Int.J.Curr.Microbiol.App.Sci (2018) *7*(11): 2461-2472

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 11 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.711.281

A Study on the Incidence of Shiga Toxin Producing E*scherichia coli* in Dairy Cattle and Its Environment

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Keywords

Escherichia coli, Shiga toxin, PCR, Faecal samples and water samples

Article Info

Accepted:
18 October 2018
Available Online:
10 November 2018

A B S T R A C T

The present study was designed to study the prevalence and characterization of shiga toxin producing *Escherichia coli* from dairy animals, water samples in dairy farms and from humans who were in close contact with animals. A total of 508 faecal samples from dairy animals, 4 water samples from dairy farms and 28 Human faecal samples, who were in close contact with animals were subjected to cultural and biochemical tests for confirmation of *E. coli.* The molecular characterization of positive isolates with *stx1*, *stx2*, *eaeA* and *hlyA* genes and multiplex PCR for confirmation. A total 522 samples were positive for *E. coli* out of 540 (96.67%) samples by culture method, 319 (61.11%) positive on blood agar plate assay and various biochemical characteristics were studied. Out of 522 positive E. coli, 56 (10.72%) are positive for STEC by PCR method. On multiplex PCR of *stx1*, *stx2*, *eaeA* and *hlyA* genes 6 were positive 6 out of 56 (8.92%). The frequent isolation of STEC strains from non human sources like animals, food and other products along with the identification of multidrug resistance and virulence genes across the Indian subcontinent poses a serious threat of the outbreaks in humans that can occur in the future.

Introduction

E. coli is an important member of the coliform group. Based on the pathogenicity and variation in biochemical characteristics, *E. coli* has been classified into 6 categories, viz. enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EaggEC) and diffusely adherent *E. coli* (DEAC), of which the EHEC is considered as most dangerous group and also referred as shiga toxin producing *E. coli* (STEC). STEC is an important group of *E. coli* that can cause severe diarrhoea and responsible for a number of food borne outbreaks worldwide. The STEC family is diverse and more than 200 serotypes of STEC have been identified so far

and more than 160 of these have been recovered from humans with haemorrhagic colitis or haemolytic uremic syndrome (Abassi K and Elahe T, 2015).

STEC strains produce cytotoxins known as shiga toxins (stx) which have been classified into two major classes they are shiga toxin1(stx1) *and* shiga toxin2(stx2) and coded by *stx1* and stx2 genes respectively. In addition to shiga toxins, most disease-causing STEC strains produce a protein called intimin that is encoded by the *eae* gene and is involved in the enterocyte attaching and effacing phenotype (Bakhshi *et al.,* 2014). Besides, a specific plasmid encoded haemolysin called EHEC haemolysin, which is encoded by *ehxA*gene, might contribute to the virulence of STEC for humans (Bergy *et al.,* 1984).

Contamination of feeds such as grain pellets, soybean meal, silage grasses and grass hay with STEC may occur due to contaminated water used for cultivation of crops, spreading of manure and slurry as fertilizers or via wild birds or mammalian faeces (Brunen *et al.,* 2004).

The ability of zoonotic STEC to survive and persist in faeces, manure and soil in the environment can be considered as a risk factor for the infection of animals and humans. It has been shown that STEC can survive for several months in water or sediment from drinking water troughs. STEC can also survive in soil for long periods particularly in the presence of manure, and during rain fall can be leached out of the soil and travel below the top layers of the soil for more than two months, increasing the probability of contamination of ground water which is recycled for crop irrigation, vegetable cleaning or as drinking water for animals and humans (Collins C and Green AJ, 2010).

Hence using run-off water from manure piles contaminated with zoonotic STEC as a water source for raising the fodder crops for animal grazing, silage production for animal feed or food crops for human consumption may result in persistent animal infection and a great risk of human exposure.

The first documentation of outbreak of STEC was produced by an episode involving strain O157:H7, in 1982 causing haemorrhagic colitis (HC), since then the incidence of this strain in the disease has increased annually (Dastmalchi and Ayremlou, 2012). Further, significant morbidity and mortality secondary to infection be attributed to the development of haemolytic uremic syndrome (Dhanashree and Mallya, 2008) due to STEC because of its association with several large outbreaks of human illness with severe manifestations.

Humans are infected with zoonotic STEC mostly through the consumption of under cooked hamburgers and ground beef products, raw milk and milk products such as cheese, curd, butter and ice creams. Person to person transmission via the faeco-oral route has been an important mode of transmission.

Water borne outbreaks of STEC associated with recreational waters have been increasingly reported since the early 1990's. Since, 1996 outbreaks of STEC, resulting from a new mode of transmission have been recognized i.e. direct contact between humans and cows or calves at farms and fairs etc (Duris *et al.,* 2009).

Keeping in view of the public health significance of STEC the present study was designed to isolate and identify *Escherichia coli* from farm water and faeces of dairy animals and humans and molecular characterization of the isolates to find out the shiga toxin producing *Escherichia coli* by using specific primers.

Materials and Methods

Collection of samples

The faecal samples from cattle, water sources of the concerned dairy farm and from the persons who are working in the farms were collected by using sterile cotton swabs for cattle, sterilized glass bottles for water samples and the persons were given sterilized plastic containers to collect their faeces. A total of 508rectal swabs from cattle, 4 water samples and 28 human faecal samples were collected aseptically in sterile normal saline tubes. The collected specimens were processed within 2 to 4 hours of collection. The source and number of samples collected in this study is given in Table 1.

Isolation and identification

Tryptic soy broth was used for enrichment of inoculum. MacConkey agar and Eosine Methylene Blue (EMB) agar were used for isolation of *Escherichia coli*. Loop full of inoculum from the normal saline tubes was transferred to tryptic soy broth tubes and the tubes were incubated at 37^0C for 24 hours. After incubation a loop full of inoculum from tryptic soy broth tubes was streaked over MacConkey agar and Eosine Methylene Blue agar plates and the plates were incubated at 37^0C for 24 hours. Identification of the isolate was carried out by making a smear was from the growth on MacConkey agar and Eosine Methylene Blue agar plates and it was stained with Grams method of staining. Gram negative bacteria were identified up to genus level as *Escherichia* based on morphology. All the isolates were identified up to species level based on biochemical tests (Fagan *et al.,* 1998). Test for haemolysis for the isolates of *Escherichia coli* grown over night on Eosine methylene blue agar plates were spot inoculated on tryptose soya agar plates containing 5% of disseminated sheep blood cells. The plates were incubated at 37°C for 24 hours and appearance of haemolytic zone around the colony was recorded. Biochemically confirmed isolates were stored in sterile Luria-Bertani glycerol broth vials and mixed well in vortex mixer. The vials were then labelled and stored for molecular characterization of the isolates.

For confirmation of Shiga Toxin producing *Escherichia coli* from the isolates Sorbitol Mac Conkey agar part I and Part II were used as a ready to use dehydrated media from M/s Hi-Media Laboratories, Mumbai and prepared as recommended by the manufacturer. All the isolates were streaked on Sorbitol McConkey agar plates and the plates were incubated at 37°C / 24 hours. After incubation the plates were observed for pink colour colonies (Non O157:H7) and colour less colonies (O157:H7).

Molecular characterization of isolates by multiplex PCR

For the molecular characterization of the isolates the reference strain for Shiga toxin producing *Escherichia coli* MTCC 1699 was obtained from Department of Veterinary Public Health & Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh.

During the molecular characterization of the isolates preparation of template DNA from *Escherichia coli* strains was carried and isolated. The primers used in the study were custom synthesized by M/s Eurofins Genomics, Banglore (India).The details of the primers are given in Table.2.

A multiplex PCR assay was developed through synthesis of specific primers targeting *stx1, stx2, eaeA* and *hlyA* were used in our study (10) with slight modifications. After rapid DNA extraction, 5 µl of bacterial DNA was added to a 25µl PCR mixture containing 50 mMKCl, 20mM Tris-HCl (pH 8.4), 2.5mM

MgCl2, 0.2mM each deoxynucleoside triphosphate (dATP, dUTP, dGTP, and dCTP) (Thermo, USA), 2.0 µl of each primer, and 1.0 U of *Taq*DNA polymerase (Thermo, USA). Amplification was performed by using a Thermal cycler (Corbett Research, Germany). Amplification was carried out at an initial denaturation of 94°C for 3 min followed by a cyclic denaturation at 94°C for 30 seconds, 55°C for 35 seconds and 72°C for 1 min for 35 cycles. Final extension step carried out at 72°C for 10 min. On completion of the reaction, tubes with PCR products were held at 4°C until further analysis/confirmation.

DNA amplified by PCR was subjected to 1.5% agarose gel electrophoresis (11). About 5 µl of each PCR product was mixed with 2 µl of bromophenol blue (6X) loading dye and loaded into each well. Electrophoresis was performed at 5 v/cm and the motility was monitored by the migration of the dye. After sufficient migration, the gel was observed under UV trans illumination using Alpha innotech gel documentation system to visualize the bands. The PCR product size was determined by comparing with a standard low molecular weight ladder marker.

Results and Discussion

The particulars of the samples which were positive for *Escherichia coli* were given in Table 3 and it's characterization on cultural characteristics on blood agar and MacConkey and EMB agar.

The isolates were subjected to the biochemical tests like IMViC tests, urease test and triple sugar iron agar tests. For further confirmation of Escherichia coli, all the isolates were streaked on blood agar plates and incubated at 37°C/24 hours. The results revealed that 319 isolates produced β haemolysis on blood agar plates.

For the phenotypic detection of shiga toxin producing *Escherichia coli*, all the *Escherichia coli* isolates from different sources were streaked on Sorbitol MacConkey agar plates and the plates were incubated at 37°C / 24 hr. The results revealed that none of the isolates have shown white colonies on Sorbitol MacConkey agar plates but a total of 158 isolates have shown pink colonies.

In the present study an oligonucleotide primer set was used which encodes the shiga toxins, intimin and entero haemolysins produced by the bacteria. Primers were selected on the basis of published nucleotide sequence of the 180 bp for *stx1*, 255 bp for *stx2*, 384 bp for *eaeA*and 534 bp for *hlyA* genes (Paton JC and Paton AW, 1998). With the desired PCR amplification of 180 bp for *stx1*, 255 bp for *stx2*, 384 bp for *eaeA*and 534 bp for *hly A*gene of shiga toxin producing *Escherichia coli* (Figure 1).

In the present investigation, *E.coli* isolated from the faecal samples of cattle from the dairy farm of College of Veterinary Science, Tirupati exhibited *stx1* (4.34%), *stx2* (2.89%) and *hlyA*(1.44%) genes, *E.coli* isolates from private dairy farm 1 revealed *stx1* (8.00%), *stx2* (4.00%) and *hlyA* (12.00%) genes, whereas the *E.coli* isolates from private dairy farm 2 exhibited all the four genes in various combinations viz., *stx1* (1.34%), *stx2 (*1.00%),, *eaeA* (1.34%), *hlyA* (1.00%), individually and a combination of *stx1* + *stx2* + *eaeA* (1.34%), and *stx1* + *stx2* + *eaeA* + *hlyA* (1.34%), In the private dairy farm 3 the *E.coli* isolates from the faecal samples of cattle revealed *stx1* (3.70%), *stx2* (2.77%), *eaeA* (0.92%) and *hlyA* (3.70%) genes individually and also a combination of *stx1* + *stx2* + *eaeA* + *hlyA* (1.85%) genes (Fig. 1). The percentage of toxigenic genes of the isolates of Shiga Toxin producing *Escherichia coli* of different sources was represented in Table 4.

From this study it is clear that pathogenesis of STEC is multifactorial and involves several virulence attributes of the organism. Rapid and sensitive methods for detection of STEC are now in force; especially there has been advance in PCR technology, which has increased the speed and has made it possible to quantitate the number of STEC organisms present in a suspected sample. These results substantiate those obtained by other methodological approaches followed by Fagan *et al.,* 1998, who detected the shiga toxin gens (*stx1* and stx_2), intimin (*eaeA*) gene and haemolysin (*hlyA*) genes in animal faeces by multiplex PCR assay and reported that 19.45% samples positive for *stx1* and 6.7% samples carried *stx2*, 35.9% samples positive for hlyA gene and 6.7% samples positive for the *eaeA* genes, Jamhidi *et al.,* (2015) observed the prevalence of shiga toxin producing *E. coli* in healthy cattle and reported that 15% isolates carried *stx1*, 19% possess *stx2* alone and 8% were positive for both *stx1* and *stx2* only, Hallwell *et al.,* (2016) detected shiga toxin genes in cattle faecal samples and reported that 85.8% positive for *stx1*, 60.0% carried *stx2* and 52.5% were posess *eaeA* genes, Mahanti *et al.,* (2013) analyzed the presence of shiga toxins by multiplex PCR and reported that 13.3% isolates carried *stx1* gene and 12.12% isolates positive for *stx2*. Sridhar *et al.,* (2017) reported the prevalence of shiga toxins in *E.coli* isolates from the cattle and reported that 48.4% isolates carried *stx1* gene, 22.4% possess *stx2* gene, and 29.2% samples positive for the both *stx1* and *stx2*, Paula and Marin (2008) observed the occurrence of non-O157 STEC in dogs with diarrhoea and they reported that 7.6% isolates positive for *stx1*, 5.4% for *stx2*, 9.8% possess *eaeA* genesand62% isolates carried *stx1, stx2, hlyA* and *eaeA* genes, Islam *et al.,* (2008) analyzed the prevalence and genetic characterization of STEC in slaughtered animals faecal samples of cattle, buffalos and goats and reported that buffalo and cattle faecal samples showed more prevalence for both *stx1* and *stx2* genes than *stx1* or *stx2* alone, Parul *et al.,* (2016)analyzed the STEC in cattle calves faeces samples and reported that 37.5% samples were positive for *stx1*,43.75% carried *stx2* and 18.75% possess both *stx1* and *stx2*, 18.75% and 34.37% isolates positive for *eaeA* and *hlyA* genes. Dastmalchi and Ayremlou (2012) observed the prevalence and characterization of STEC in faeces of health and diarrhoeic calves, and reported that, 23.1% of the isolates were positive for only *stx1*, 26.92% isolates carried *stx2* alone, 26.92% samples were positive for *eaeA* gene and 57.69% isolates were positive for *hlyA* gene. Bakshi *et al.,* (2014) observed the presence of shiga toxin genes (*stx1* and *stx2*) and intimin (*eaeA*) genes in the faecal samples of calves with diarrhoea, and reported that 21 isolates were positive for the *stx1, stx2* and *eaeA* genes, Kumar *et al.,* (2014) reported that among the 600 *E.coli* isolates, the *stx1*(117) gene was significantly more prevalent than *stx2*(53) and his study further revealed that the *eaeA* and the *hlyA* gene were significantly more prevalent in animal faecal isolates. In this study, an attempt was made to identify, isolate and characterize STEC from animals with the help of available modern method i.e. PCR. The samples were collected from different sources like organized dairy farms and local dairy units. This study showed PCR to be more sensitive to characterize STEC. The isolation of STEC was more in diarrhoeic cattle in the present study which correlated with the study of Brunen's *et al.,* (2004) as rearing of cattle is a very common practice in Andhra Pradesh particularly in the rural areas. Again, proper hygienic practices and sanitary measures are lacking while handling of the cattle in these areas. Thus, it may be presumed that the diarrhoeic cattle's particularly can be an important source of STEC causing human enteritis. Therefore, emphasis should be given to screen the people suffering from diarrhoea for presence of STEC as a part of surveillance system. This will

enable to reveal the actual magnitude of the problem caused by STEC and also give early warning regarding any outbreaks in future.

Four water samples from four different dairy farms were screened for *E.coli* in the present investigation and the results revealed that all the four samples were positive for *E.coli* and among them the isolates from the water source of private dairy farm 2 exhibited *stx1* and *stx2* genes. Shojaei M (2017) studied about the virulence factors of STEC in drinking water and reported that out of 200 water samples 10 were positive for STEC among these one isolate was positive for *stx1, eaeA* and *ehlyA* genes and 4 isolates were positive for *stx1* and one for *stx2*. Lascowki *et al.,* (2013) reported through their investigations that *Stx* has been implicated as a possible pathogenic agent in drinking water responsible for gastrointestinal illness outbreaks and further in all of these cases, the presence of either *stx1* and /or *stx2* genes were confirmed, but the actual amount of bacterial isolates that contained these genes in the water source was not identified. They examined 1850 water samples among these 300 were positive for *E. coli* in these 300 also only 12 isolates were positive for STEC i.e. two positive for *stx1*, 5 positive for *stx2* and 5 positive for both *stx1* and *stx2* at the same time no isolate was possess *eaeA* gene but all 12 were positive for *ehxA* gene. Ram *et al.,* (2007) studied about the Prevalence of multi-antimicrobial-agent resistant, Shiga toxin and enterotoxin producing *Escherichia coli* in surface waters of river Ganga and reported that 30% of the 60 screened *E. coli* isolates from these water sources contained either *stx1* or *stx2*, Halabi *et al.,* (2008) studied about the prevalence of Shiga toxin-, intimin- and haemolysin genes in *Escherichia coli* isolates from drinking water supplies in a rural area of Austria and 200 *E. coli* isolates were obtained from various drinking water sources and reported that only one was found to contain *stx2,* Ram *et al.,* (2007) have analyzed *stx* gene presence in bacterial isolates obtained from water samples indicate an even higher percentage of *stx* gene presence in recreational waters and their results revealed that, *stx* genes were present in 22.7% of *E. coli* isolates obtained from the river Ganga. Duris *et al.,* (28) have analysed *stx* gene presence in bacterial isolates obtained from water samples and revealed that greater than 50% of faecal coliform isolates tested positive for *stx2* DNA in river water in Maryland and river water samples obtained in Michigan and Indiana. Waste water has been attributed to pollution of recreation and drinking water in specific cases (Lienemann *et al.,* 2011) the general efflux of these to a water supply remains unknown, especially in cases where water treatment facilities are in effect. However, Walters *et al.,* (2011) stated that attempts to relate *stx* gene or *stx*-producing organism presence to indicators of wastewater or faecal pollution have failed to show a correlation in many cases. This suggests that other factors besides general wastewater efflux explain the presence of microbes harbouring or expressing *stx* in aquatic environments. These limited studies set a wide range for which to compare *stx* distribution and abundance in other drinking water systems, will give information which is important to know the prevalence of *stx*-dependent illness arising from drinking water both in animals and human beings.

In the present investigation 28 human faecal samples were screened for *E.coli* and 18 isolated could be identified as *E.coli.* Among these 18 isolates *stx1, eaeA* and *hlyA* genes were carried by one isolate, 1 isolate carried only *hlyA* gene and another isolate carried *stx2* and *hlyA* genes. The findings of this study are in association with Kumar *et al.,* (2014) isolated 11 STEC from 600 human faecal samples with bloody and mucus diarrhoea and reported that the *stx*1 gene was the most common virulence gene, present in 11 (100%) isolates followed by *stx*2 in six (54.5%)

isolates and further they reported that the *eaeA* gene was present in isolate from HUS patients only, Kumar et. al.(2014) reported that among the 600 *E.coli* isolates, the *stx2* gene was significantly more prevalent in animal faecal samples than in human stool isolates, and his study further revealed that the *eaeA* gene was present in 4 human stool isolates and the *hlyA* gene was significantly more prevalent in animal faecal isolates than in human stool isolates. Abassi and Elahe (2015) observed the prevalence of STEC in urine samples of patients suspected with UTI, by using multiplex PCR assay by targeting s*tx1* and s*tx2* genes and reported that 76 samples were positive for *E. coli* and 5 STEC strains were isolated by multiplex PCR and further reported that 5 isolates positive for *stx1*, 2 carried *eaeA* and no isolate was positive for *stx2*. Haugum *et al.*, (2014) screened 12,651 stool samples of patients for a 5 years period among these 138 patient were infected with STEC in 138 positive STEC 108 isolates positive for *eaeA* gene, 57 isolates carried *stx1*, 50 possess *stx2* and 21 were positive for both *stx1* and *stx2*. Virpari *et al.*, (2013) reported that 59 *E. coli* isolates that were obtained from 100 faecal samples, of which 10.16% were positive for *stx1*, 15.25% possess *stx2* and 5.08% were positive for *eaeA* gene. Sehgal *et al.*, (2008) conducted 10 years epidemiological survey of *E. coli* O157 across India in humans and among 5678 human samples, the survey results showed that only 30 (0.5%) samples were positive for *E. coli* O157.

Table.1 Source and number of samples collected

S.No	Source of the sample	Number collected
1.	Dairy farm, College of Veterinary Science, Tirupati	73
2.	Private dairy farm 1	25
3.	Private dairy farm 2	302
4.	Private dairy farm 3	108
5.	Water samples from the dairy farms	04
6.	Human faecal samples	28
	TOTAL	540

Table.2 Details of oligonucleotide primers used in this study

Primer	Target gene	Primer sequence (5^1 -3^1)	Expected amplicon size(bp)	Reference
***stx 1*: F**	*stx1*	ATAAATCGCCATTCGTTGACTAC	23	**Paton and paton (1998)**
***stx 1*: R**		AGAACGCCCACTGAGATCATC	21	
***stx 2*: F**	*stx2*	GGCACTGTCTGAAACTGCTCC	21	
***stx 2*: R**		TCGCCAGTTATCTTGACATTCTG	22	
***eaeA*: F**	*eaeA*	GACCCGGCACAAGCATAAGC	20	
***eaeA*: R**		CCACCTGCAGCAACAAGAGG	20	
***hlyA*: F**	*hlyA*	GCATCATCAAGCGTACGTTCC	21	
***hlyA*: R**		**AATGAGCCAAGCTTGTTAAGCT**	**22**	

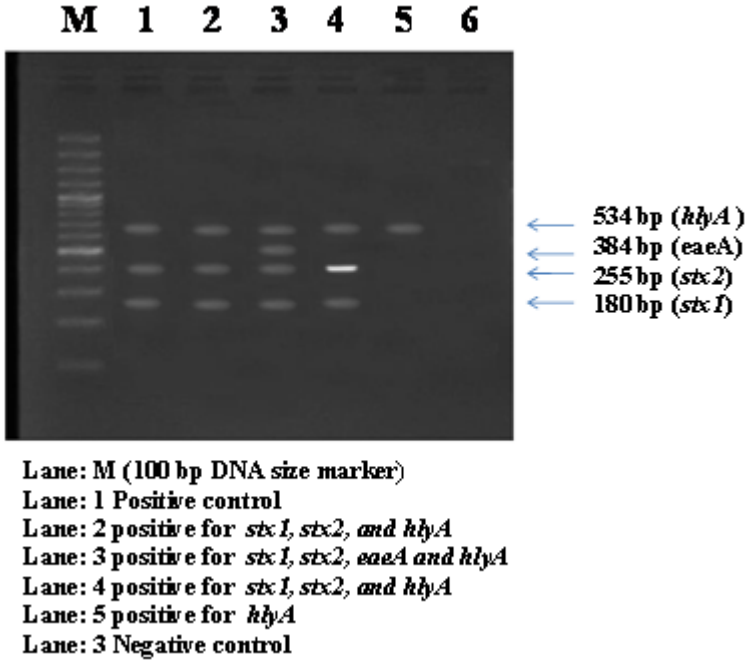
Table.3 Prevalence of *Escherichia coli* from various sources

S.No	Source	No. of samples screened	No. of samples positive for *E.coli*	No. of samples positive for blood agar plate test
1	Dairy farm College of Veterinary Science, Tirupati	73	69 (94.52%)	44 (63.76%)
2	Private dairy farm 1	25	25 (100.00%)	12 (48.00%)
3	Private dairy farm 2	302	298 (98.67%)	173 (58.04%)
4	Private dairy farm 3	108	108 (100.00%)	86 (79.62%)
5	Water samples	04	04 (100.00%)	00 (00.00%)
6.	Human faecal samples	28	18 (64.28%)	04(22.22%)
	TOTAL	540	522 (96.66%)	319 (61.11%)

Table.4 Screening and comparison of culture method for detection of *Escherichia coli* and multiplex PCR for detection of Shiga toxin producing *E.coli*

S. No	Source	No. of samples screened	No. of *E. coli* by culture method	Multiplex PCR for *stx1*, *stx2*, *eaeA* and *hlyA* genes					
				stx1	***stx2***	***eaeA***	***hlyA***	***stx1, stx2, eaeA***	***stx1, stx2, eaeA* and *hlyA***
1	Dairy farm C.V.Sc., Tirupati	73	69	3 (4.34%)	2 (2.89%)	---	1 (1.44%)	---	---
2	Private dairy farm 1	25	25	2 (8.00%)	1 (4.00%)	---	3 (12.00%)	---	---
3	Private dairy farm 2	302	298	4 (1.34%)	3 (1.00%)	4 (1.34%)	3 (1.00%)	4 (1.34%)	4 (1.34%)
4	Private dairy farm 3	108	108	4 (3.70%)	3 (2.77%)	1 (0.92%)	4 (3.70%)	---	2 (1.85%)
5	Water samples	04	04	1 (25.00%)	1 (25.00%)	---	---	---	---
6	Human faecal samples	28	18	1 (5.55%)	1 (5.55%)	1 (5.55%)	3 (16.66%)	---	---
Grand total		540	522	15	11	6	14	4	6

Figure.1 Detection of *Stx*$_1$, *Stx2*, *eae* and *hlyA* genes in samples collected from cattle in different private farms



Dhanashree and Mallya (2008) screened the diarrhoeagenic stool samples for STEC by culture methods and PCR and reported that 2 were positive for *hlyA* and belonging to serogroup O8 and also 110 *eaeA* positive *E. coli* isolated recovered from stool samples.

In India various studies conducted have found either no or rare occurrence of STEC in humans in India. Except for diarrhoeal outbreaks (Kang, 2001), STEC does not pose a major threat to human health in India. It is not clear that why the incidence of STEC is low in India and other developing countries, despite having all the reservoirs and STEC in the food chain. Some researchers have argued that under reporting of the incidence is the cause, where as other showed that due to the presence of pre-existing *stx*1, IgG antibodies in asymptomatic cases offers positive protection against STEC infection (Karmali, 1989). But the frequent isolation of STEC strains from non human sources like animals, food and other products along with the identification of multidrug resistance and virulence genes across the Indian subcontinent poses a serious threat of the outbreaks that can occur in the future.

Acknowledgement

The authors are thankful to the Sri Venkateswara Veterinary University for providing necessary fund and facilities to do research in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati.

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How to cite this article:

Venkateswara Rao, K., A. Jagadeesh Babu, T. Madhava Rao, Chinta Siva Swetha, P. Ramya, Deepak and Somasekhar, S. 2018. A Study on the Incidence of Shiga Toxin Producing *Escherichia coli* in Dairy Cattle and Its Environment. *Int.J.Curr.Microbiol.App.Sci.* 7(11): 2461-2472. doi: https://doi.org/10.20546/ijcmas.2018.711.281