

Original Research Article

Distribution of the *stx1*, *stx2* and *hlyA* genes: Antibiotic profiling in Shiga-toxigenic *E. coli* strains isolated from food sources

M.U.Rasheed^{1*}, Kaiser Jamil¹, N.Thajuddin², Mukesh Pasupuleti³,
Parveez Ahamed², and K.P.Muthukumaresan³

¹Centre for Biotechnology & Bioinformatics, Jawaharlal Nehru Institute of Advanced Studies, 6th Floor, Buddha Bhavan, M.G Road, Secunderabad - 500 003, India.

²Department of Microbiology, Bharathidasan University, Tiruchirappalli- 620 024, India.

³SRM Research Institute, SRM University, Kattankulathur 603 203, Chennai, India.

*Corresponding author

ABSTRACT

Keywords

Shiga-Toxigenic *E. coli*, virulent genes, PCR, Food, Antibiotic resistance.

The purpose of this study was to determine the distribution of virulent genes, *stx1*, *stx2* and *hlyA* as well as antibiotic profiling among Shiga-Toxigenic *E. coli* isolated from food samples. One hundred and thirty four strains of *E. coli* isolated from 300 food samples were subjected to PCR analysis. Antibiotic sensitivity profiling was also performed. Of the 31 STEC positive strains, 25.8% exhibited only *stx1* gene, 54.8% exhibited both *stx1* and *stx2* genes, 9.6% exhibited *stx1*+*hlyA* genes, 3.2% exhibited *stx2*+*hlyA* genes and 7.1% isolates were positive for all the three genes *stx1*+*stx2*+*hlyA* studied. About 32.3% of STEC elicited eight different antibiotic resistance patterns ranging from one to eight widely used antibiotics. There was an overall uneven distribution of all the three virulence genes in the STEC strains. The *stx1* gene and the *stx1*+*stx2* combination were the most prevalent in the STEC strains. This study indicates that the bacteria which contain both types of shiga toxin were resistant to most of the antibiotics tested. Efficient cooking and other good hygienic prophylactic measures are needed to decrease the incidence of STEC in food items.

Introduction

Even though *E. coli* is a part of the normal human gut flora, there are certain subsets within these species which have acquired virulence genes that enable them to cause diarrhoea and other associated diseases (Wani et al., 2004). Diarrhoeagenic *Escherichia coli* (*E. coli*) are an important group of zoonotic human pathogens.

Diarrhoeagenic class includes enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and shiga toxin producing *E. coli* (STEC) (Murase et al., 2012). Among all the strains, those which produce shiga toxin

are of considerable concern. STEC strains are increasingly recognized as the causative agent for life threatening conditions such as haemorrhagic colitis and/or haemolytic-uremic syndrome (HUS). Shiga toxin, also called the verotoxin, is produced by enterohemorrhagic *E. coli*. Shiga toxin is multi-subunit protein with a molecular weight of 68 KD and acts on the lining of the blood vessels. It first binds to a component of the cell membrane known as Gb3 and enters into the cell. Once inside it interacts with ribosomes and inactivates them, thus halting the protein synthesis machinery and leading to the apoptosis and death of the cell (Karmali, 1989; Sandvig, 2001).

STEC strains are typically characterized by their ability to cause the attaching and effacing lesions due to the production of shiga toxin (Schmidt and Karch, 1996). Even though both the Shiga toxins (Stx1 and Stx2) are encoded on a lambdoid bacteriophage, they are genetically and immunologically distinct from each other. Stx2 shows 55–60% genetic and amino acid identity to Stx1 (Sandvig, 2001; Lee et al., 2007). *E. coli* in addition to Stx also produces the plasmid encoded enterohaemolysin (encoded by enterohemorrhagic *E. coli* (EHEC) *hlyA*) (Schmidt and Karch, 1996). Strains which carry both the genes are considered potentially more dangerous to humans than the ones with only either *stx* or *hlyA* alone. Interestingly, Shiga toxin type 2 strains (Stx2, encoded by *stx2*) appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1 (Stx1, encoded by *stx1*) (Kleanthous et al., 1990). Shiga toxin genes are found in more than 200 serotypes of different strains of *E. coli* strains, of which O157 and non O157: H

are quite well known for their disease causing ability (Page and Liles, 2013). O157: H serotype remains the prototype for the STEC and is found in 70-80% of clinical cases of diarrhoea (Shaikh and Tarr, 2003).

Ruminants are the primary reservoirs of STEC and human infection is through consumption or contact with contaminated foods such as vegetables, fruits (Beutin et al., 1995), undercooked burgers/fast-food, unpasteurised milk or milk products and drinking or swimming in contaminated water. STEC can survive in faeces for several months therefore animal environments (eg. pastures) can remain contaminated for considerable time. STEC can also survive for some time in water and soil (Ram et al., 2008; Fremaux et al., 2008a, 2008b).

Food-borne outbreaks associated with STEC have been well documented worldwide especially those that are caused by *E. coli* O157:H7 strain (Ho et al., 2013). Recently in 2011, a new strain, O104 was also found to be responsible for the outbreak of STEC in Germany (Page and Liles, 2013). Surprisingly, most reported outbreaks of food borne illnesses of STEC are from the developed countries (Griffin and Tauxe, 1991). It is not very clear whether this situation is due to under reporting in developing countries or to the relatively infrequent incidence of such organisms in foods in these countries. In India, it was previously thought that STEC was not a major etiologic agent of diarrhea though recently *E. coli* strain O157 and other O serogroups that exhibit cytotoxic activity in vero cells have been reported from patients suffering with the diarrhoea (Gupta et al., 1992). Various researches have reported the isolation of STEC strains from various foods, suggesting that

this enteropathogen may be a public health concern (Pal et al., 1999; Wani et al., 2004; Rajendran et al., 2009; Dutta et al., 2011; Kumar et al., 2012; Mahanti et al., 2013).

Antimicrobial resistance in *E. coli* has been reported worldwide. Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents (Sabat'e et al., 2008). Determining the susceptibility pattern of the bacterial strains is of critical value in order to make a strategic plan for drug selection and treatment in medicine and in agriculture as well as to contain the further spreading of the drug resistance to other bacteria.

PCR based methods have been widely used by various researchers all round the world to detect the toxin genes (Fratamico et al., 1995; Paton and Paton, 1998; Rahman, 2002; Moore et al., 2008; Gerritzen et al., 2012; Kumar et al., 2012). Information on the distribution of the *stx1*, *stx2* and *hlyA* genes among STEC strains is totally lacking from Hyderabad city of Andhra Pradesh, India. This study used, PCR, a well established method to generate data from different food sources collected in and around the city of Hyderabad.

Materials and Methods

Sample collection and transport:

A total of 300 samples each comprising 30 numbers; vegetable salad (carrot, cucumber, cabbage, tomatoes, spinach, lettuce, beet root and radish), raw egg-surface, raw chicken, unpasteurized milk of buffalo, fresh raw meat of sheep, cooked chicken fried rice from street vendors, cooked chicken noodles from street vendors, snacks such as chaat/pav

bhaji-street vendors, drinking water – street vendors and hand washing water-street vendors were collected randomly from twelve different localities of Hyderabad. In this work, the street-vended foods are defined as those foods prepared on the street ready to eat, or prepared at home and consumed on the street without further preparation (Bryan et al., 1997).

All samples were collected packaged in sterile polythene zip bags and then delivered to the laboratory under aseptic conditions in a cold box within 2 hours. Duplicate samples were obtained when possible. In the laboratory all samples were analyzed under sterile conditions within 2-4 hours after reception. A sharp sterile knife was used to cut-up the samples (vegetable salad, raw meat, raw chicken etc) on sterile trays. Liquid samples were measured in sterile volumetric cylinders.

Bacteria isolation

To isolate bacteria, a 25 gm sample portion (in case of eggs, each egg separately) was inoculated into 225 ml sterile Tryptic Soy Broth (TSB) and incubated for 6-8 h at 37⁰C. The TSB culture was then streaked onto the MacConkey's agar (MAC) plates and incubated for 18-24 h at 35 ± 2⁰C. Lactose fermenting colonies (one colony and for some samples with different colony morphologies at most two colonies) were identified and confirmed as *E.coli* by using gram stain, motility and standard biochemical tests, viz., catalase, oxidase, fermentation of lactose and glucose using triple sugar iron agar, production of indole, methyl red test, voges proskauer test, urease test and utilization of citrate (Cheesbrough, 1993). Colonies which were identified as *E. coli* were preserved

in 20 % glycerol at -20°C for further characterization. Biochemically confirmed isolates of *E. coli* were grown on Sorbitol Mac Conkey agar, Xylose Lysine Deoxycholate (XLD) agar & Deoxycholate Citrate agar (DCA) medium to check their growth characteristics.

DNA isolation using the boiling method

Biochemically confirmed *E. coli* strains were grown over night in nutrient broth at 37°C; this was then centrifuged at 8000 rpm for 5 min. The resultant bacterial pellet was washed twice in sterile distilled water and re-suspended in 250 µl sterile distilled water. The samples were then placed in a water bath maintained at 100°C for 10 minutes and then incubated on ice for 5 min. The lysate was centrifuged at 8000 rpm for 5 min and the supernatant then used as a template for PCR amplification (Shetty et al., 2012).

PCR Reaction

Crude DNA was obtained from all the 57 isolates using the boiling method. Sequence spanning the *stx1*, *stx2* and *hlyA* was amplified by PCR method using Taq DNA polymerase. The reaction mixture of total volume of 20µl reaction mixture contained 2 µl of 10X PCR buffer (100 mM Tris with pH 9.0, 500 mM KCl, 15 mM MgCl₂ and 1% Triton X-100), 2 µl of 10mM of 4-deoxynucleotide triphosphate mix, 1 U of Taq DNA polymerase (3B BlackBio Biotech, India), 2 µl each of 4 picomoles/µl of forward and reverse primers and 5µl of crude bacterial cell lysate.

The PCR primers were as stated below

stx1 Forward primer – 5'-
ACACTGGATGATCTCAGTGG-3'

Reverse primer - 5'-
CTGAATCCCCCTCCATTATG-3'
stx2 Forward primer - 5'-
CCATGACAACGGACAGCAGTT-3'
Reverse primer - 5'-
CCTGTCAACTGAGCAGCACTTTG-3'
hlyA O157-3 Forward primer - 5'-
GTAGGGAAGCGAACAGAG-3'
Reverse primer - 5'-
AAGCTCCGTGTGCCTGAA-3'

These primers were selected based on previously published reports (Wang et al., 1997; Fagan et al., 1999) for the detection of STEC virulence genes. A 35 cycles PCR reaction program was carried out in a thermocycler (*Veriti® Thermal Cycler*, Applied Biosystems, USA) using the above primer pairs. The cycling conditions were: initial denaturation at 94°C for 5 min, 35 cycles at 58°C for 1 min, 72°C for 2 min, 94°C for 1 min with final extension at 72°C for 10 min. *E. coli* O157:H7 strain EDL933, which produces *stx1*, *stx2* and *hlyA* genes, was used as a positive control. Sterile distilled water served as the negative control. The PCR products were separated on 1.5% agarose gel electrophoresis, stained with ethidium bromide (0.5µg/ml) and visualized by an UV transilluminator.

Antimicrobial Susceptibility Testing

Susceptibility tests on Mueller-Hinton agar were performed according to the Kirby-Bauer method in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (NCCLS 2007) (Thomas, 2007; Schwarz et al., 2010) by using 19 antibacterial agents: Ampicillin (10 mcg), Amoxicillin (25 mcg), Amoxyclav (30 mcg), Aztreonam (30 mcg), Cefotaxime (30 mcg), Ceftazidime (30 mcg), Ceftriaxone (30 mcg), Chloramphenicol

(30 mcg), Ciprofloxacin (5 mcg), Colistin (10 mcg), Co-trimoxazole (1.25/23.75 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg), Ofloxacin (5 mcg), Piperacillin+tazobactam (100/10 mcg), Streptomycin (10 mcg), Tetracycline (30mcg), and Tigecycline (15 mcg).

The *E. coli* isolates of STEC strains were inoculated in nutrient broth and incubated at 35+2°C for 5 hours. The broth was then diluted in normal saline solution to a density of 0.5 McFarland turbidity standard. Cotton swabs were used for streaking the diluted broth onto Mueller-Hinton agar plates. After air drying, antibiotic discs were placed 30 mm apart and 10 mm away from the edge of the plate. Plates were inverted and incubated aerobically at 35+2°C for 16 to 18 hours. The zone of inhibition and resistance was measured, recorded, and interpreted according to the recommendation of the CLSI (NCCLS, 2002). The ATCC strain of *E. coli* 25922 was used as a control strain.

Extended Spectrum β - Lactamase (ESBL) Confirmatory Test

Double Disc Synergy Test (DDST)

Isolated colonies of STEC strains were inoculated into nutrient broth at 35 \pm 2°C for 5h. The turbidity was adjusted to 0.5McFarland standard and lawn cultures were made on Mueller-Hinton agar using sterile swabs. Augmentin discs (20/10 mcg) were placed in the centre of plate. On both sides of the Augmentin disc, a disc of cefotaxime (30 mcg) and ceftazidime (30 mcg), were placed with centre to centre distance of 15mm to centrally placed disc. The plates were incubated at 35 \pm 2°C overnight. ESBL

production was interpreted as the 3rd-generation cephalosporin disc, inhibition was increased towards the augmentin disc or if neither discs were inhibitory alone but bacterial growth was inhibited where the two antibiotics diffused together (Jarlier et al., 1988).

All STEC virulence genes positive isolates were serotyped by 0157 antisera (Denka Seiken Co. Japan) using slide agglutination method. All the bacteriological media and antimicrobial discs were purchased from HiMedia Laboratories, Mumbai, India.

Results and Discussion

A total of 134 biochemically confirmed strains of *E. coli* were isolated from 300 different food samples (Table 1). Food samples were also processed to isolate other medically important food borne pathogens like *Salmonella species*, *Staphylococcus aureus* and *Bacillus cereus*.

PCR studies performed on all the 134 biochemically confirmed *E. coli* isolates showed that only 31 (10.3%) of them belonged to STEC class (Table 1). The incidence rates of STEC were 30%, 26.7% and 16.7% for raw chicken, raw meat and vegetable salads respectively. Surprisingly *hlyA* gene was found only in these three sources of food. The lowest incidence of STEC noted was at 6.7% from unpasteurized milk. The overall incidence of STEC was 10.7%.

Of the 31 STEC positive strains, 8 (25.8%) exhibited only *stx1* gene (two isolates from raw egg outer surface, and three isolates each from raw sheep meat and hand-washing water from street vendors),

17 (54.8%) exhibited both *stx1* and *stx2* genes (seven isolates from raw chicken, four from vegetable salad, and 2 each from raw sheep meat, raw egg-surface and unpasteurized milk), 3 (9.6%) exhibited *stx1*+ *hlyA* genes (two isolates from raw sheep meat and one from vegetable salad), 1 (3.2%) exhibited *stx2*+*hlyA* genes (from raw meat) and two (7.1%) isolates were positive for all the three genes *stx1*+*stx2*+*hlyA* studied (from raw chicken).

All STEC isolates in this study carried the *stx 1* gene except the one (3.2%) isolate from raw meat. Eleven (35.5%) isolates were without *stx 2* gene and 6 (19.35%) exhibited *hlyA* gene in combination with *stx* genes. On the whole the most prevalent (54.8%) virulence genes were *stx1*+*stx2*. Two (6.45%) of the STEC strains (*stx1*+*stx2*+*hlyA*; raw chicken, and *stx1*+*stx2*; raw meat) were of serotype 0157.

None of the isolate of *E. coli* from Chicken fried rice-street vendors, Chicken noodles- street vendors Chaat/pav bhaji and Drinking water-street vendors were confirmed as STEC by PCR.

It was interesting to observe that the overall distribution of all the three virulence genes in the STEC strains were not uniform (Table 1).

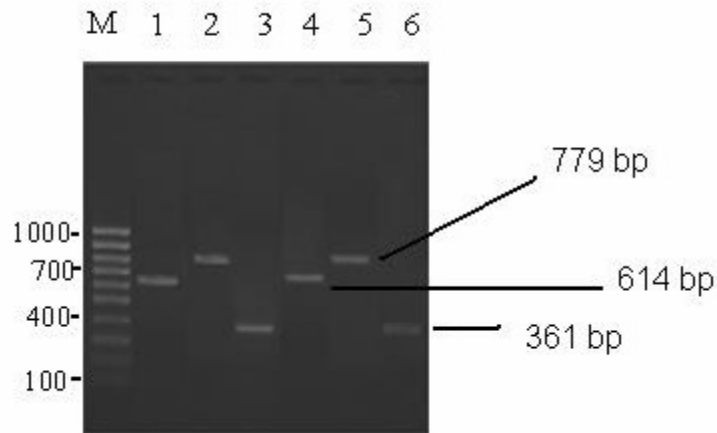
It is accepted world-wide that the STEC serotype 0157 is of epidemiologic significant concern. The 0157 serotyping of the 14 confirmed STEC isolates in this study showed that only two isolates belonged to the serotype 0157.

All 31 confirmed strains of STEC were tested for their antibiotic profile against 19 different antimicrobial agents. A single and multi drug resistance patterns to antimicrobial agents was observed in 10 of 31 isolates of STEC strains of *E. coli* as shown in Table 2. Resistance was observed in the isolates as: to ampicillin, amoxicillin and co-trimoxazole each in 8 isolates, tetracycline in 7, streptomycin in 4, ciprofloxacin, ofloxacin and gentamicin in 1 each.

Ten (32.25%) STEC strains elicited eight different patterns of antibiotic resistance to the agents used in this study (Table 2). The number of antibiotics against which each isolate showed resistance ranged between 1 and 8. Among all the tested strains, two exhibited resistance to one each different antibiotics; two to 3 antibiotics, three to 4 antibiotics, two to 5 antibiotics and one to 8 different antibiotics. On a closer look at the genes present, all the ten resistant strains of STEC were seen to carry the *stx1* gene, 7 carried the *stx1* gene in combination with *stx2* and one carried the *stx1* gene in combination with the *hlyA* gene. Only two carried the single gene *stx1*. Fortunately none of the isolated strains was an ESBL producer and neither of them was found resistant to new/reserved type of antibiotics of Amoxyclav, Aztreonam, Cephalosporins, Chloramphenicol, Colistin, Carbapenems, Piperacillin+tazaobactam, and Tigecycline.

Worldwide epidemiological studies show that not all the strains producing shiga toxin are pathogenic, it is believed that accessory genes may be responsible for or

Figure.1 Agarose gel electrophoresis of *stx1*, *stx2* and *hlyA* PCR products



Lane M: 100 bp molecular size marker, Lanes 1, 2 & 3: isolate from raw chicken sample positive for *stx1*, *stx2* and *hlyA* genes respectively. Lanes 4, 5 & 6: positive control (*E. coli* EDL 933) positive for *stx1*, *stx2* and *hlyA* genes respectively.

Table.1 Incidence of STEC and virulence gene combination

S. No.	Type of food	No. of samples	Incidence of <i>E. coli</i> (%)	Incidence of STEC (%)	Virulence gene/s combination for isolates of STEC
1	Raw chicken	30	25 (83.3)	9 (30)	<i>stx1+stx2</i> (7n) <i>stx1+stx2+hlyA</i> (2n)
2	Raw sheep meat	30	20 (66.7)	8 (26.7)	<i>stx1</i> (3n) <i>stx1+stx2</i> (2n) <i>stx1+hlyA</i> (2n) <i>stx2+hlyA</i> (1n)
3	Vegetables salad	30	23(76.7)	5 (16.7)	<i>stx1+stx2</i> (4n) <i>stx1+hlyA</i> (1n)
4	Raw egg - surface	30	18 (60)	4 (13.3)	<i>stx1</i> (2n) <i>stx1+stx2</i> (2n)
5	Unpasteurized milk	30	13 (43.3)	2 (6.7)	<i>stx1+stx2</i> (2n)
6	Hand washing water	30	18 (60)	3 (10)	<i>stx1</i> (3n)
7	Chicken fried rice	30	7 (23.3)	0	-
8	Chicken noodles	30	4 (13.3)	0	-
9	Chaat/pav bhaji	30	3 (10)	0	-
10	Drinking water	30	3 (10)	0	-
Total No. of Samples		300	134 (44.7)	31 (10.3)	

Table.2 Antibiotic resistance profile of isolates of STEC strains

S. No.	Strain ID	Antibiotic resistance profile	STEC Genotype ^{\$}			Source of isolation
			<i>stx1</i>	<i>stx2</i>	<i>hlyA</i>	
1	STEC01	A, Am, Co, S, T	+	+	-	Raw chicken
2	STEC02	A, Am, Cip, Co, G, Of, S, T	+	+	-	Raw chicken
3	STEC03	A, Am, Co, T	+	+	-	Raw Chicken
4	STEC10	Co	+	-	-	Raw mutton
5	STEC13	A, Am, Co, T	+	+	-	Raw mutton
6	STEC15	A, Am, Co, S, T	+	-	+	Raw mutton
7	STEC18	A, Am, T	+	+	-	Vegetable salad
8	STEC19	T	+	+	-	Vegetable salad
9	STEC26	A, Am, Co, S	+	+	-	Raw -egg surface
10	STEC30	A, Am, Co	+	-	-	Hand-washing water

A: Ampicillin, Am: Amoxicillin, Cip: Ciprofloxacin, Co: Co-trimoxazole, G: Gentamicin, Of: Ofloxacin, S: Streptomycin, T: Tetracycline; \$ +, gene present; -, gene absent.

contribute to human pathogenic state (Espie et al., 2006; Bradley et al., 2011; Brandt et al., 2011; Vally et al., 2012). Many researchers have established that strains of STEC causing bloody diarrhoea are positive for the enterohemolysin gene. Enterohemolysin liberates haemoglobin from the RBCs and is linked to the severity of the disease. It is widely accepted that *E. coli* strains harbouring both *stx* and *hlyA* may pose a higher risk to human health (Manna et al., 2006). PCR methods are increasingly gaining attention among the researchers and clinicians for the identification of microbes which cannot be easily differentiated by simple culture techniques (Rahman, 2002; Moore et al., 2008). Direct PCR analysis with primers specific to *stx* provided the presence of STEC strains in the sample;

however it did not provide any information about the viability of the strain and thus its ability to cause infection (Moore et al., 2008). Previously, it was routine to use the DNA extracts obtained directly from the faeces and food samples as templates for PCR amplifications (Dhanashree and Mallya, 2008). However, in the cases of enteric pathogens, it was observed that best results were usually obtained by testing the extracts of primary cultures. The culture enrichment step, as little as 4 hours can significantly increase the sensitivity by diluting the inhibitors and also increases the number of target bacterial species (Dhanashree and Mallya, 2008; Gerritzen et al., 2012). Hence the culture of sample is utmost important before carrying out the PCR.

Although Fratamico et al. (1995) reported that PCR primers targeting the *hlyA* gene are suitable for detection of STEC belonging to serogroup O157, later studies indicated that these primers recognized the EHEC *hlyA* gene: this is not confined to the O157 serotype, and is therefore suitable for detection of all STEC (Paton and Paton, 1998). In this study 19.35% (6/31) STEC strains harboured *hlyA* gene but only 6.45% (2/31) were serotyped as O157: the rest of the isolates could thus be considered to be STEC non-O157 serotypes.

This study found the highest incidence (30%) of STEC carrying all the three genes studied in these combinations; *stx1+stx2* and *stx1+stx2+hlyA* in raw chicken which were collected from the poultry shops cum butcheries. The lowest prevalence of *E. coli* was found in the cooked food items (Chicken fried rice & noodles) and no STEC was detected among these *E. coli* strains. This indicates efficient cooking procedures within the street vendor's kitchens. In order to draw a concrete conclusion on this point a study with a larger sample size of fast food items from street vendors is recommended.

The high number of STEC strains isolated could be due to cross contamination during handling at butcheries. In a study to compare the fate of STEC O157:H7 and non-O157:H7 in blade-tenderized steaks cooked on a gas grill showed that both the pathogens were susceptible to heat in a similar fashion (Luchansky et al., 2012) and the cooking eliminated significant numbers of both pathogen types. Furthermore, the results of this study are in agreement with those obtained in a study conducted in Argentina (Alonso et al., 2012) which found a greater proportion of enteropathogenic *E. coli*

(EPEC) in chicken hamburgers than STEC, while the chicken samples collected in butcheries contained more number of STEC.

The second highest incidence (26.7%) of STEC as well as presence of *hlyA* gene was found in raw meat and 23.3% were non-O157 STEC and 3.3% was O157 (non-*hlyA*). Various global studies have shown the presence of STEC strains in meat products (Hwang and Juneja, 2011; Rebecca et al., 2011; Díaz-Sánchez et al., 2012; Tomat et al., 2013). This wide presence could be attributed to a symptomatic carriage or cross contamination from the faeces during slaughter (Omisakin et al., 2003).

Wide advertising has lead to increased consumption of ready-to-eat (RTE) salads worldwide. Consequently, the number of outbreaks caused by food-borne pathogens, including diarrheagenic *E. coli* phenotypes (DEPs), associated with the consumption of RTE-salads have also increased especially in areas where fresh crops continue to be irrigated with untreated sewage water. In a study carried out in Hidalgo, Mexico by Castro-Rosa J et al., (2012) out of 130 salad samples purchased from restaurants, 85% harboured *E. coli* and 7% of them accounted for the diarrheagenic *E. coli* phenotype. Even though geographically different and widely separated, our results have shown somewhat similar trends. In this study, the overall incidence of *E. coli* and STEC from salad vegetables was 76.7% and 16.7% respectively; the percentage of STEC was the third highest among all the food items and none of the isolate was serotyped as O157 STEC. As in raw chicken and meat, the isolates from vegetable salads carried all the three targeted genes but none of the isolates

carried all three genes; each isolate carried two genes in two different combinations.

In this study, four (13.3%) STEC isolates were isolated from the surface of raw eggs. The genotype of two isolates was *stx1* and for other two *stx1+stx2* which was similar to the seven strains of STEC isolated from the raw chicken. Poultry are considered an important source of food borne diseases and the illnesses were associated with the consumption of contaminated eggs (Sharma and Carlson, 2000).

There is significant health risk of human infection when unpasteurized milk/products are consumed. Such consumption has been the source of a series of severe infections associated with pathogens such as STEC (Liptakova et al., 2004). This study has documented a lowest incidence of STEC (6.7%) with the most common type of genotype noted (*stx1+stx2*) which were isolated from unpasteurized milk.

Only 3 (10%) *E. coli* and no STEC were isolated from drinking water available at street vendors whereas from the same vendors the water for hand washing showed a high incidence of *E. coli* (60%) and the presence of three (10%) STEC with a genotype of *stx1*. This indicates that the importance was not given to quality of water which was not used for drinking purpose; hand washing water may indirectly pose significant danger to public health.

Interestingly, we observed no specific difference in the growth characteristics of all the 134 isolates of *E. coli* grown on XLD and DCA agars thus showing the inability of the biochemical based methods

to differentiate STEC strains from non-STECS (Sharma and Carlson, 2000). It is known that XLD and DCA media are used for the isolation of *Salmonellae* and *Shigellae* species and in this study these media were used to check if there are any strains of *E. coli* that might grow in a different form.

In conventional methods for the study of STEC, sorbitol-containing medium is used and only sorbitol non-fermenters colonies are picked up for further testing (Shaikh and Tarr, 2003). Interestingly, two of the fourteen *stx*-positive isolates were positive for sorbitol fermentation when grown on Sorbitol Mac Conkey agar medium. The results of this study suggest the possibility of sorbitol fermenting STEC in a significant number of samples in India; other authors have reported similar findings (Karch and Bielaszewska, 2001). This finding supports the need to consider selection methods for STEC that are independent of the sorbitol phenotype.

Antimicrobial resistance has been recognized as an emerging worldwide challenge in human and veterinary medicine (Amundson et al., 1988; Cohen, 2000; McKeon et al., 1995; Bager and Helmuth, 2001; Hammerum and Heuer, 2009). A variety of foods and environmental sources harbour bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and in food-animal production (Kleanthous et al., 1990; Fratamico et al., 1995; Pal et al., 1999; Bager and Helmuth, 2001; Wani et al., 2004; Rajendran et al., 2009). This study noted that about 32.3% of STEC elicited eight different patterns of antibiotic resistance ranging from one to eight antibiotics that are widely used. The indiscriminate use of antibiotics in agriculture and medicine is accepted as a

major selective force in the high incidence of antibiotic resistance among gram-negative bacteria (McKeon et al., 1995). The results of this study indicated that the bacterium which contains both types of shiga toxin genes (*stx1+stx2*) were resistant to most of the antibiotics tested. Bacteriophages contain antibiotic resistance genes and are potentially excellent vehicles for the propagation of bacterial resistance genes in the environment (Colomer-Lluch M et al., 2011). The phage which codes for the shiga toxin might probably have the resistance genes. A study starting with the isolation of the phages, followed by molecular characterisation might provide some further insight?

Except for diarrhoeal outbreaks (Kang et al., 2001), STEC does not pose a major threat to human health in India. It is not clear 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 *stx1*, IgG antibodies in asymptomatic cases offers positive protection against STEC infection (Karmali, 1989).

Of considerable concern is that in this study, two isolates from raw chicken and one from raw meat carried both the *hlyA* and *stx2* genes; this combination is potentially dangerous to human beings (Manna et al., 2006). PCR is a very specific and rapid method of detecting the virulent STEC. The detection of STEC in foods of animal and plant origin in this study warrants that human diarrhoeal stools during foodborne outbreaks must be routinely examined for STEC. The overall

distribution of all the three virulence genes in the STEC strains was ununiform. The most prevalent gene was *stx1* and the most prevalent gene combination was *stx1+stx2* found in the STEC strains isolated from the food items. Efficient cooking and other good hygienic prophylactic measures are needed to decrease the incidence of STEC in food items. Furthermore, there is a need to accentuate on the rational use of antibiotics in agriculture and medicine. The study also indicates that the bacterium which contains both types of shiga toxin were resistant to most of the antibiotics tested.

The results of this study will be of considerable interest internationally as well as locally as this is the first report about the distribution of genes of STEC isolated from different food items of Hyderabad city.

References

- Alonso M.Z., Lucchesi, P.M.A., Rodríguez, E.M., Parma, A.E. and Padola, N.L. 2012. Enteropathogenic (EPEC) and Shigatoxigenic *Escherichia coli*(STEC) in broiler chickens and derived products at different retail stores. *Food. Contr.* 23(2): 351–355.
- Amundson D., Lindholm, C., Goyal, S.M. and Robinson. R.A. 1988. Microbial pollution of well water in southeastern Minnesota. *J. Environ. Sci. Health. Part A: Environ. Sci. Engi.* 23(5): 453-468.
- Bager F. and Helmuth R. 2001. Epidemiology of resistance to quinolones in Salmonella. *Vet. Res.* 32(3-4): 285-290.
- Beutin L., Geier, D., Zimmermann, S. and Karch, H. 1995. Virulence markers of Shiga-like toxin-producing *Escherichia coli* strains originating from healthy domestic animals of different species. *J. Clin. Microbiol.* 33: 631–635.
- Bradley K.K., Williams, J.M., Burnsed, L.J., Lytle, M.B., McDermott, M.D., Mody, R.K., Bhattarai, A., Mallonee, S., Piercefield, E.W.,

- McDonald-Hamm, C.K. and Smithee, L.K. 2011. Epidemiology of a large restaurant-associated outbreak of Shiga toxin-producing *Escherichia coli* O111:NM. *Epidemiol. Infect.* 140(9): 1644-1654.
- Brandt S.M., King, N., Cornelius, A.J., Premaratne, A., Besser, T.E. and On, S.L. 2011. Molecular risk assessment and epidemiological typing of Shiga toxin-producing *Escherichia coli* by using a novel PCR binary typing system. *Appl. Environ. Microbiol.* 77(7): 2458-2470.
- Bryan F.L., Jermini, M., Schmitt, R., Chilufya, E.N., Michael, M., Matoba, A. and Mfume, E.C. 1997. Hazards associated with holding and reheating foods at vending sites in a small town in Zambia. *J. Food. Protect.* 60: 391-398.
- Castro-Rosas J., Cerna-Cortés, J.F., Méndez-Reyes, E., Lopez-Hernandez, D., Gómez-Aldapa, C.A. and Estrada-Garcia, T. 2012. Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *Int. J. Food. Microbiol.* 156(2):176-180.
- Cheesbrough M. 1993. Medical Laboratory Manual for Tropical Countries. Vol. II. Microbiology, first ed. ELBS Butterworth-Heinemann Ltd., Oxford.
- Cohen M.L. 2000. Changing patterns of infectious disease. *Nature* 406 (6797): 762-767.
- Colomer-Lluch M, Imamovic L, Jofre JM, Muniesa. 2011. **Bacteriophages Carrying Antibiotic Resistance Genes in Fecal Waste from Cattle, Pigs, and Poultry.** *Antimicrob. Agents. and Chemother.* 55 (10): 4908-4911.
- Dhanashree B. and Mallya, P.S. 2008. Detection of shiga-toxigenic *Escherichia coli* (STEC) in diarrhoeagenic stool & meat samples in Mangalore, India. *Ind. J. of Med. Res.* 128(3): 271-277.
- Díaz-Sánchez S., Sánchez, S., Sánchez, M., Herrera-León, S., Hanning, I. and Vidal, D. 2012. Detection and characterization of Shiga toxin-producing *Escherichia coli* in game meat and ready-to-eat meat products. *Int. J. Food. Microbiol.* 160(2): 179-182.
- Dutta T.K., Roychoudhury, P., Bandyopadhyay., Wani, S.A. and Hussain, I. 2011. Detection & characterization of Shiga toxin producing *Escherichia coli* (STEC) & enteropathogenic *Escherichia coli* (EPEC) in poultry birds with diarrhoea. *Ind. J. Med. Res.* 133: 541-545.
- Espie E., Grimont, F., Vaillant, V., Montet, M.P., Carle, I., Bavai, C., De Valk, H. and Vernozy-Rozand, C. 2006. O148 Shiga toxin-producing *Escherichia coli* outbreak: microbiological investigation as a useful complement to epidemiological investigation. *Clin. Microbiol. Infect.* 12(10): 992-998.
- Fagan P.K., Hornitzky, M.A., Bettelheim, K.A. and Djordjevic, S.P. 1999. Detection of Shiga-Like Toxin (*stx*₁ and *stx*₂), Intimin (*eaeA*), and Enterohemorrhagic *Escherichia coli* (EHEC) Hemolysin (EHEC *hlyA*) Genes in Animal Feces by Multiplex PCR. *Appl. Environ. Microbiol.* 65(2): 868-872.
- Fratamico P.M., Sackitey, S.K., Wiedmann, M. and Deng, M.Y. 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. *J. Clin. Microbiol.* 33: 2188-2191.
- Fremaux B., Prigent-Combaret, C., Delignette-Muller, M.L., Mallen, B., Dothal, M., Gleizal, A. and Vernozy-Rozand, C. 2008a. Persistence of Shiga toxin-producing *Escherichia coli* O26 in various manure-amended soil types. *J. App. Microbiol.* 104(1): 296-304.
- Fremaux B., Prigent-Combaret, C. and Vernozy-Rozand, C. 2008b. Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: an updated review. *Vet. Microbiol.* 132(1-2): 1-18.
- Gerritzen A., Wittke, J.W. and Wolff, D. 2012. Rapid and sensitive detection of Shiga toxin-producing *Escherichia coli* directly from stool samples by real-time PCR in comparison to culture, enzyme immunoassay and Vero cell cytotoxicity assay. *Clin. Lab.* 57(11-12): 993-998.
- Griffin P.M. and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli* and the associated hemolytic uremic syndrome. *Epidemiol. Rev.* 13: 60-98.
- Gupta S., Soni, N.K., Kaur, P. and Sood, D.K. 1992. Verocytotoxic activity of *Escherichia coli* O157 and other 'O' serogroups isolated from patients of diarrhoea. *Ind. J. Med. Res.* 95: 71-76.
- Hammerum A.M. and Heuer, O.E. 2009. Human

- health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clin. Infect. Dis.* 48(7): 916-921.
- Ho N.K., Henry, A.C., Johnson-Henry, K. and Sherman, P.M. 2013. Pathogenicity, host responses and implications for management of enterohemorrhagic *Escherichia coli* O157:H7 infection. *Can. J. Gastroenterol.* 27(5): 281-285.
- Hwang C.A. and Juneja, V. 2011. Effects of salt, sodium pyrophosphate, and sodium lactate on the probability of growth of *Escherichia coli* O157:H7 in ground beef. *J. Food. Protect.* 74(4): 622-626.
- Jarlier V., Nicolas, M.H., Fournier, G. and Philippon, A. 1988. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev. Infect. Dis.* 10(4): 867-878.
- Kang G., Ramakrishna, B.S., Daniel, J., Mathan, M. and Mathan, V.I. 2001. Epidemiological and laboratory investigations of outbreaks of diarrhoea in rural South India: implications for control of disease. *Epidemiol. Infect.* 127 (1): 107-112.
- Karch H. and Bielaszewska, M. 2001. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(-) strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *J. Clin. Microbiol.* 39(6): 2043-2049.
- Karmali M.A. (1989) Infection by verotoxin-producing *Escherichia coli*. *Clin. Microbiol. Rev.* 2: 15-38.
- Kleanthous H., Smith, H.R., Scotland, S.M., Gross, R.J., Rowe, B., Taylor, C.M. and Milford, D.V. 1990. Haemolytic uremic syndromes in the British Isles, 1985-8: association with verocytotoxin producing *Escherichia coli*. Part 2: Microbiological aspects. *Arch. Dis. Child.* 65(7): 722-727.
- Kumar A., Taneja, N., Kumar, Y. and Sharma, M. 2012. Detection of Shiga toxin variants among Shiga toxin-forming *Escherichia coli* isolates from animal stool, meat and human stool samples in India. *J. App. Microbiol.* 113(5): 1208-1216.
- Lee J.E., Reed, J., Shields, M.S., Spiegel, K.M., Farrell, L.D. and Sheridan, P.P. 2007. Phylogenetic analysis of Shiga toxin 1 and Shiga toxin 2 genes associated with disease outbreaks. *BMC. Microbiol.* 7: 109.
- Liptakova A., Siegfried, L., Rosocha, J., Podracka, L., Bogyiiova, E. and Kotulova, D. 2004. A family outbreak of haemolytic uraemic syndrome and haemorrhagic colitis caused by verocytotoxigenic *Escherichia coli* O157 from unpasteurised cow's milk in Slovakia. *Clin. Microbiol. Infect.* 10: 576-578.
- Luchansky J.B., Porto-Fett, A.C., Shoyer, B.A., Call, J.E., Schlosser, W., Shaw, W., Bauer, N. and Latimer, H. 2012. Fate of Shiga toxin-producing O157:H7 and non-O157:H7 *Escherichia coli* cells within blade-tenderized beef steaks after cooking on a commercial open-flame gas grill. *J. Food. Protect.* 75(1): 62-70.
- Mahanti A., Samanta, I., Bandopaddhay, S., Joardar, S.N., Dutta, T.K., Batabyal, S., Sar, T.K. and Isore, D.P. 2013. Isolation, molecular characterization and antibiotic resistance of Shiga Toxin-Producing *Escherichia coli* (STEC) from buffalo in India. *Lett. Appl. Microbiol.* 56(4): 291-298.
- Manna S.K., Brahmane, M.P., Das, R., Chandana, M. and Batabyal, S. 2006. 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.
- McKeon D.M., Calabrese, J.P. and Bissonnette, G.K. 1995. Antibiotic resistant gram-negative bacteria in rural groundwater supplies. *Wat. Res.* 29(8):1902-1908.
- Moore J.E., Watabe, M., Millar, B.C., Rooney, P.J., Loughrey, A. and Goldsmith, C.E. 2008. Direct molecular (PCR) detection of verocytotoxigenic and related virulence determinants (eae, hyl, stx) in *E. coli* O157:H7 from fresh faecal material. *Br. J. Biomed. Sci.* 65(3): 163-165.
- Murase K., Ooka, T., Iguchi, A., Ogura, Y., Nakayama, K., Asadulghani, Md., Islam, MR., Hiyoshi, H., Kodama, T., Beutin, L. and Hayashi, T. 2012. Haemolysin E- and enterohaemolysin-derived haemolytic activity of O55/O157 strains and other *Escherichia coli* lineages. *Microbiol.* 158: 746-758.
- Omisakin F., MacRae, M., Ogden, I.D. and Strachan, N.J.C. 2003. Concentration and prevalence of *Escherichia coli* O157 in cattle

- feces at slaughter. *Appl. Environ. Microbiol.* 69(5): 2444-2447.
- Page A.V. and Liles, W.C. 2013. Enterohemorrhagic *Escherichia coli* Infections and the Hemolytic-Uremic Syndrome. *Med. Clin. North. Am.* 97(4): 681-695.
- Pal A., Nair, G.B., Ghosh, S., Yamasaki, S., Bhattacharya, S.K., Ramamurthy, T., Tsukamoto, T. and Takeda, Y. 1999. Shiga-toxin producing *Escherichia coli* from healthy cattle in a semi-urban community in Calcutta, India. *Ind. J. Med. Res.* 110: 83-85.
- Paton A.W. and Paton, J.C. 1998. Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohaemorrhagic E.coli hlyA, rfbO111, and rfb O157. *J. Clin. Microbiol.* 36: 598-602.
- Rahman H. 2002. Multiplex PCR for detection of stx genes of *Escherichia coli*. *Ind. J. Med. Res.* 115: 251-254.
- Rajendran P., Rajan, D.P., Kang, G. and Thorpe, C.M. 2009. Shiga toxin-producing *Escherichia coli* infection in South India. *J. Med. Microbiol.* 58(11): 1525-1526.
- Ram S., Vajpayee, P. and Shanker, R. 2008. Contamination of potable water distribution systems by multiantimicrobial-resistant enterohemorrhagic *Escherichia coli*. *Environ. Health. Perspec.* 116(4): 448-452.
- Rebecca H., Jill, F., Lucas, G.R., Stephanie, C., Paul, L., Jerry, T., Tim, G., Frank, K., Barry, N., Edan, H., Paul, N., Jennifer, R. and Mark, N.M. 2011. Reveal *E. coli* 2.0 method for detection of *Escherichia coli* O157:H7 in raw beef. *J. AOAC. Intern.* 94(6):1835-1845.
- Sabaté M., Prats, G., Moreno, E., Ballesté, E., Blanch, A.R. and Andreu, A. 2008. Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. *Res. in Microbiol.* 159: 288-293.
- Sandvig K. (2001) Shiga toxins. *Toxicon.* 39(11): 1629-1635.
- Schmidt H. and Karch, H. 1996. Enterohemolytic phenotypes and genotypes of Shiga toxin-producing *Escherichia coli* O111 strains from patients with diarrhea and hemolytic-uremic syndrome. *J. Clin. Microbiol.* 34: 2364-2367.
- Schwarz S., Silley, P., Simjee, S., Woodford, N., Duijkeren, E.V., Johnson, A.P. and Gaastra, W. 2010. Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* 65(4): 601-604.
- Shaikh N. and Tarr, P.I. 2003. *Escherichia coli* O157:H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *J. Bacteriol.* 185(12): 3596-3605.
- Sharma V.K. and Carlson, S.A. 2000. Simultaneous detection of *Salmonella* strains and *Escherichia coli* O157:H7 with fluorogenic PCR and single - enrichment-broth culture. *Appl. Environ. Microbiol.* 66: 5472-5476.
- Shetty V.A., Kumar, S.H., Shetty, A.K. and Karunasagar, I. 2012. Prevalence and characterization of diarrheagenic *Escherichia coli* isolated from adults and children in Mangalore, India. *J. Lab. Physicians.* 4: 24-29.
- Thomas R.S. 2007. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard, M100-S17. National Committee for Clinical Laboratory Standards 6.
- Tomat D., Migliore, L., Aquili, V., Quiberoni, A. and Balagué, C. 2013. Phage biocontrol of enteropathogenic and shiga toxin-producing *Escherichia coli* in meat products. *Front. Cell. Infect. Microbiol.* 3: 20.
- Vally H., Hall, G., Dyda, A., Raupach, J., Knope, K., Combs, B. and Desmarchelier, P. 2012. Epidemiology of Shiga toxin producing *Escherichia coli* in Australia, 2000-2010. *BMC. Pub. Health.* 12: 63.
- Wang R.F., Pao, W.W. and Cerniglia, C.E. 1997. A universal protocol for PCR detection of 13 species of foodborne pathogen in foods. *J. Appl. Microbiol.* 83:727-736.
- Wani S.A., Pandit, F., Samanta, I., Bhat, M.A. and Buchh, A.S. 2004. Molecular epidemiology of Shiga toxin-producing *Escherichia coli* in India. *Curr. Sci.* 87(10): 1345-1353.