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Detection of Antimicrobial Resistance Genes from Shiga Toxin Producing *Escherichia coli* by Multiplex PCR

Ramya Putturu[1], Manyam Shashi Kumar[1], Angalakudithi Jagadeeshbabu[1], Sujatha Singh[1], Eevuri Tirupathi Reddy[1], Alla Gopala Reddy[1]

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ABSTRACT

Background: Shiga toxin-producing *Escherichia coli* (STEC) strains are considered to be most common food-borne enteric zoonotic pathogen, causing various disease conditions in both animals and humans and are highly pathogenic to human in low infectious doses. Resistance against antibiotics by STEC is also a big concern now a days. Hence in view of the public health significance of STEC, present work was planned to know the combination of phenotypic and genotypic resistance patterns against certain most commonly used antibiotics by using disk diffusion method and multiplex PCR respectively.

Methods: A total of 426 PCR confirmed STEC isolates isolated from pooled samples (animal faecal(179), farm water(122) and human faecal samples (125) of different livestock farms in and around Proddatur andhra Pradesh, were subjected to antibiotic sensitivity test by using disc diffusion method. The isolates that showed resistance for tetracycline, streptomycin, sulphonamides and ampicillin were selected and subjected to multiplex PCR for the detection of resistance genes.

Result: Disk diffusion assay revealed highest phenotypic resistance for STEC isolates against Cepahlothin (100%), followed by Ampicillin (99.06%), Tetracycline (97.2%), Streptomycin (94.3%), Sulphonamides (90.8%) and Trimethoprim (84.5%). Pooled samples also revealed the presence of antimicrobial resistance genes like *tetA*(59.2%), *tetB*(43.5%), *tetC* (9.2%), *strA* (39.3%), *strB*(54.1%), *sul1* (40.8%), *sul2* (58.7%), *sul3* (3.8%) and *blaTEM*(83.4%). These findings indicate the highest prevalence of antimicrobial resistance among the STEC isolates, which alarms indiscriminate use of antibiotics both for therapeutic purpose and as growth promoters. Strict hygienic, sanitation and HACCP programmes should be applied to counter *STEC* prevalence.

Key words: Antimicrobial resistance, Genotype, PCR, Shiga toxin producing *Escherichia coli*.

INTRODUCTION

Escherichia coli is one of the main inhabitants of the intestinal tract of most mammalian species, including humans. Hence recovery of *Escherichia coli* from livestock products and environmental samples like water is used as reliable indicator of faecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms, which constitute a public health hazard. Shiga toxin producing *Escherichia coli* (STEC) forms major food borne pathogen that produces shiga 1 and shiga 2 toxins that affects human health (Koutsoumanis *et al*., 2020).

Most pathogenic *E. coli* are transmitted by fecal-oral route from food materials, water, animals and environment. Humans may also acquire STEC infections primarily from consumption of undercooked beef, raw milk, meat and dairy products, vegetables, unpasteurized fruit juices and water contaminated with faeces of animal (Molina *et al*., 2003). These STEC isolates, specially those with *stx2*, cause a variety of human illnesses ranging from diarrhoea to hemorrhagic colitis (HC), thrombotic thrombocytopenia purpurea (TTP) and hemolytic uremic syndrome (HUS) with fatal consequences (Walker *et al*., 2012).

Antibiotic usage is probably the most important factor that promotes the emergence, selection and dissemination of antibiotic resistance in both veterinary and human medicine. The increase in antimicrobial resistance in STEC is an emerging problem worldwide, as this resistant bacteria disseminates resistance to human pathogens. The resistance genetic mechanisms involved in the transfer of resistance from one strain of STEC to other may be by transfer of plasmids or by mobile genetic elements (Schwarz and Dancla, 2001). As much work has not carried on the antibiotic resistance of STEC isolates from animal and human sources of our area, the present work was carried out to study the antibiogram of STEC isolates and the genes responsible for specific antibiotic resistance from faecal samples of animals and humanbeings along with water samples of different farms as a part of PhD research work from 2018-2020.

[1]Department of Veterinary Public Health and Epidemiology, Sri Venkateswara Veterinary University, Tirupathi-517 502, Andhra Pradesh, India.

Corresponding Author: Ramya Putturu, Department of Veterinary Public Health and Epidemiology, Sri Venkateswara Veterinary University, Tirupathi-517 502, Andhra Pradesh, India.
Email: puttururamya@gmail.com

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MATERIALS AND METHODS

Antibiotic susceptibility testing

Out of 838 *E. coli* isolates isolated from pooled samples, (426-animal faecal, 196-farm water and 216- human faecal samples) 426 isolates (179 animal faecal, 122 farm water and 125 human faecal samples) were confirmed as STEC by multiplex PCR in a previous study conducted at Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Proddatur, YSR Kadapa District andhrapradesh, India. In the present study, all the confirmed STEC isolates were subjected to antibiotic sensitivity test by using disc diffusion method (Bauer *et al*., 1966) with Muller Hinton agar, against 10 different antibiotics (Hi MEDIA Laboratories) and the results were documented as sensitive (S), intermediate (I) and resistant (R).

Genomic DNA extraction

STEC isolates that showed resistance for tetracycline, streptomycin, sulphonamides and ampicillin were tested for the presence of AMR genes by PCR for which template is needed, extracted by Boiling and Snap chilling method (Lee, 2003). The suspensions were boiled, cooled and centrifuged and the supernatant was used as template for multiplex PCR.

Detection of antibiotic resistance genes

The multiplex PCR protocol was standardized in a volume of 25 ìl of reaction mixture. Thermal cycling was performed using the 96-Well Q-Sat 96 Thermal Cycler® and the PCQB software. Cyclic conditions followed are an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for *tetA*, *tetB* and *tetC*, 55°C for *strA* and *strB*, 68°C for *sul1*, 66°C for *sul2*, 51°C for *sul3*, 55°C for *blaTEM* for 1 min and elongation at 72°C for 1 min and final elongation at 72°C for 7 min (Boerlin *et al*., 2005). The primers used for the detection of different genes are listed in the Table 1. The multiplex PCR products were visualized after agarose gel electrophoresis using UV transilluminater.

RESULTS AND DISCUSSION

Antibiotic resistance/sensitivity

The present study evaluated prevalence of antimicrobial resistance and molecular profile of STEC isolates from animal, human and environmental origin and it is noteworthy to find resistance for different antibiotics (Table 2) along with the presence of resistance genes for antibiotics like ampicillin, tetracycline, streptomycin and sulphonamides. The results in the present study were similar to the reports of Collelo *et al*. (2018), Gentle *et al*. (2020), Parvez-Munoz *et al*. (2021) and many other researchers. Antimicrobial treatment for STEC infections is controversial (Begum *et al*., 2018) and it is generally not recommended, but the indirect selection for multiresistant strains could occur due to the antibiotic induced selection pressure on other diseases causing bacteria. In addition, antimicrobials are widely used prophylactically, metaphylactically and as growth promoters in animal husbandry (Cabello and Godfrey, 2016).

Genotypic identification of tetracycline resistance genes

Phenotypically tetracycline resistant STEC isolates from different samples were subjected for mPCR to detect *tetA* (Fig 1), *tetB* and *tetC* genes (Table 3). Among 171, 121, 123 tetracycline resistant STEC isolates respectively from pooled animal faecal samples, water samples and human faecal samples, highest prevalence of *tetA* was noticed ranging from 53.7% to 65%. The prevalence (59.1%) of *tetA* observed in the present study was almost similar to the prevalence (60%) reported by Rao *et al*. (2011), higher than the prevalence (50%) observed by Hameed *et al*. (2017) and lower than the prevalence (65.1%) observed by Meselle *et al*. (2017). In the present study *tetA* was dominant gene and similar observations were made by Li *et al*. (2013), whereas on contrary Tang *et al*. (2011) reported higher prevalence of *tetB* (49.8%) than *tetA* (24.0%) in *E. coli* isolates from pigs raised under overuse of antimicrobials in China. Bryan *et al*. (2004) and Srinivasan *et al*. (2007) reported dominance of *tetB* and *tetC,* whereas Velusamy *et al*. (2007) reported the higher prevalence of *tetA* and *tetC* than *tetB*. Diarra *et al*. (2009) reported the prevalence of *tetB* only. Rao *et al*. (2011) reported lower prevalence (27%) of *tetB* and slightly higher prevalence (12%) of *tetC* compared to the present findings. Bok *et al*. (2015) reported a very low prevalence of *tetA*, *tetB* and *tetC* genes as 25.7%, 2.9% and 2.9%. Velusamy *et al*. (2007) reported that 79.8% of STEC O157H7 and 91.7% O157H7- isoalates carried one or more antimicrobial resistant genes regardless of whether phenotypically resistant or susceptible.

All most all tetracycline resistant isolates harbored *tetA* and/or *tetB* as the tetracycline has been used worldwide in both human and veterinary medicine due to being able to select resistance strains (Maynard *et al*., 2003). Resistance to tetracycline is encoded by more than 40 genes (tet-genes) and they are divided into 11 classes, with a majority of classes (60%) encoding for membrane-associated efflux proteins. These efflux pumps selectively transport tetracycline from the cytosol to the periplasm, thereby limiting the access of tetracycline to the ribosomes in the cell (Tuckman *et al*., 2007). *Tet* (A) is the most common efflux pump type found in commensal and clinical *Escherichia coli* animal isolates (Zhang *et al*., 2012). The absence of all the above three genes in any of the phenotypically tetracycline resistant isolates might have carried other genes like *tetM* (Chopra and Roberts, 2001).

Genotypic identification of streptomycin resistance genes

Phenotypically streptomycin resistant STEC isolates were subjected for mPCR to detect *strA* and *strB* (Fig 2) genes (Table 3). Out of 167, 116, 119 streptomycin resistant STEC isolates respectively from pooled animal faecal samples, farm water samples and human faecal samples, highest prevalence of *strB* (53.8% to 56.9%) was noticed from all

the three types of samples. Very low prevalence of 17% for both the genes among STEC isolates was reported by Kruger *et al*. (2015). Interestingly Velusamy *et al*. (2007) reported higher prevalence of both *strA* and *strB* (88.8%) along with *aadA* in animal faecal samples compared to the present study. The present study is in consistent with study of Lanz *et al*. (2003), who indicated the presence of both *strA* and *strB* genes to make *Escherichia coli* strains streptomycin resistance. In contrast to the present study, Chiou and Jones (1995) reported absence of both *strA* and

Table 1: Primers used for the detection of resistant genes among *STEC* isolates.

Antibiotic	Gene	Primer sequence	Amplified product	Reference
Tetracycline	*tetA*	F: 5′GGCGGTCTTCTTCATCATGC3′	502 bp	Boerlin *et al.* (2005)
		R: 5′CGGCAGGCAGAGCAAGTAGA3′		
	tetB	F: 5′CATTAATAGGCGCATCGCTG3′	930 bp	Boerlin *et al.* (2005)
		R: 5′TGAAGGTCATCGATAGCAGG3′		
	tetC	F: 5′GCT GTA GGC ATA GGC TTG GT3′	888 bp	Lanz *et al.* (2003)
		R: 5′GCC GGA AGC GAG AAG AAT CA3′		
Streptomycin	*strA*	CCT GGT GAT AAC GGC AAT TC	546 bp	Madsen *et al.* (2000)
		CCA ATC GCA GAT AGA AGG C		
	strB	ATC GTC AAG GGA TTG AAA CC GGA	509 bp	Madsen *et al.* (2000)
		TCG TAG AAC ATA TTG GC		
Sulphonamides	*sul1*	GTG ACG GTG TTC GGC ATT CT	779 bp	Lanz *et al.* (2003)
		TCC GAG AAG GTG ATT GCG CT		
	sul2	CGG CAT CGT CAA CAT AAC CT	721 bp	Lanz *et al.* (2003)
		TGT GCG GAT GAA GTC AGC TC		
	sul3	GAG CAA GAT TTT TGG AAT CG	880 bp	Pereten and Boerlin (2003)
		CAT CTG CAG CTA ACC TAG GGC TTT GGA		
Ampicillin	*blaTEM*	F: ATCAGCAATAAACCAGC	516 bp	Colom *et al.* (2003)
		R: CCCCGAAGAACGTTTTC		

Table 2: Antibiotic resistance of *STEC* positive pooled samples (n=426) from three sources by phenotypic methods.

Antibiotic	Sensitive	Intermediate	Resistance
Ampicillin (10 mcg)	02 (0.47%)	02 (0.47%)	422 (99.06%)
Cephalothin (30 mcg)	0	0	426 (100%)
Chloramphenicol (30 mcg)	294 (69)	56 (13.1)	76 (17.8)
Colistin (10 mcg)	310 (72.7)	40 (9.3)	76 (17.8)
Gentamycin (10 mcg)	364 (85.4)	36 (8.4)	26 (6.1)
Kanamycin (30 mcg)	194 (45.5)	50 (11.7)	125 (29.3)
Sulphonamides (300 mcg)	11 (2.5)	28 (6.5)	387 (90.8)
Streptomycin (10 mcg)	05 (1.1)	19 (4.4)	402 (94.3)
Tetracycline (30 mcg)	6 (1.4)	6 (1.4)	414 (97.2)
Trimethoprim (5 mcg)	27 (6.3)	39 (9.1)	360 (84.5)

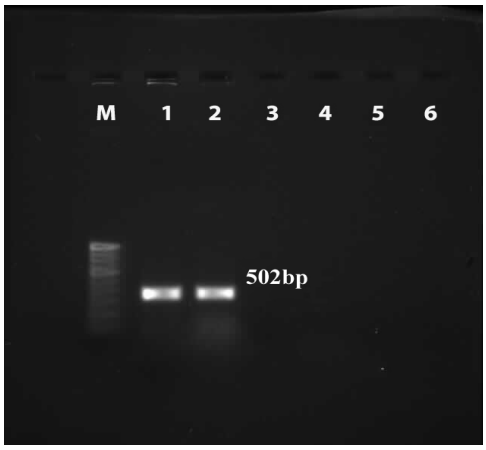
Table 3: Genotypic identification of antibiotic resistance from pooled samples.

Antibiotic	Gene	Animal faecal samples			Farm water samples			Human faecal samples		
		no.	+	%	no.	+	%	no.	+	%
Tetracycline	*tetA*	171	101	59.1	171	101	59.1	171	101	59.1
	tetB	171	71	41.5	171	71	41.5	171	71	41.5
	tetC	171	16	9.3	171	16	9.3	171	16	9.3
Streptomycin	*strA*	167	70	41.9	167	70	41.9	167	70	41.9
	strB	167	90	53.9	167	90	53.9	167	90	53.9
Sulphonamides	*sul1*	161	66	41	161	66	41	161	66	41
	sul2	161	97	60.2	161	97	60.2	161	97	60.2
	sul3	161	6	3.73	161	6	3.73	161	6	3.73
Ampicillin	*blaTEM*	179	144	80.4	179	144	80.4	179	144	80.4

strB genes among phenotypically streptomycin resistant isolates, suggesting that such resistance might be mediated by other yet undescribed genes. A lower prevalence than the present study for *strA* (15.6%) and *strB* (15.6%) was reported by Day *et al*. (2017).

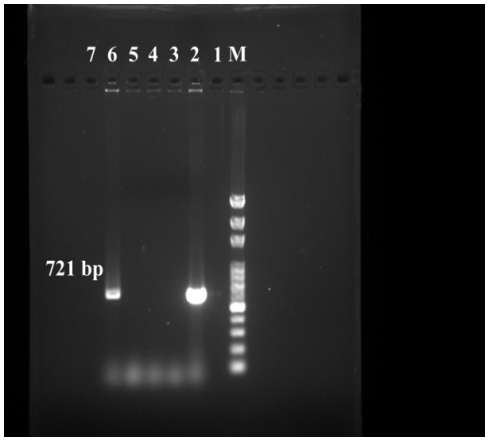
Genotypic identification of sulphonamide resistance genes

The sulfonamides resistant *Escherichia coli* is generally attributed to the presence of *sul1, sul2* (Fig 3) and/or *sul3* genes (Table 3) (Hammerum *et al*., 2006). So phenotypically sulphonamide resistant STEC isolates were subjected for mPCR to detect *sul*1, *sul*2 and *sul*3 genes. Out of 161, 112 and 114 sulphonamide resistant STEC isolates respectively from animal faecal, farm water and human faecal samples, most of the isolates have shown the presence of *sul2* (57.9% to 60.2%). The results were in consistant with the findings of Trobos *et al*. (2008) and Li *et al*. (2013). On contrary, Momtaz *et al*. (2012) reported higher prevalence of *sul*1 (47.36%), whereas Katakweba *et al*. (2014) reported high prevalence of *sul*3 among buffalo faecal samples compared to other animal samples. Higher prevalence (59% and 54%) of *sul1* than the present study (40.9%) was reported by Guerra *et al*. (2006) and Meselle *et al*. (2017) respectively, whereas Li *et al*. (2013) reported very low prevalence of *sul1* (5.5%). Low prevalence (3.7%) of *sul3* was observed in the present study, which was slightly higher than the prevalence (2.3%) reported by Li *et al*. (2013). *sul1, sul2* and *sul3* plays equal importance for sulphonamides resistance in *E. coli* strains (Ho *et al*., 2009; Zhang *et al*.,



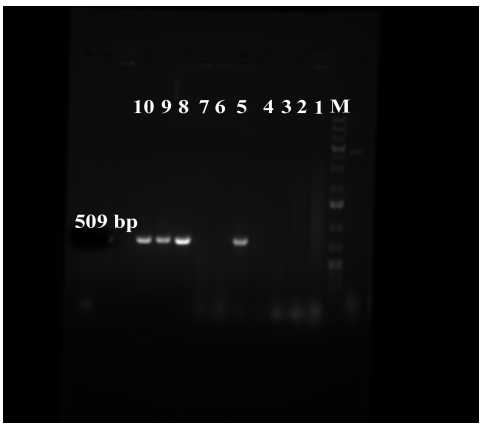
Lane M: 100 bp DNA ladder.
Lane 1: Positive control.
Lane 2: STEC isolates showing *tetA* gene.

Fig 1: Results of STEC isolates possessing *tetA* gene.



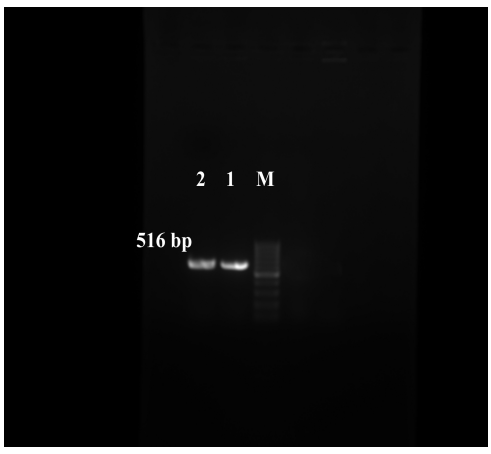
Lane M: 100 bp DNA ladder.
Lane 6: Positive control.
Lane 2: STEC isolates showing *sul2* gene.

Fig 3: Results of STEC isolates possessing having *sul2* gene.



Lane M: 100 bp DNA ladder.
Lane 1: Positive control.
Lane 5,8,9: STEC isolates showing s*trB* gene.
Lane 10: Positive control.

Fig 2: Results of STEC isolates possessing *strB* gene.



Lane M: 100 bp DNA ladder.
Lane 1: Positive control.
Lane 2: STEC isolates showing *blaTEM* gene.

Fig 4: Results of STEC isolates possessing *blaTEM* gene.

2012). This result agrees with the fact that the *sul2* gene is part of the 30CS in class 1 integron. The sulphonamide resistance genes may be present in diverse mobile genetic elements (such as integrons) that can be easily disseminated to other bacteria. The use of this antimicrobial in veterinary medicine or food animals may contribute to their maintenance of *Escherichia coli* strains (Srinivasan *et al*., 2007).

Genotypic identification of ampicillin resistance genes

Phenotypically ampicillin resistant STEC isolates were subjected for mPCR to detect *blaTEM* gene. Among 179,118 and 125 Ampicillin resistant STEC isolates respectively from pooled animal faecal samples, farm water samples and human faecal samples, almost all the samples (80.4% to 89.6%) had shown the presence of *blaTEM* gene (Fig 4) (Table 3). Guerra *et al*. (2006) reported higher prevalence (94%) of *blaTEM* in pooled animal faecal samples than the present study, whereas Bok *et al*. (2015) reported lower prevalence (19.4%) than the present study. The â-lactamase gene TEM-1 (blaTEM-1) is the most prevalent â-lactamase in gram-negative bacteria which is usually located on conjugative plasmids facilitating its spread among different species (Arvand *et al*., 2015). Some authors reported blaTEM-1 in STEC, not only isolated from food or animals but also from humans (Cergolle-Novella *et al*., 2011). In contrary to the present study, Day *et al*. (2017) and Elsayed *et al*. (2021) reported a very low prevalence of (2% and 5% respectively) *blaTEM* gene among the STEC isolates.

CONCLUSION

In summary antibiotic resistance towards most commonly used antibiotics was developed by most of the STEC isolates isolated from animal, human and environmental samples of our region, which alarms indiscriminate use of antibiotics both for therapeutic purpose and as growth promoters. This study also concluded with similar pattern of distribution of resistance genes among the isolates of different origin which indicates the interrelation between the components of epidemiological triad. Although antimicrobials are not usually used in the treatment of STEC infections, the presence of MDR in isolates collected from farm and other sources represents a risk for animal and human health as they can spread their resistance genes to other bacteria. Therefore, some measures must be taken to ensure a reasonable use of antimicrobials in the animal husbandry and strict hygienic, sanitation and HACCP programmes should be applied to counter *STEC* prevalence.

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Conflict of interest: None.

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