shown that sheep could be equally important reservoir of O157:H7 and STEC as cattle[19, 25]. In this study, results for *stx*1 production were high compared to *stx*2 production. Most of the published test studies also revealed the higher production of *stx*1 than *stx*[28,29] and also that >50% of sheep carry STEC in the intestines[28] but some studies showed higher incidence of *stx*2 compared to *stx*1[31].

Since the *E. coli* strains carrying the shiga toxin genes along with enterohaemolysin (*hly*A) gene are potentially dangerous to human health, PCR is a very specific and rapid method of detecting the virulent STEC in animal faeces, human stools and food samples for early diagnosis and helps in control of food borne outbreaks.

CONCLUSION

This study emphasizes the prevalence of virulent STEC in sheep faecal and farm water samples in and around Hyderabad, India. Keeping in view the very low infectious dose of *E. coli* O157 and other STEC (< 10 viable bacteria), much greater efforts are required for detection of these STEC. The possible ways of entry of various *E. coli* serotypes could be faecal contamination of carcasses during the slaughter and improper handling and processing of meat and meat products. Therefore, good livestock management practices and proper hygienic measures for the handling of various food products need to be adopted in order to bring down the presence of these organisms in ~~to~~ meat, thereby safeguarding the human beings from health hazards.

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REFERENCES

1. Bettelheim KA., The isolation of *E. coli* from a sheep slaughtering line in an abattoir. Comp. Imm. Microbiol. Infect. Dis. 4: 93-100, (1981).
2. Beutin L., Gleier D., and Zimmerman S., *et. al.,* Epidemiological relatedness and clonal types of natural populations of *E. coli* strains producing shiga toxins in separate populations of cattle and sheep. Appl. Environ. Microbiol. 63: 2175-2180, (1997).
3. Beutin L., Krause G., Zimmermann S., Kaulfuss S., Gleier K., Characterization of Shiga toxin-producing *E. coli* strains isolated from human patients in Germany over a 3 year period. J. Clin. Microbiol. 42: 1099-1108, (2004).
4. Blanco JE., Blanco M., Alonso MP., Mora A., Dahbi G., Coira MA., and Blanco J., Serotypes, virulence genes and intimin types of Shiga toxin (verotoxin)-producing *E. coli* isolates from human patients: prevalence in Lugo (Spain) from 1992 through 1999. J. Clin. Microbiol. 42: 311-319, (2004a).
5. Blanco M., Blanco JE., Mora A., Dahbi G., Alonso MP., Gonzalez EA., Bernardez MI., and Blanco J., Serotypes, virulence genes and intimin types of Shiga toxin (verotoxin)-producing *E. coli* isolates from cattle in Spain: identification of a new intimin variant gene (eae-ε). J. Clin. Microbiol. 42: 645-651, (2004b).
6. Brian MJ., Frosolono M., Murray BE., Miranda A., Lopez EL., Gomez HF., and Cleary TG. Polymerase chain reaction for diagnosis of enterhaemorrhagic *Escherichia coli* infection and haemolytic uremic syndrome. J. Clin. Microbiol. 30: 1801-1806, (1992).
7. Carroll PJ., Woodward MJ., and Wray C., Detection of LT and STIa toxins by latex and EIA tests. Vet. Rec. 127: 335-336, (1990).
8. Cerqueira A.M.F., Guth B.E.C., Joaquim R.M., and Andrade J.R.C., High occurrence of shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle in Rio de Janeiro State, Brazil. Veterinary Microbiology. 70: 111-121, (1999).
9. Dean AG., Ching YC., Williams RG., and Harden LB., Test for *E. coli* enterotoxin using infant mice. Application in a study of diarrhea in children in Honolulu. J. Infect. Dis. 15: 407-411, (1972).
10. Fluit A.D.C., Widjojoatmodjo M.N., Box A.T.A., Torensma R., and Verhoef J., Rapid detection of *Salmonellae* in poultry with the magnetic immuno-polymerase chain reaction. Appl. Environ. Microbiol. 58: 3809-3815, (1993).
11. Fode-Vaughan K.A., Maki J.S., Benson J.A., and Collins M.L.P., Direct PCR detection of *Escherichia coli* O157:H7. Letters in Appl. Microbiol. 37: 239-243, (2003).
12. Fraenkel G., Giron JA., Valmassoi J., and Schoolnik GK., Multigene amplification simultaneous detection of three virulence genes in diarrhoeal stool. Mol. Microbiol. 3: 1729-1734, (1989).
13. Griffin P.M., and Tauxe R.V., The epidemiology of infections caused by *Escherichia coli* O157, other enterohaemorrhagic *E.coli*, and the associated haemolytic uraemic syndrome. Epidemiol. Rev. 13: 60-98, (1991).
14. Gyles C., Johnson R., Anli A., Ziebell K., Pierard D., Aleksic S., and Boerlin P., Association of enterohaemorrhagic *Escherichia coli* haemolysin with serotypes of shiga like toxin producing *Escherichia coli* of human and bovine origins. Appl. Environ. Microbiol. 64: 4134-4141, (1998).
15. Karch H., and Meyer T., Single primer pair for amplifying segments of distinct shiga-like toxin genes by polymerase

chain reaction. J. Clin. Microbiol. 27: 2751-2757, (1989).
16. Kleanthous H., Smith H.R., Scotland S.M., Grass R.J., Rowe B., Taylor C.M., and Milford D.V., Haemolytic Uraemic Syndromes in the British Isles 1985-8 association with Verocytotoxin producing *Escherichia coli*. Part 2: Microbiological aspects. Arch. Dis. Child. 65: 722-777, (1990).
17. Konowalchuk J., Spiers JI., and Stavric D., VERO cell response to a cytotoxin of *E. coli*. Infect. Immun. 18: 755-779, (1977).
18. Kudva IT., Hatfield PG., and Hodve CJ., Characterization *E. coli* O157:H7 and other shigatoxin-producing *E. coli* serotypes isolated from sheep. J. Clin. Microbiol. 35: 892-899, (1997).
19. Kudva I.T., Hatfield P.G., and Hovde C.J., *Escherichia coli* O157:H7 in microbial flora of sheep. J. Clin. Microbiol. 34: 431-433, (1996).
20. Manna S.K., Brahmane M.P., Das R., Manna Chandana and Batabyal S., Detection of *Escherichia coli* O157 in foods of animal origin by culture and multiplex polymerase chain reaction. J. Food Sci. Technol. 43(1): 77-79, (2006).
21. Meng, J., and Doyle M.P., Microbiology of shiga toxin producing *Escherichia coli* in foods. In: Kaper, J.B. and O'Brien, A.D. (eds), *Escherichia coli* O157:H7 and other shiga toxin producing *E.coli* strains. ADM, Washington DC, p: 92-108, (1998).
22. Nataro J.P., and Kaper J.B., Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11: 142-201. (1998).
23. Paton J.C., and Paton A.W., Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11: 450-479, (1998).
24. Restaino L., Catillo H.J., Stewart D., and Tortorello M.L., Antibody-direct Epifluorescent filter technique and Immun-magnetic separation for 10-h screening and 24-h confirmation of *Escherichia coli* O157:H7 in beef. *J. Food Prot.,* 59(10): 1072-1075, (1996).
25. Robins-Browne RM., *Escherichia coli* strains that cause diarrhea. Models of bacterial pathogenesis. In: Recent advances in microbiology. The Australian Society for Microbiology, Melbourne, Australia. 292-335, (1994).
26. Saiki RK., Gelfand DH., Stoffel S., Scharf SJ., Higuchi R., Horn GT., Mullis KB., and Ellich HA., Primer-directed enzymatic amplification of DNA with a Thermostable DNA polymerassse. Science. 239: 487-491, (1988).
27. Schrank I.S., Mores M.A.Z., Frazzon A.P.G., Sonchi R., Schrank A., Vainstein M.H., and Silva S.C., Influence of enrichment media and application of a PCR based method to detect *Salmonella* in poultry industry products and clinical samples. Vet. Microbiol. 82: 45-53, (2001).
28. Sidjabat-Tambunan H., and Bensink J.C., Verotoxin-producing *E.coli* from the faeces of sheep, calves and pigs. Aust. Vet. J. 75: 292-293, (1997).
29. Sidjabat-Tambunan H., Bensink J.C., and Bettelheim K.A., Isolaition of verocytotoxins-producing *Escherichia coli* from mutton carcasses. Aus. Vet. J. 76(5): 364-365, (1998).
30. Wang R.F, Pao W.W, Cerniglia C.E. (1997 A universal protocol for PCR detection of 13 species of foodborne pathogen in foods. Journal of Appl Microbiol 83: 727-736.
31. Yilmaz A., Gun H., Ugur M., Turan N., and Yilmaz H., Detection and frequency of *VT*1, *VT*2 and *eae*A genes in *Escherichia coli* O157 and O157:H7 strains isolated from cattle, cattle carcasses and abattoir environment in Istanbul. Int. J. Food Microbiol. 1: 213-217, (2006).
32. Zweifel C., Blanco JE., Blanco M., Blanco J., Stephan R., Serotypes and virulence genes of ovine non-O157 Shiga toxin-producing *E. coli* in Switzerland, Int. J. Food Microbiol. 95: 19-27, (2004).