Original Research

Molecular Characterization of Shiga Toxin Producing *Escherichia coli* from Raw Milk Samples

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Abstract

Shiga toxin-producing E. coli (STEC) has been associated with both outbreaks and sporadic cases of human disease, ranging from uncomplicated diarrhea to haemorrhagic colitis and haemolytic uremic syndrome (HUS). The dominant STEC serotype is O157:H7, which is also most commonly involved in large number of outbreaks in the world. Keeping in view of the public health significance of STEC a small work was designed to detect the presence of STEC from milk samples that are available in Proddatur town of YSR Kadapa district, Andhra Pradesh, India. Among the 50 samples 22 isolates were found, Escherichia coli by culture method. PCR assay of the isolates revealed that out of 22 samples 3 were positive for STEC. Among the three positive samples two isolates carried stx_1 gene and one isolate carried stx_2 gene. The results indicated that the samples revealed the presence of STEC in the tested milk samples they were non O157:H7. The findings of this study revealed that there may be a danger of transmission of STEC to consumers through consumption of not properly sterilized milk and milk products.

Key words: *Escherichia coli*, Milk, Molecular Characterization, Shiga Toxin

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Introduction

Escherichia coli is a Gram-negative, nonsporulating, rod shaped, flagellated, and facultative anaerobic bacteria belonging to the Enterobacteriaceae family (Bavaro, 2012), which considered a reliable indicator of contamination by manure, soil and contaminated water (Todar, 2008). Most *E. coli* are harmless, but some are known to be pathogenic bacteria, causing severe intestinal and extra intestinal diseases in man (Kaper *et al*., 2004). Based on its virulence, this species is classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), attaching and effacing *E. coli* (AEEC) and Shiga toxinproducing *E. coli* (STEC) (Bavaro, 2012). The broad group of *E. coli* are known as enterohemorrhagic *Escherichia coli* (EHEC), including *E. coli* O157:H7 and non-O157 (Louie *et al.,* 1993). EHEC refers to a subset of Shiga toxin-producing *Escherichia coli* (STEC) strains found to cause human and sometimes animal disease (Terrance *et al.,* 2002). Shiga toxin-producing *Escherichia coli* (STEC) present two kinds of interactions with animals: (i) intestinal disease in newborn and (ii) carriage by healthy animals as a source of infection to humans (Paton and Paton, 1998b).

Cattle are considered the primary reservoir of both O157:H7 and non-O157 STEC bacteria (Terrance *et al.*, 2002). Cattle frequently excrete the bacteria in their feces (Molina *et al*., 2003). Transmission of this food-borne pathogens occur through consumption of under cooked meat, unpasteurized dairy products, vegetables or water contaminated by ruminant feces. Contact with infected animal or human has also been documented (Jamshidi, 2008). Raw milk is a well-known good medium that supports the growth and multiplication of many kinds of microorganisms due to its complex biochemical composition and high water activity (Oliver *et al*., 2005). Many microorganisms can get access to raw milk and among these are *Escherichia coli,* which is an environmental pathogen found in the immediate surroundings of the cow such as the soil, grass, manure and the bedding of housed cows. It is therefore easy for the organism to be found in the udder of cow thereby gaining entrance to the milk. Majority of cases STEC outbreaks were associated with consumption of raw seafood products, traditional dairy products, unpasteurized milk, contaminated food with pollution sources such as feces, contaminated water, contaminated equipments, infected water and even infected chief, fast-food, contaminated plants' food and finally raw or even undercooked foods (Madic *et al*., 2011). It produces toxins that destroy cell membrane and can directly damage milk-producing tissues which can lead to bovine mastitis (Jones *et al*., 1998). The presence of bacteria in milk has many undesirable effects on the quality and safety of milk and its products (Gruetzmacher and Bradle 1999). Milk contaminated by high levels of bacteria usually becomes unsuitable for further processing (Nanu *et al*., 2007). Therefore, insufficient heat-treatment of raw milk forms a potential infection risk (Betts, 2000), and the processing conditions for different milk products are very important, for the risk of survival of the bacterium.

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Martin *et al*. (1986) reported two cases of hemolytic uraemic syndrome which provide evidence that raw milk may be a vehicle of transmission of *E. coli* O157: H7, both affected person consumed raw milk. Recovery of *E. coli* from food is an indicative of possible presence of enteropathogenic and/or toxigenic micro-organism which could constitute a public health hazard. They are linked to development of hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) (Hussein, 2003) and thrombotic thrombocytopenic purpura (TTP), which requires hospitalization and intensive care (Riley,1983). STEC O157:H7 strains were first isolated from cattle in Argentina in 1977, although the strains were identified as such 10 years later (Bonardi, 1999).

Shiga toxin-producing *Escherichia coli* (STEC), also called Verotoxin-producing *E. coli* (VTEC), are a subgroup of *Escherichia coli* capable of producing potent toxins called two phage-encoded cytotoxins called Shiga toxin (Stx1, Stx2), Shiga toxin 1 (Ludwig *et al*., 2001) is 98% homologous to the Stx produced by *Shigella dysenteriae* type 1, while Stx2 is about 60% homologous with Stx1 and is antigenically different (Nataro & Kaper, 1998; Paton & Paton, 1998), and may also possess additional putative virulence factors such as Intimin which is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa (Paton&Paton, 1998. Karmali, 1989) and the plasmid-encoded enterohaemolysin or enterohaemorrhagic *E. coli* haemolysin (*ehly*) (Law, 2000). According to Nataro and Kaper, 1998, in addition to Stx, typical EHEC strains carry on their chromosomes the locus for enterocyte effacement (LEE), a large pathogenicity island that is shared with enteropathogenic *E. coli* (EPEC). The LEE is responsible for attaching and effacing (A/E) lesions on enterocytes. This pathotype is a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic uremic syndrome (HUS), which is the main cause of acute renal failure in children (Nataro and Kaper, 1998). But if we administer antimicrobials early in the course of infection, may prevent disease progression to HUS (Fukuma *et al*., 1998; Ikeda *et al*., 1999).

Detection and isolation of STEC in foodstuffs by traditional culture methods is rather laborious and time-consuming and is complicated by the lack of common biochemical characteristics distinguishing most STEC from other *E. coli* strains. Development of rapid methods for the detection of the most pathogenic STEC strains is essential to ensure the safety of food products.

Materials and Method

Isolation and Identification

A total of 50 samples were collected from local dairy farms and local markets in and around Proddatur town, YSR Kadapa district. The samples were collected in sterile containers and transferred under aseptic conditions (on icepack) to the laboratory for bacterial isolation, each sample was pre enriched in 90ml

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buffered peptone water (BPW). Then a loopful of inoculum was transferred from each tube onto selective media plates (EMB agar plates) and incubated at 37^0C for 24 h. Colonies with typical metallic sheen were taken and subjected for biochemical tests like IMViC (Indole, MethylRed, Voges- Prosverker, Citrate) tests, Triple sugar iron agar test, and urease test for confirmation of *E. coli.* For the detection of Shiga toxin producing *Escherichia coli* all the isolates were streaked on Sorbitol Mac Con key agar plates and incubated at 37^0C for 24 h.

DNA Isolation

1.5 ml of each enriched broths were transferred to eppendorf tubes then bacterial contents were pelleted by centrifugation at 8000 rpm for 10 min. After the supernatant was discarded. 50 µl of sterile distilled water was added to each tube and boiled at 100^0C for 10 min in a water bath, then immediately snap chilled to release DNA. Each eppendorf tube was centrifuged at 13,000 rpm for 5 min then the supernatants were used as DNA templates for PCR analysis.

PCR Assay

Bacterial DNA amplification was done in 20µl reaction mixture containing 2 µl of 10x Taq DNA polymerase buffer (100mM tris PH 9.0, 500 mM KCl, 15 mM $MgCl_2$ and 1% triton X-100), 2 µl of 10 mM dNTPs mix, 0.9U/µl Taq DNA polymerase (Genei), 2 µl of each primer (4pmol/µl) and 5 µl bacterial DNA. Complete mixture volume to 20 µl using nuclease free water. Amplification was done in thermal cycler (Eppendorf, MC gradient) following standardized conditions. The amplified DNA fragments were resolved by agarose gel electrophoresis (B.Genei), stained with ethidium bromide (0.5 µg/ml) and visualized with an UV transilluminater (B.genei, MD-20, 312nm).

Cyclic Conditions

S. No.	Step	Temperature (^{0}C)	Duration	No. of Cycles
1	Initial denaturation	94^0c	3 min	1
2	Final denaturation	94^0c	30 sec	35
3	Annealing	55^0c	35 sec	
4	Initial extension	72^0c	1 min	
5	Final extension	72^0c	10 min	1
6	Hold	4^0c	10 min	---

Results

Among the 50 raw milk samples 22(44%) isolates were found, *Escherichia coli* by culture method. Gram's staining of the isolates revealed that they are Gram negative cocobacillary rods. All the 22 isolates were negative for Voges-Proskauer and Citrate utilization tests, whereas the same isolates produced bright red color in methyl red test and red color ring in Indole test. Further all the 22 isolates were subjected to urease test and triple sugar iron agar test. The results revealed that all the isolates were

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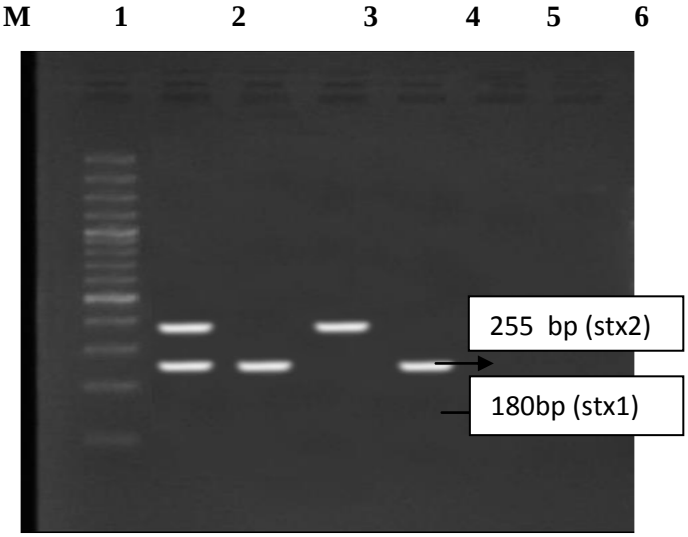
positive for urease test and triple sugar iron agar test as the isolates produced acid butt and slant and also gas. All the biochemical reactions confirmed the presence of *Escherichia coli*. For the detection of Shiga toxin producing *Escherichia coli* all the isolates were streaked on Sorbitol Mac Con key agar plates and the results revealed that all the isolates have shown pink colonies and none have shown colourless colonies. PCR assay of the isolates revealed that out of 22 samples 2(9.09%) were positive for STEC. Among the two positive samples two isolates carried stx_1 gene and one isolate carried stx_2 gene.



Lane: M (100 bp DNA size marker); Lane: 1 Positive control; Lane: 2 positive for stx1; Lane: 3 positive for stx2; Lane: 4 positive for stx1

Fig.: PCR assay Detection of STEC in milk samples using *stx1 and stx2* gene primers pair, where the obtained amplicons sized 180 and 255 bp respectively.

Discussion

In the present study out of 50 milk samples tested for the presence of *E. coli*, 22samples(44%) were identified to have *E. coli* by cultural method. These results were in agreement with the reports of Rasheed *et al.* (2014) (43.33%). Higher prevalence than the present study was reported by Neher *et al.* (2015), Ali & Abdelgadir (2011) Lingathurai & Vellathurai (2010) and Shahzad *et al.* (2013) as 58.82%, 63%, 70% and 76% respectively. Lower prevalence than the present study was reported by Bali *et al.* (2013) as 32.5%. The present study reported 9.09% of STEC among 22 *E. coli* isolates by using PCR assay, which was lower than the reports of Vendramin *et al.* (2014) (31.1%), Neher *et al.* (2015)(23.33%) and Mohammadi *et al.* (2013)(17.47%). Almost similar results to the present study were reported by Rasheed *et al.* (2014) as 6.7%. Presence of even single STEC in food sample may lead to gastrointestinal disorder due to their multiplication in the body or the food itself during storage in poor condition (Gyles, 2007). Prevalence of STEC in milk may show seasonal variation. Mohammadi *et al.* (2013) reported prevalence of STEC as 17.47% in milk samples that were collected during summer months of the year (Paton *et al.*, 1996). Numerous factors along with season that are likely to contribute to the variation observed are

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geographical location, farm size, number of animals in farm, hygiene, farm management practices, variation in sampling, variation in types of samples evaluated and differences in detection methodologies used (Jelacic *et al.*, 2003).

In the present study out of 2 isolates of STEC that were subjected to PCR amplification for virulence factor (Stx1 and stx2), one is possessing stx1 gene (4.54%) and the other isolate is having stx2 gene (4.54%). Higher percentage of STEC with stx1 gene were reported by Shahzad *et al.* (2013), Mohammadi *et al.* (2013), Lingathurai & Vellathurai (2010), and Neher *et al.* (2015) as 73%, 43.59%, 25.8% and 23.33% respectively. Lower percentage of STEC with stx1 gene than the present study was reported by Vendramin *et al.* (2014) (1.9%) and Parisi *et al.* (2010) (5.7%). STEC harboring stx1 and stx2 genes were reported to be typical cattle colonizers (Bretta *et al.*, 2003, Vukhac & Cornick, 2008). Present study reported 4.54% of STEC possessing stx2 gene as virulent factor, which is lower than the reports of Mohammadi *et al.* (2013) (56.4%), Shahzad *et al.* (2013) (62%) and Vendramin *et al.* (2014) (28.3%). In humans epidemiologic data suggested that *E. coli* O157 strains that express stx2 are more important than stx1 in the development of Haemolyric Uraemic syndrome(HUS) and that strains that express stx2 alone are more likely to be associated with the progression to HUS than strains that produce both stx1 and stx2(Griffin, 1995).

After doing several studies some authors have observed a significant association of stx1 and stx2 genes with bovine meat and milk products. They compared the properties of food borne STEC with published data on fecal STEC from food producing animals and found that virulence profiles and serotypes of STEC from food showed remarkable similarities to those of fecal STEC that were from the same animal species and they also pointed out that food producing animals represent the most important source for the entry of STEC in the food chain (Martin and Beutin, 2011).

Conclusion

The present study has investigated and reported the prevalence of *Escherichia coli* in the raw milk samples, which was may be due to fecal contamination of raw milk from different sources. This suggests that raw milk should be considered as a vehicle for the transmission of potentially pathogenic bacteria. So its better to follow strict hygienic practices before, during and after milking process and to create awareness among the people to avoid raw milk consumption**.**

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