RESEARCH ARTICLE

Molecular Characterisation and Antimicrobial Resistance Patterns of Shiga Toxin Producing *Escherichia coli* Isolated from Farm Water Samples

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) strains are considered the most common food-borne zoonotic pathogen and are highly pathogenic to humans in low infectious doses, causing food-borne diseases through consumption of contaminated water or food. Resistance against antibiotics by STEC is a big concern nowadays. Two hundred farm water samples (Cattle-40, Buffaloes-40, Sheep-30, Goat-20, Pig-20 and poultry-50) were collected aseptically from different livestock farms in and around Proddatur, Andhra Pradesh, India and Processed for E. coli isolation, identification with culture method and molecular characterization by PCR. E. coli was characterized as STEC with two genes i.e. stx1 and stx2. All the STEC isolates were subjected to an antibiotic sensitivity test by disc diffusion method against ten antibiotics. Results showed that out of 200 farm water samples, 196 were positive for *E. coli* with an overall prevalence of 98% (196/200) and 62.2% (122/196) for STEC by PCR. Antimicrobial susceptibility test by disc diffusion method against ten antibiotics revealed the higher resistance to Cepahlothin (100%) followed by Tetracyclin (98.4%), Ampicillin (96.7%), Streptomycin (95%), Sulphonamides (91.8%), Trimethoprim (84.4%), Kanamycin (34.4%), Chloromphenicol (17.2%), Colistin (9.0%) and least resistance to Gentamycin (4.9%).

Keywords: Antimicrobial resistance, Escherichia coli, Farm water samples, Shiga toxin producing

Ind J Vet Sci and Biotech (2022): 10.48165/ijvsbt.18.4.15

Introduction

Shiga toxin-producing *E. coli* (STEC) strains are considered the most common food-borne zoonotic pathogen causing various disease conditions in animals and humans (Kumar *et al.,* 2014). Ruminants are considered as an important source of STEC and cattle are regarded as the primary reservoir (Perera *et al.*,2015). STEC strains are highly pathogenic to human in low infectious doses, causing food-borne diseases through consumption of contaminated water or food (Dweik *et al.,* 2012). Shiga toxin (stx1 & stx2) is the key factor in STEC pathogenesis (Koutsoumanis *et al.,* 2020) which is toxic to human colonic, ileal epithelial and endothelial cells. These STEC strains, especially those with stx2, cause a variety of human diseases ranging from diarrhea (Bruyand *et al.,*2018) to hemorrhagic colitis (HC), thrombotic thrombocytopenia purpurea (TTP), and hemolytic uremic syndrome (HUS) with fatal consequences (Walker *et al.,* 2012).

The detection of STEC strains by conventional methods is laborious and time consuming and there is a possibility of getting false results (Orth *et al.,* 2009). Molecular methods are sensitive, specific, and rapid as it is less laborious, time saving compared to immunology-based techniques. The development of PCR-based methods for detecting pathogens or virulence factors (Shahi *et al.,*2013) can be used for the initial screening of the presence of microorganisms from different samples.

Hence, considering the above facts, the present study was designed to detect the prevalence and antimicrobial

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How to cite this article: Putturu, R., Kumar, M.S., Babu, A.J., Singh, S. & Reddy, A.G. (2022)., Molecular Characterisation and Antimicrobial Resistance Patterns of Shiga Toxin Producing Escherichia coli Isolated from Farm Water Samples. Ind J Vet Sci and Biotech. 18(4), 68-72.

Source of support: Nil

Conflict of interest: None.

Submitted: 30/03/2022 **Accepted:** 23/07/2022 **Published:** 10/09/2022

resistance patterns of STEC isolates from water samples of different livestock farms in and around Proddatur, Andhra Pradesh, India.

MATERIALS AND METHODS

A total of 200 samples (randomly collected) of farm water (Cattle-40, Buffaloes-40, Sheep-30, Goat-20, Pig-20 and poultry-50) were collected from different livestock farms in and around Proddatur, Andhrapradesh and analyzed for the presence of E.coli by cultural method and STEC by PCR. All the samples were collected aseptically and pre-enriched in buffered peptone water, incubated at 37^0C for 24 h. After pre-enrichment 1 mL of each inoculum was transferred into selective broth (HI media)i.e., Tryptic soy broth and incubated at 37^0C for 24 h. All the enriched samples were subjected to selective isolation by MacConkey agar and Eosine Methylene Blue (EMB) agar and confirmed by using biochemical tests.

PCR protocol

The biochemically confirmed *E.coli* isolates were subjected to PCR to confirm STEC by using primers specific to stx1 and stx2 (Manna *et al.,* 2006) (Table-1), synthesized from Merck GeNeiTM. The DNA from the selective broth cultures was extracted by Boiling and snapchilling (Arora *et al,* 2006). The amplification protocols of PCR for stx1 and stx2 gene was standardized as described by (Arora *et al.* 2006). The multiplex PCR protocol was standardized in a volume of 25 µL of reaction mixture containing 2 µL of TaqDNA Polymerase (1 unit/ µL), 2.0 µL of DNA template solution, 2.5 µL of 10 x reaction buffer, 0.5 µM of deoxynucleoside triphosphates (dNTPs), 0.2 µM each of the 4 primers (2 primer pairs) and magnesium chloride (MgCl2). Sterile nuclease free water was added to make up the volume 25 µL of the reaction mixture.

The cycling conditions for PCR included an initial denaturation of DNA at 94°C for 5 min, followed by 35 cycles of 94°C for 30s denaturation, 50°C for 1 min annealing, extension at 72°C for 1 min, followed by the final extension of 10 min at 72°C and hold at 4°C. The final amplified product was analyzed by agarose gel electrophoresis on 2% agarose gel and visualized under gel documentation system. The samples that showed the presence of anyone of stx1, stx2 genes were considered positive for STEC, and the samples without any genes were considered non-STEC isolates.

All the positive STEC isolates by PCR from farm water samples were subjected to antibiotic sensitivity test by using disc diffusion method against 10 different antibiotics (HI-Media Laboratories) such as Ampicillin (AMP) 10 µg/ disc, Cephalothin (CEP) 30 µg/disc, Chloromphenicol (C) 30 µg/ disc, Colistin (CL) 10 µg/disc, Gentamycin (GEN) 10 µg/disc, Kanamycin (K) 30 µg/disc, Sulphonamides (SSS) 300 µg/disc, Streptomycin (S) 10 µg/disc, Tetracycline (TE) 30 µg/disc and Trimethoprim (TR) 5 µg/disc.

RESULTS AND DISCUSSION

Prevalence of *E. coli*

The prevalence of *Escherichia coli* was 100% (40/40; 20/20 and 50/50) in buffaloes, pigs, and poultry farm water samples, followed by 97.5% (39/40) in cattle farm water samples, 96.66% (29/30) in sheep farm water and 90% (18/20) in goat farm water samples with overall prevalence of *E. coli* in water samples from various farms as 98%(196/200). Rao (2015) reported 100% prevalence in cattle farm water samples from private dairy farm, which was almost similar to the prevalence in the present study, whereas Makhado *et al.* (2020) reported an overall prevalence of 92% of *E.coli* from pooled farm water samples, which was slightly lower than the present study. On the contrary, a low prevalence of 29.7%, *E. coli* from drinking water samples and 30.2% from poultry farm water samples was reported by Rao *et al.* (2011) and Ahmad *et al.* (2009) respectively.

Prevalence of STEC

Out of 196 *Escherichia coli* isolates from farm water samples,

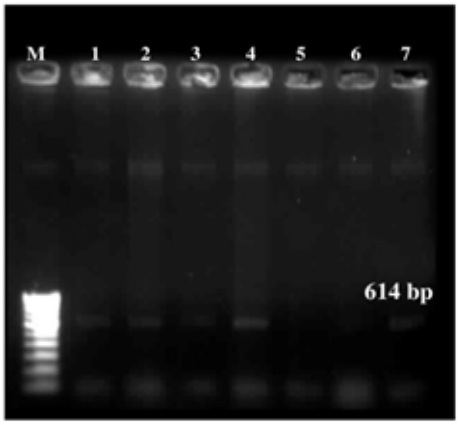

Fig. 1: Results of Farm Water samples for stx1 Lane M : 100 bp DNA Ladder Lane 1,2,4 & 7 Positive farm water samples for stx1 Lane 3,5,6 **:**Negative farm water samples for stx1

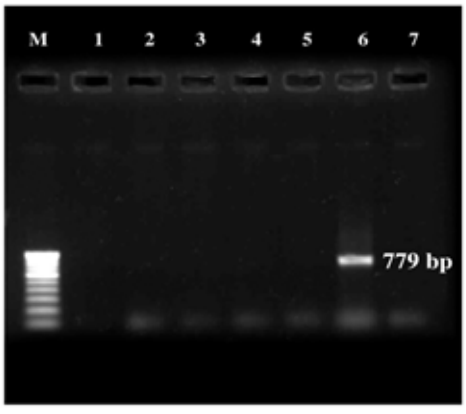

Fig. 2: Results of Farm Water samples for stx1or stx2 Lane M : 100 bp DNA Ladder Lane 6 : Positive farm water samples for stx2 Lane 1,2,3,4,5,7 : Negative farm water samples for stx2

the overall prevalence of STEC was 62.24%(122/196), with 13.11%(16/122) isolates harbouring stx1 (Fig. 1), 55.73%(68/122) harbouring stx2 (Fig. 2) and 31.14% (38/122) harboring both stx1 and stx2 (Table-2). Our study indicated that the highest prevalence (85%) (17/20) of STEC was noticed from pig farm water samples, followed by 66.66%(12/18) in goat farm water samples, 64.1% (25/39) in cattle farm water samples, 62% (31/50) in poultry farm water samples, 55.17% (16/29) in sheep farm water samples and least 52.5% (21/40) in buffalo farm water samples. El-Shatoury *et al.* (2015) reported a slightly higher prevalence of (66%) STEC in pooled water samples than the present study (62.24%), whereas Moore *et al.* (2004), Benedict *et al.* (2014), Kiranmayi and Krishnaiah (2011) and Rao (2015) reported prevalence of 0.4%, 7%, 40% and 50% of STEC respectively in cattle farm water samples compared to the prevalence in the present study (64.1%).

Higher prevalence of stx2 than stx1 in pooled water samples was observed in the present study and similar trend was reported by Padola *et al.* (2004), Manna *et al.* (2006), Yilmaz *et al.* (2006) and Ongor *et al.* (2007), whereas contrary to the present findings Kiranmayi and Krishnaiah (2011) reported higher prevalence of stx1 than stx2.

In cattle farm water samples, 16, 52, and 32% of STEC positive isolates harbored stx1, stx2 and both stx1 and stx2, respectively. These results are nearly similar to Kiranmayi and Krishnaiah (2011), who reported a higher prevalence (40%) of stx1 and lower prevalence of 13.3% and 6.6% of stx2 and both stx1 and stx2, respectively from cattle farm water samples. Rao (2015) reported a higher prevalence of 50 and 33.3% of stx1 in Government and private cattle farms, respectively, whereas low prevalence of 33.3 and 16.7% of stx2 in Government and private cattle farms, respectively. El-Jakee *et al.* (2009) reported a higher prevalence (57.1%) of stx1 and a lower prevalence (28.6%) of stx2 than the present study.

Our study revealed that 14.2, 52.3 , and 33.3% of STEC positive isolates harbored stx1, stx2 and both stx1 and stx2 genes were observed in buffaloes. In sheep farm water samples, 12.5, 56.3 and 31.3% of STEC positive isolates possessed stx1, stx2 and both stx1 and stx2 genes, respectively, whereas Kiranmayi and Krishnaiah (2011) reported almost similar prevalence (13.6%) of stx1 and lower prevalence of 4.5% and 4.5% of stx2 and both stx1 and stx2, respectively from sheep farm water samples compared to the prevalence in the present study.

In goat farm water samples, 8.3, 58.3, 33.3% of STEC positive isolates harbored stx1, stx2 and both stx1 and stx2 genes respectively. In Pigs 11.7, 58.8 and 29.4% of STEC positive isolates harbored stx1, stx2 and both stx1 and stx2 genes, respectively.

In poultry farm water samples 12.9, 58, 29% of STEC positive isolates harbored stx1, stx2 and both stx1 and stx2 genes, respectively, whereas Kiranmayi and Krishnaiah(2011) reported higher prevalence (40.0%) of stx1 and lower prevalence of 20.0% and 0% of stx2 and both stx1 and stx2 respectively from poultry farm water samples compared to the prevalence in the present study. Variations in the distribution of stx toxins among the animal species might be due to the makeup of the species, habits during feeding and behavior during changes in the environmental conditions (Ateba and Mbewe, 2011).

Antibiotic Susceptibility of STEC Isolates

The positive STEC isolates by PCR from water samples of different livestock farms were highly resistant to Cepahlothin (100%), followed by Tetracyclin (98.4%), Ampicillin (96.7%), Streptomycin (95%), Sulphonamides(91.8%), Trimethoprim (84.4%), Kanamycin (34.4%), Chloromphenicol (17.2%),

Table-1:Sequence of the oligonucleotide primers used for identification of STEC

Primer	Target gene	Primer sequence (51-31)	Expected amplicon size(bp)
stx 1: F	stx1	ATAAATCGCCATTCGTTGACTAC	614
stx 1: R		AGAACGCCCACTGAGATCATC	
stx 2: F	stx2	GGCACTGTCTGAAACTGCTCC	779
stx 2: R		TCGCCAGTTATCTTGACATTCTG	

Table-2: Prevalence of STEC from water samples of different farms

S.No	Source	No.of samples positive for *E.coli*	STEC by PCR(%)	Presence of shiga toxin (stx)		
				stx1	stx2	Both stx1 and stx2
1.	Cattle	39	25(64.1)	04	13	08
2.	Buffaloes	40	21(52.5)	03	11	07
3.	Sheep	29	16(55.17)	02	09	05
4.	Goat	18	12(66.66)	01	07	04
5.	Pig	20	17(85)	02	10	05
6	Poultry	50	31(62.0)	04	18	09
	Total	196	122(62.24)	16(13.11)	68(55.73)	38(31.14)

Table-3: Antibiotic Resistance of STEC isolated from water samples (122) from different livestock farms

S. No.	Antibiotic	No. of Sensitive isolates (%)	No. of Intermediate resistant isolates (%)	No. of Resistant isolates (%)
1.	Ampicillin(10µg)	02 (1.6)	02(1.6)	118 (96.7)
2.	Cephalothin(30µg)	0	0	122 (100)
3.	Chloramphenicol(30µg)	81 (66.4)	20 (16.4)	21 (17.2)
4.	Colistin(10µg)	105 (86)	06 (4.9)	11 (9.0)
5.	Gentamycin(10µg)	106 (86.8)	10 (8.2)	6 (4.9)
6.	Kanamycin (30µg)	64 (52.4)	16 (13.1)	42 (34.4)
7.	Sulphonamides(300µg)	02 (1.6)	08 (6.5)	112 (91.8)
8.	Streptomycin (10µg)	01 (0.8)	05 (4)	116 (95)
9.	Tetracycline (30µg)	01 (0.8)	01 (0.8)	120 (98.4)
10.	Trimethoprim (5µg)	07 (5.7)	12 (9.8)	103 (84.4)

Colistin (9.0%). At the same time, they were least resistant to Gentamycin (4.9%) and highly sensitive to Gentamycin (86.8%), followed by Colistin (86%), Chloromphenicol (66.4%), Kanamycin (52.4%), Trimethoprim (5.7%), Ampicillin and sulphonamides (1.6%), Streptomycin and Tetracycline (0.8%) and no sensitivity to Cephalothin in the present study (Table 3).

High resistance (96.7%) against ampicillin by the STEC positive isolates from farm water samples was observed in the present study, whereas Fashina *et al.* (2018) observed only 23.8% of the resistance.

Fashina *et al.* (2018) observed 100% resistance against cephalothin for STEC-positive farm water samples; similar resistance was observed in the present study. Resistance to cephalothin in E. coli strains isolated from different sources is consistent worldwide (Colello *et al.,* 2015), indicating the widespread and lengthy use of cephalothin for treating human diseases and as a growth promoter in animals.

Fashina *et al.* (2018) observed 30.7% resistance against gentamycin for STEC-positive farm water samples, which was higher than the resistance (4.9%) observed in the present study. Higher sensitivity (86.8%) for gentamycin was observed in the present study.

Higher resistance of 98.4% against tetracyclines by the STEC isolates from farm water samples was observed in the present study, whereas zero resistance was reported by Fashina *et al.* (2018) and very low resistance (7%) by El-Shatoury *et al.* (2015). Resistance to tetracycline in E. coli strains isolated from different sources is consistent worldwide (Colello *et al.,* 2015), indicating the widespread and lengthy use of tetracycline for treating human diseases and as a growth promoter in animals.

Conclusion

This study revealed a high prevalence of shiga toxin-producing *E. coli, i.e.,* 62.2% from farm water samples which indicates recent sewage or animal waste contamination. Taking proper personal hygiene and environmental sanitation measures, further transmission can be avoided/ minimized. The present study also showed resistance of STEC isolates towards Cepahlothin, Tetracyclin, Ampicillin, Streptomycin, Sulphonamides, Trimethoprim etc., which may be due to misuse of antibiotics.

Acknowledgment

The authors are grateful to the PVNRTVU, Hyderabad and SVVU, Tirupathi for providing facilities to carry out the work.

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