

# Multi drug resistance patterns of Shiga toxin – producing *Escherichia coli* (STEC) and non – STEC isolates from meats, RTE meat foods, drinking water and human diarrhoeic samples of Punjab, India

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## Abstract

Antimicrobial susceptibility testing was performed on a total of 253 [203 shiga toxin-producing *Escherichia coli* (STEC) and 50 non-STEC] *Escherichia coli* isolates from different raw meats, RTE meat foods, drinking water and human diarrhoeic samples in Punjab, India. Among the 20 antimicrobial agents tested, resistance was most frequent for penicillin (253/253, 100%) followed by linezolid (249/253, 98.4%), erythromycin (245/253, 96.8%), streptomycin (232/253, 91.7%), tetracycline (223/253, 88.1%), ampicillin 123/253, 48.2%), cephotaxime (106/253, 41.9%), trimethoprim (93/253, 36.8%), co-trimoxazole (85/253, 33.6%), cefaclor (84/253, 33.2%), amoxicillin (79/253, 31.3%), ciprofloxacin (78/253, 30.8%), kanamycin (76/253, 30.0%), norfloxacin (62/253, 24.5%), ofloxacin (60/253, 23.7%), chloramphenicol (35/253, 13.8%), Polymyxin-B (27/253, 10.6%), colistin (25/253, 9.9%), amikacin (22/253, 8.7%) and gentamycin (17/253, 6.7%). Out of 203 STEC isolates 73 (35.9%) showed resistance to more than 50% of the antibiotics tested. Only one isolate from pork showed resistance to 90% of the antibiotics tested. Cluster analysis also revealed that human isolates were different from other *E. coli* isolated from meat and meat products sources. The distribution of resistance determinants for tetracycline and streptomycin was assessed by PCR in resistant isolates. The most common resistance determinants were *tetA* (60%) and *tetB* (27%). Forty seven per cent of the isolates contained both *strA* and *strB* genes, 33% and 10% isolates carried *strA* and *strB* genes, respectively and 10% of isolates did carry neither *strA* nor *strB*. Cloning and sequencing of *tetA* and *tetB* genes of O69 shiga toxin-producing *E. coli* isolate from buffalo meat showed 99-100% homology with published sequences of related isolates in GenBank. Antibiotic susceptibility studies revealed that meat, RTE meat products, drinking water and human diarrhoeic samples from Punjab, India contains multiple drug resistant strains of *E. coli* which may serve as a reservoir for antibiotic resistance genes in the food environment and may transmit to humans through food chain.

**Key words:** STEC, non-STEC, Multidrug resistance, PCR, *tetA*, *tetB* and *tetC*, *strA* and *strB*.

## Introduction

*Escherichia coli* is a common inhabitant of the intestinal tract of a wide variety of animals and humans [1] and this intestinal bacteria can be easily disseminated in different ecosystems through the water, soil, food and others [2]. The increase in antimicrobial resistance and the impact on human health is an emerging problem worldwide. Exposure to resistant bacte-

ria through food chain has gained increased attention, as the presence of resistant bacteria in food and water might have an impact on the development and dissemination of resistance to antimicrobial agents in human bacterial pathogens. A recent study conducted by Johnson *et al* [3] suggests that retail foods, especially meat and meat products, may be an important vehicle for community-wide dissemination of antimicrobial resistant *Escherichia coli* and extraintestinal pathogenic *Escherichia*

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*coli*. Antibiotic usage is probably the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant in both veterinary and human medicine [4; 5]. In addition to their therapeutic use in human and veterinary medicine, antimicrobials are routinely used for disease prevention and growth promotion agents in animal production. It has been shown that repeated sublethal exposure to antimicrobial agents not only promotes adaptive resistance but also confers decreased sensitivity to antibiotics [6].

The aim of the present study was to assess the actual frequency of antimicrobial resistance in shiga toxin-producing *Escherichia coli* (STEC) and non-shiga toxin-producing *E. coli* from various meats and ready-to-eat (RTE) meat products, water and human sources in Punjab, India at the phenotype level. The second objective was to assess the diversity and distribution of the major resistance genes to tetracyclines and streptomycin in these STEC isolates and other non-STEC isolates from the above sources.

## Materials and methods

### Sample collection

A total of 675 samples comprising 392 raw meats (pork n=106, buffalo meat n= 69, chevon n=68, mutton n=55, fish n=54 and chicken=40), 167 RTE meat products (buffalo meat n=40, chicken n=40, fish n=27, pork n=25, mutton n=25 and chevon n=10) from different retail meat shops in Punjab, drinking water samples (n=64) from different localities in Punjab and human diarrhoeic samples (n=52) from different Government and corporate hospitals in Punjab were collected between June 2008 and December 2009. Approximately 50g of the raw meat and RTE meat products, 500 ml of drinking water samples and 10g of human diarrhoeic samples were collected aseptically in sterile wide mouth screw capped disposable polyethylene containers and transported to the laboratory on ice and processed the sample for isolation of *E. coli* within 24h of sample collection

### Bacterial isolates

Two hundred fifty three *E. coli* isolates were isolated from different raw meats (n=192), RTE meat foods (n=28), drinking water (n=19) and human diarrhoeic samples (Children below 10 years) (n=14) by standard procedure. The samples were enriched in Tryptic soy broth (TSB), then streaked on EMB and McConkey lactose (MLA) agar plates. Isolates showing greenish metallic sheen growth on EMB and pink colour colonies with central depression on MLA were picked up (single colony) and streaked on Nutrient agar slants and stored at 4°C for further biochemical studies and for antimicrobial susceptibility tests. The identification of each isolate was confirmed by using the

following tests: lack of oxidase activity, catalase, indole, methyl red (MR) and nitrate reduction positivity, voges proskauer (VP), citrate, urease, H<sub>2</sub>S production on triple sugar iron agar, fermentation of cellobiose, raffinose and adonitol negativity [7]. Out of 253 *E. coli* isolates 203 were identified as STEC by multiplex PCR as per the method of Paton and Paton 1998 [8] and 50 were non-STEC. Isolates possessing either *stx*<sub>1</sub> or *stx*<sub>2</sub> or both genes by multiplex PCR were considered as STEC and isolates without any genes were considered as non-STEC. Of 203 STEC isolates, 159 from raw meat, 19 from RTE meat foods, 18 from drinking water and 7 from human diarrhoeic samples. The STEC isolates from raw meat include 23 from buffalo meat, 21 from mutton, 38 from chevon, 41 from pork, and 21 from fish and 15 from chicken. The STEC isolates from RTE meat foods include, 7 from chicken products, 6 from mutton products, 4 from pork products and 1 from each chevon and buffalo meat products.

### Antimicrobial susceptibility testing

A total of 253 *E. coli* (203 STEC and 50 non-STEC) isolates were tested for antibiotic resistance by standard disk diffusion technique [9] on Mueller Hinton agar using commercial discs (HiMedia, Mumbai, India). The following antibiotics with the disc strength in parentheses were used: amikacin (30 µg), amoxicillin (30 µg), ampicillin (10 µg), cefaclor (30 µg), cephalexin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), colistin (10 µg), co-trimoxazole (1.25/23.75 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), linezolid (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), penicillin G (10 µg), polymyxin B (300 units), streptomycin (10 µg), tetracycline (30 µg) and trimethoprim (5 µg).

### Cluster analysis

To evaluate the strain diversity of STEC isolates from different raw meat, RTE meat foods, water and human diarrhoeic samples, dendrograms of antibiotic resistance patterns were constructed by using Treecon software.

### Polymerase chain reaction for the detection of tetracycline and streptomycin resistance genes

Isolated bacterial colonies were suspended in 100 µl of sterilized distilled water. The suspensions were boiled, cooled and centrifuged, and the supernatants were used as template. All the PCR experiments were performed in 25 µl volumes containing 3 µl of prepared sample supernatant as template in a PCR mix consisting of 10mM Tris pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.005% Tween 20, 0.005% Nonidet NP40 (Fermentas), 0.25 µM of each primer and 200 µM of each dNTP, 1 U of *Taq* DNA polymerase. All amplifications consisted of 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* and 55°C for *strA* and *strB* for 1 min and elongation at 72°C for 1 min and final elongation at 72°C for 7 min. The primers were used for the detection of *tetA*, *tetB*, *tetC*, *strA* and *strB* genes were as per the method of Boerlin *et al* 2005 [10]. In each PCR reaction the positive and negative controls were included.

### Cloning and sequencing of *tetA* and *tetB* genes of shiga toxin-producing *E.coli* O69 serotype of buffalo meat isolate

The *E. coli* O69 serotype was chosen for sequencing studies for the following reasons. Firstly, O69 is the most common STEC serotype recovered from buffalo meat and buffalo meat RTE products. Secondly the majority of the O69 serotypes carried both *tetA* and *tetB* tetracycline resistant determinants. The PCR amplicons (502 bp for *tetA* and 930 bp for *tetB*) were recovered from low melting point agarose, purified, and cloned with a pUCm-T vector. White colonies of ampicillin-resistant transformants were screened for the presence of *tetA* and *tetB* fragments by PCR with the same primer sets (TetA-F 5'GGCGGTCTTCTTCATCATGC3';TetA-R5'CGGCAGGCAGAGCAAGTAGA3'and TetB-F5'CATTAATAGGC-GCATCGCTG3';TetB-R 5' TGAAGTCATCGATAGCAGG3' ) used for amplification. DNA sequence of *tetA* and *tetB* genes were performed using the Big Dye terminator cycle sequencing method with Ampli *Taq* DNA polymerase in ABI PRIZM 377 DNA sequencer(Perkin-Elmer).The fragments were sequenced at least twice with each primer to reduce possibility of sequencing artefacts. Compilation and analysis of DNA sequence data were performed using Auto Assembler software 9(Perkin-Elmer). Nucleotide and amino acid homology analysis was performed using the BLAST (Basic Local Alignment Search Tool) in GenBank [11].

**Nucleotide sequence accession number:** The sequences of *tetA* and of O69 serotype of shiga toxin-producing *E. coli* of buffalo meat were submitted to the GenBank database and have been given the accession numbers AF497970 and FJ7917423 respectively.

## Results

### Antibiotic resistance patterns

Of the 253 *E. coli* isolates used in this study, 203 (81.2%) were STEC and 50 (19.8%) were non STEC. To detect the resistance/sensitivity pattern of STEC and non-STECC isolates from different sources, in vitro antibiotic sensitivity test was carried out by disk diffusion method using 20 commercially available Himedia (India) antibiotic discs. All the isolates of STEC and non-STECC were resistant to at least one of the antibiotics tested.

Globally, the level of resistance to each of the 20 antimicrobial agents assayed among STEC and non-STECC proved to be very similar.

Antimicrobial resistance patterns study revealed that STEC and non-STECC isolates were completely resistance to penicillin (253/253, 100%) followed by linezolid (249/253, 98.4%), erythromycin (245/253, 96.8%), streptomycin (232/253, 91.7%), tetracycline (223/253, 88.1%), ampicillin 123/253, 48%), cephotaxime (106/253, 41.9%), trimethoprim (93/253, 36.8%), cotrimoxazole (85/253, 33.6%), cefaclor (84/253, 33.2%), amoxicillin (79/253, 31.3%), ciprofloxacin (78/253, 30.8%), kanamycin (76/253, 30%), norfloxacin (62/253, 24.5%), ofloxacin (60/253, 23.7%), chloramphenicol (35/253, 13.8%), colistin (25/253,9.9%), polymyxin-B (27/253, 10.7%), amikacin (22/253, 8.7%) and gentamycin (17/253, 6.7%)

Out of 203 STEC isolates 73 (35.9%) isolates showed resistance to more than 50% of the antibiotics tested. Only one STEC isolate from pork showed resistance to 90% of the antibiotics tested.

On source wise analysis of the resistant STEC isolates 17 pork isolates, 16 goat meat isolates, 9 chicken isolates, 9 water isolates, 6 isolates from RTE meat foods, 6 human isolates, 5 fish isolates, 3 mutton isolates, and 2 isolates from buffalo meat showed resistance to 10 to 17 antibiotics tested.

Out of 50 non-STECC isolates, 24 (48%) showed resistance to more than 50% of the antibiotics tested. Only one isolate from humans showed resistant to 18 (90%) antibiotics tested. Among the 24 non - STECC isolates, 7 human isolates, 6 pork isolates, 4 RTE products isolates, 2 chicken isolates, 2 fish isolates, 1 goat meat isolate, 1 mutton isolate and 1 water isolate showed resistance to more than 50% of the antibiotics tested.

Sourcewise details of antibiotic resistance patterns of STEC and non-STECC are presented in **Table 1** and **2**. Of the 253 isolates from various sources in this study, the highest frequencies of antimicrobial resistance phenotypes were observed for STEC and non-STECC isolated from humans. When compared with the food isolates human STEC isolates were resistant to cefaclor, erythromycin, linezolid, penicillin G, streptomycin and tetracycline (100% each), ampicillin, amoxicillin, cephotaxime and kanamycin (85.7%), ciprofloxacin, norfloxacin, ofloxacin, trimethoprim and co-trimoxazole (71.4%), gentamycin (57.1%), polymyxin B (28.6%) and amikacin (8.9%).

Resistance profiles among STEC isolates from buffalo meat, chevon, pork and fish were almost similar to each other. Hundred percent of STEC isolates from buffalo meat were resistant to penicillin G followed by 95.7% to erythromycin and linezolid, 69.6% to tetracycline, 65.2% to streptomycin, 26.1% to ampicillin, 21.7% to polymyxin B and 17.4% to colistin. Hundred per-

**TABLE 1.** Antibiotic resistance among Shiga toxin-producing E.coli (STEC)

Antibiotics	Resistant strains/total investigated																			
	Total STEC		Humans		Water		Buff. Meat		Chevon		Mutton		Chicken		Fish		Pork		RTE Foods	
Ak	18/203	8.86%	1/7	14.28%	1/18	5.55%	2/23	8.69%	0/38	0%	2/21	9.52%	3/15	20%	1/21	4.76%	8/41	19.51%	0/19	0%
Am	61/203	30.04%	6/7	85.71%	10/18	55.55%	2/23	8.69%	11/38	28.94%	5/21	22.80%	7/15	46.6%	5/21	23.80%	9/41	21.95%	6/19	31.5% <sup>6</sup>
A	97/203	47.78%	6/7	85.71%	11/18	61.11%	6/23	26.08%	17/38	44.73%	12/21	57.14%	10/15	66.6%	9/21	42.85%	19/41	46.34%	7/19	36.8%
Cj	63/203	31.03%	7/7	100%	8/18	44.44%	3/23	13.04%	11/38	28.94%	4/21	19.04%	5/15	33.3%	6/21	28.57%	15/41	36.58%	4/19	21%
Ce	85/203	41.87%	6/7	85.71%	13/18	54.16%	3/23	13.04%	3/38	7.89%	10/21	47.61%	7/15	46.6%	10/21	47.61%	28/41	68.29%	4/19	21%
C	28/203	13.79%	3/7	42.85%	0/18	0%	1/23	4.34%	5/38	13.15%	2/21	9.52%	5/15	33.3%	2/21	9.52%	9/41	21.95%	1/19	5.2%
Cf	60/203	29.55%	5/7	71.42%	8/18	44.44%	0/23	0%	12/38	31.57%	0/21	0%	6/15	40%	4/21	19.04%	21/41	51.21%	4/19	21%
Cl	20/203	9.85%	1/7	14.28%	2/18	11.11%	4/23	17.39%	4/38	10.52%	1/21	4.76%	1/15	6.6%	1/21	4.76%	5/41	12.19%	1/19	4.16%
Co	62/203	30.54%	5/7	71.42%	6/18	33.33%	0/23	0%	17/38	44.73%	6/21	28.57%	7/15	46.6%	6/21	28.57%	9/41	21.95%	7/19	36.8% <sup>6</sup>
E	196/203	96.55%	7/7	100%	18/18	100%	22/23	95.65%	37/38	97.36%	21/21	100%	5/15	33.3%	21/21	100%	41/41	100%	19/19	100%
G	14/203	6.89%	4/7	57.14%	5/18	27.77%	0/23	0%	0/38	0%	0/21	0%	0/15	0%	1/21	4.76%	1/41	2.43%	2/19	10.5%
K	60/203	29.55%	6/7	85.71%	8/18	44.44%	1/23	4.34%	10/38	26.31%	2/21	9.52%	6/15	40%	4/21	19.04%	19/41	46.34%	5/19	26.3%
Lz	200/203	98.52%	7/7	100%	18/18	100%	23/23	95.65%	37/38	97.36%	21/21	100%	15/15	100%	21/21	100%	41/41	100%	19/19	100%
Nx	45/203	22.16%	5/7	71.42%	8/18	44.44%	0/23	0%	14/38	36.84%	0/21	0%	7/15	46.6%	3/21	14.28%	4/41	9.75%	4/19	21%
Of	44/203	21.67%	5/7	71.42%	7/18	38.88%	1/23	4.34%	12/38	31.57%	0/21	0%	7/15	46.6%	3/21	14.28%	5/41	12.19%	4/19	21%
P	203/203	100%	7/7	100%	18/18	100%	23/23	100%	38/38	100%	21/21	100%	15/15	100%	21/21	100%	41/41	100%	19/19	100%
Pb	20/203	9.85%	2/7	28.51%	1/18	5.55%	5/23	21.73%	4/38	10.52%	3/21	14.28%	2/15	13.3%	0/21	0%	3/41	7.31%	1/19	4.16%
S	184/203	90.64%	7/7	100%	16/18	88.88%	15/23	65.21%	34/38	89.47%	21/21	100%	14/15	93.3%	20/21	95.23%	41/41	100%	16/19	84.21%
T	178/203	87.68%	7/7	100%	16/18	88.88%	16/23	69.56%	33/38	86.84%	20/21	95.23%	11/15	73.3%	19/21	90.47%	38/41	92.68%	17/19	89.4%
Tr	69/203	33.69%	5/7	71.42%	7/18	38.88%	1/23	4.34%	18/38	47.36%	6/21	28.57%	9/15	60%	7/21	33.33%	10/41	24.39%	7/19	3%

cent of STEC isolates from chicken were resistant to erythromycin, linezolid and penicillin G, 93.3% to streptomycin, 73.3% to tetracycline, 66.6% to ampicillin, 60% to trimethprim, and cephotaxime, 46.6% to amoxicillin, norfloxacin and ofloxacin and, 40% to ciprofloxacin and amikacin.

### Cluster analysis

To evaluate the strain diversity of STEC isolates from different raw meat, RTE meat foods, water and human diarrhoeic samples, dendograms of antibiotic resistance patterns were constructed by using unweighted pair group method with

**TABLE 2.** Antibiotic resistance among non- Shigatoxic E. coli (Non-STEC)

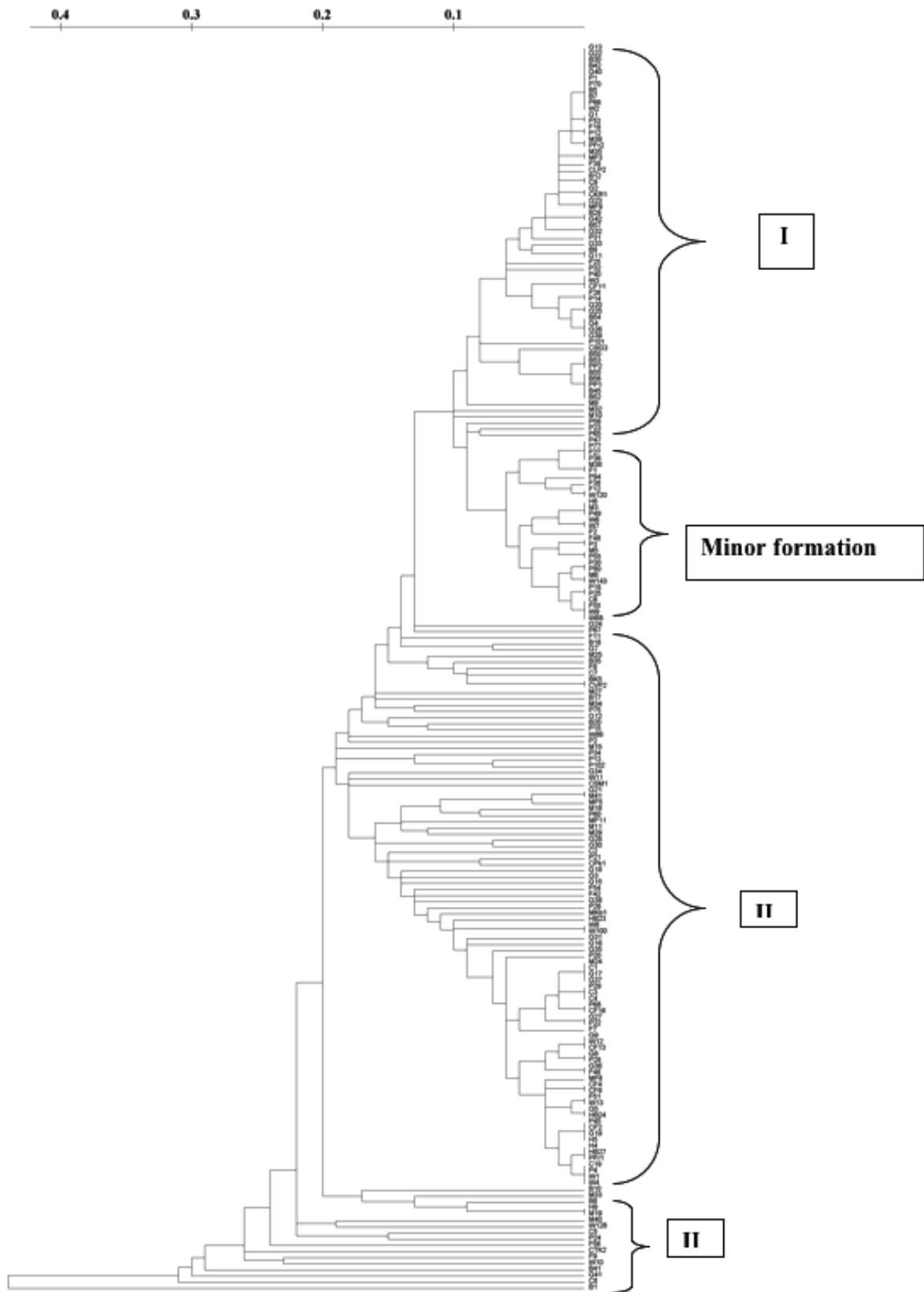
Antibiotics	Resistant strains/total investigated																			
	Total Non – STEC		Humans		Water		Buff. Meat		Chevon		Mutton		Chicken		Fish		Pork		RTE Foods	
Ak	4/50	8%	1/7	14.28%	0/1	0%	0/7	0%	0/1	0%	0/4	0%	0/2	0%	1/5	20%	2/14	14.28%	0/9	0%
Am	18/50	36%	6/7	85.71%	0/1	0%	0/7	0%	0/1	0%	1/4	25%	1/2	50%	2/5	40%	4/14	28.57%	4/9	44.44%
A	26/50	52%	7/7	100%	1/1	100%	1/7	14.28%	0/1	0%	4/4	100%	2/2	100%	2/5	40%	5/14	35.71%	4/9	44.44%
Cj	21/50	42%	6/7	85.71%	0/1	0%	1/7	14.28%	0/1	0%	1/4	25%	1/2	50%	2/5	40%	7/14	50%	3/9	33.33%
Ce	21/50	42%	5/7	71.42%	1/1	100%	0/7	0%	0/1	0%	2/4	50%	0/2	0%	1/5	20%	7/14	50%	5/9	55.55%
C	7/50	14%	3/7	42.85%	0/1	0%	0/7	0%	0/1	0%	1/4	25%	0/2	0%	1/5	20%	2/14	14.28%	0/9	0%
Cf	18/50	36%	6/7	85.71%	0/1	0%	0/7	0%	1/1	100%	1/4	25%	1/2	50%	2/5	40%	4/14	28.57%	3/9	33.33%
Cl	5/50	10%	1/7	14.28%	1/1	100%	0/7	0%	0/1	0%	1/4	25%	0/2	0%	1/5	20%	0/14	0%	1/9	11.11%
Co	23/50	46%	6/7	85.71%	0/1	0%	0/7	0%	1/1	100%	2/4	50%	1/2	50%	1/5	20%	7/14	50%	5/9	55.55%
E	49/50	98%	7/7	100%	1/1	100%	6/7	85.71%	1/1	100%	4/4	100%	2/2	100%	5/5	100%	14/14	100%	9/9	100%
G	3/50	6%	2/7	28.57%	0/1	0%	0/7	0%	0/1	0%	0/4	0%	1/2	50%	0/5	0%	0/14	0%	0/9	0%
K	16/50	32%	7/7	100%	1/1	100%	1/7	14.28%	1/1	100%	0/4	0%	1/2	50%	0/5	0%	4/14	28.57%	1/9	11.11%
Lz	49/50	98%	7/7	100%	1/1	100%	6/7	85.71%	1/1	100%	4/4	100%	2/2	100%	5/5	100%	14/14	100%	9/9	100%
Nx	17/50	34%	6/7	85.71%	0/1	0%	0/7	0%	1/1	100%	0/4	0%	1/2	50%	2/5	40%	3/14	21.42%	4/9	44.44%
Of	16/50	32%	6/7	85.71%	0/1	0%	0/7	0%	1/1	100%	1/4	25%	1/2	50%	1/5	20%	2/14	14.28%	4/9	44.44%
P	50/50	100%	7/7	100%	1/1	100%	7/7	100%	1/1	100%	4/4	100%	1/2	50%	5/5	100%	14/14	100%	9/9	100%
Pb	7/50	14%	2/7	28.57%	1/1	100%	0/7	0%	1/1	100%	1/4	25%	0/2	0%	1/5	20%	1/14	7.14%	0/9	0%
S	48/50	96%	7/7	100%	1/1	100%	7/7	100%	1/1	100%	4/4	100%	2/2	100%	4/5	80%	14/14	100%	8/9	88.88%
T	45/50	90%	7/7	100%	1/1	100%	4/7	57.14%	1/1	100%	4/4	100%	2/2	100%	4/5	100%	13/14	92.85%	9/9	100%
Tr	24/50	48%	6/7	85.71%	0/1	0%	0/7	0%	1/1	100%	2/4	50%	1/2	50%	1/5	20%	8/14	57.14%	5/9	55.55%

arithmetic means (UPGMA) tree building and significant clusters in each dendrogram were identified (Fig. 1 and 2) The dendrogram revealed that STEC isolates from different sources of the study formed 4 significant clusters (Cluster I, cluster II, cluster III and minor formations).

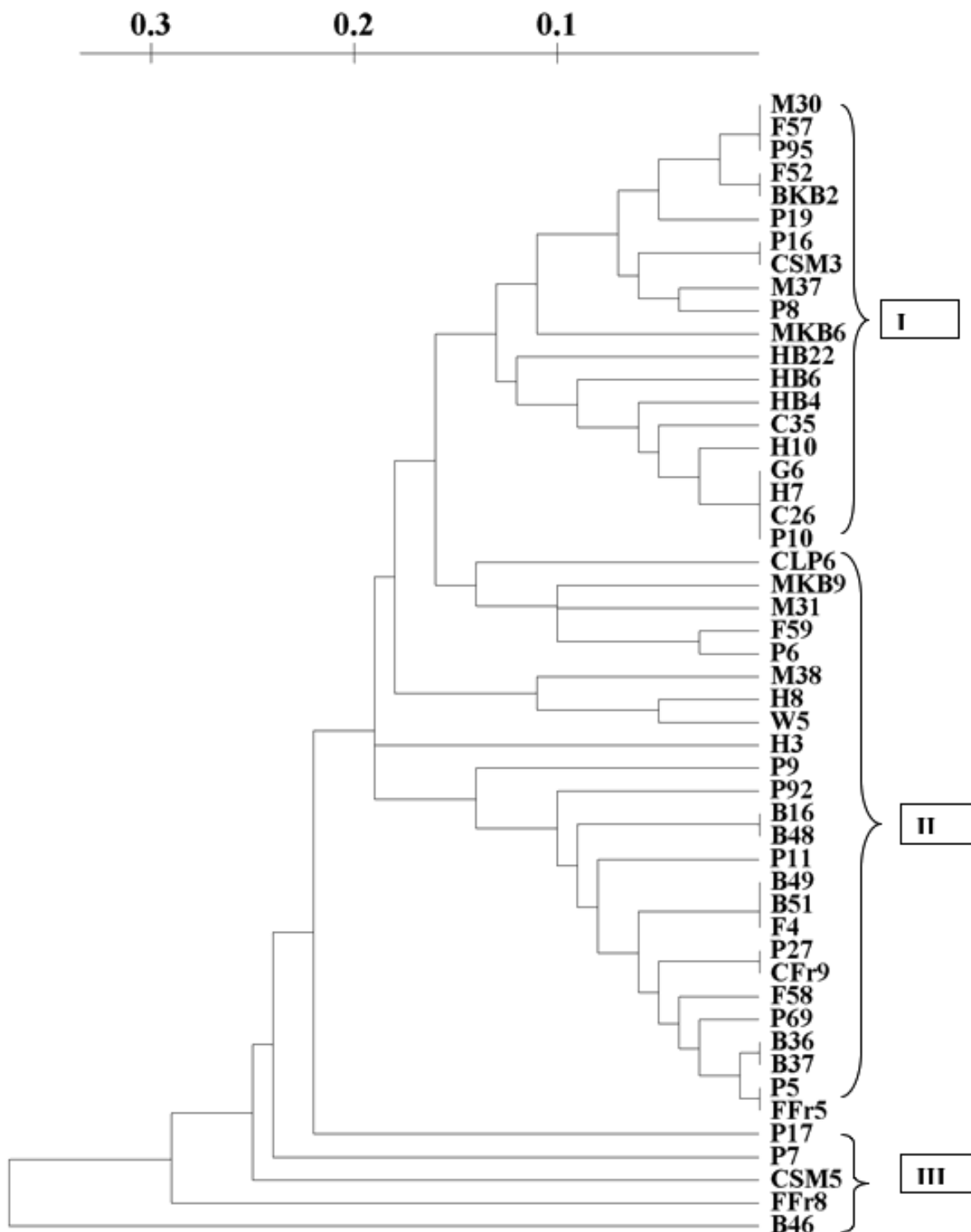
Cluster I comprised mainly STEC isolates of goat meat, buffalo meat and pork. Human STEC isolates were absent in cluster I.

In tightly knit cluster II, majority of goat meat isolates, pork isolates and RTE meat foods isolates were present. Some chicken, fish, mutton, humans and water isolates were also present.

Majority of the Pork isolates (15) formed as a separate group (minor formation). In cluster III, 17 isolates of different sources (Buffalo meat-4, mutton-3 water-2, fish-2, chicken-2, RTE meat foods-1, humans-1, chevon-1 and pork-1) were present.



**FIG. 1** Dendrogram generated by Treecon software, showing relatedness among STEC isolates from various sources according to antibiotic resistance patterns



**FIG. 2** Dendrogram generated by Treecon software showing relatedness of Non-STEC isolates from various sources according to antibiotic resistance patterns.

Cluster/proximity analysis of non-STEC isolates from different sources revealed 3 significant clusters. Pork isolates and human isolates were the major non-STEC isolates in cluster I. Some isolates from fish, mutton, chicken, goat meat and RTE meat foods were also present. No isolates from water sources were present in cluster I.

In cluster II isolates from buffalo meat and pork were the major source. Isolates from water, fish, mutton RTE meat foods and humans were also present. Chicken isolates and goat meat isolates were absent in this cluster. In cluster III only pork and RTE meat foods isolates and one isolate from buffalo meat were present. Isolates from goat meat, water, fish, mutton, chicken and humans were absent in this cluster.

### Distribution of *tetA*, *tetB* and *tetC* resistance genes

All 223 isolates (178 STEC and 45 non-STEC), which were phenotypically resistant to tetracycline by disk diffusion method were examined further by using PCR for the presence of *tetA*, *tetB*, *tetC* genes. The frequencies of the major resistance genes for tetracycline in the different *E. coli* isolates of different sources, buffalo meat, chevon, mutton, chicken, fish, humans, water and RTE meat foods are reported in Table. 3. Of the 178 shiga-toxic *E.coli* showing phenotypic resistance to tetracycline analyzed by PCR, 177 (99.5%) STEC contained at least one of three (*tetA*, *tetB*, *tetC*) tetracycline resistance genes (Fig. 3)

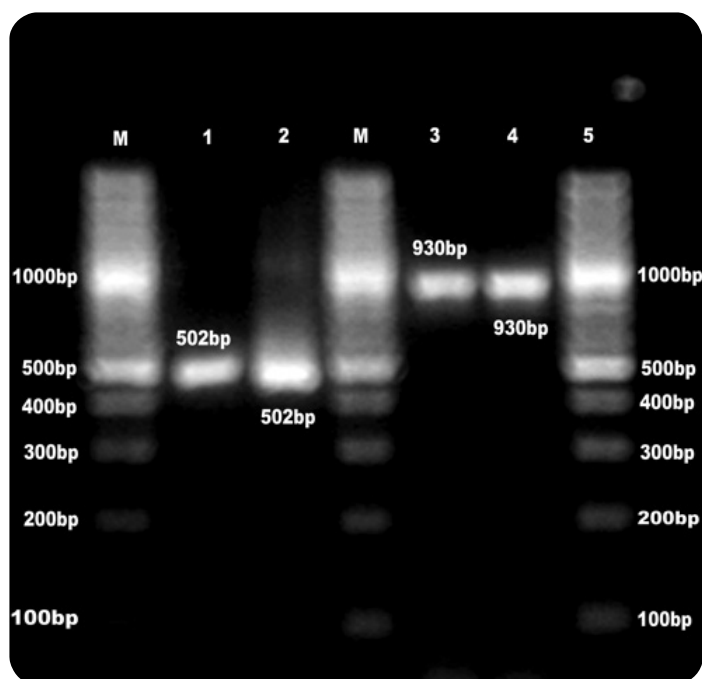
### STEC isolates

The most common resistance genes were *tetA* (107 of 178; 60%) and *tetB* (48 of 178; 27%). Twelve per cent of STEC isolates were carried both *tetA* and *tetB* resistance genes. Only one isolate from chicken sausage has shown *tetC* resistance gene. Among STEC isolates *tetA* gene was predominant in humans (100%), chicken (86%), mutton (80%), Chevon (67%), Pork 63%), buffalo meat (62.5%) RTE meat foods (36%) and water (31%) STEC isolates.

The *tetB* resistance gene was present in 50% of water, 37% of fish, 32% of pork, 27% of chevon, 14% of chicken and 12.5% of buffalo meat STEC isolates. All human STEC isolates were negative for *tetB* determinants. Both *tetA* and *tetB* genes were present in 25% of buffalo meat, 19% of water, 18% of RTE meat foods STEC isolates. None of the human and chicken STEC isolates carried both *tetA* and *tetB* resistance genes in combination.

### Non-STEC isolates

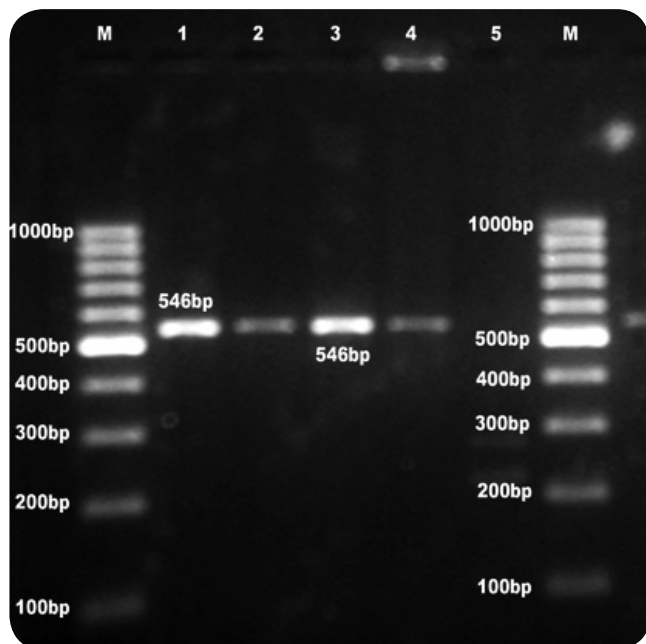
The *tetA* gene was predominant in all (100%) non-STEC isolates of buffalo meat, chevon, and water, 75% of mutton, 62% of pork, 56% of RTE meat foods, 43% of humans and 25% fish isolates. In chicken isolates *tetA* resistance gene was absent. The *tetB* resistance gene was present in 50% of chicken and fish, 23% of pork and 22% of RTE meat food isolates and was absent in buffalo meat, chevon, mutton, water and human isolates.



Lane M:	1000 bp DNA ladder
Lane 1:	<i>tetA</i> standard
Lane 2:	<i>E. coli</i> from buffalo meat positive for <i>tetA</i> gene
Lane 3:	<i>tetB</i> standard
Lane 4:	<i>tetB E. coli</i> from human positive for <i>tetB</i> gene

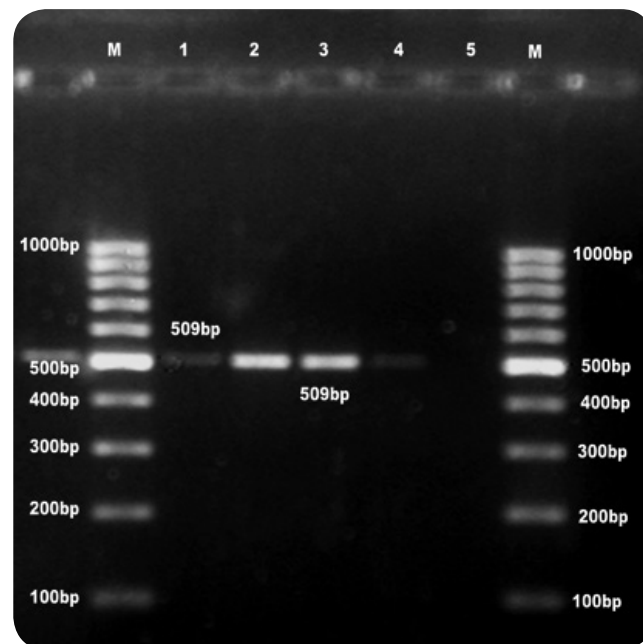
**FIG. 3.** Standardization of PCR for detection of *tetA* and *tetB* genes





Lane M:	100 bp DNA ladder
Lane 1:	<i>StrA</i> standard
Lane 2-4:	<i>E. coli</i> from buffalo meat, mutton and human positive for <i>strA</i> gene
Lane 5:	<i>E. coli</i> from RTE foods negative for <i>strA</i> gene

**FIG. 4.** Standardization of PCR for detection of *strA* gene.



Lane M:	100 bp DNA ladder
Lane 1:	<i>StrB</i> standard
Lane 2-4:	<i>E. coli</i> from buffalo meat, chevon and fish positive for <i>strB</i> gene
Lane 5:	<i>E. coli</i> from human negative for <i>strB</i> gene

**FIG. 5.** Standardization of PCR for detection of *strB* gene.

Fifty seven per cent of human, 50% of chicken, 25% of fish, 22% of RTE meat foods and 15% of pork non-STEC strains carried both *tetA* and *tetB* tetracycline resistance genes. Among 45 non-STEC isolates tested by PCR, 98% contained the tetracycline resistance genes. The most common resistance genes were *tetA* (58%), followed by *tetA* and *tetB* in combination (22%), and *tetB* (18%). All the non STEC isolates were negative for *tetC* gene.

Out of 223 *E. coli* isolates phenotypically resistance to tetracycline, only one STEC isolate from fish and one non-STEC isolate from mutton lacked all three tetracycline resistance genes.

### Distribution of *strA* and *strB* genes

All 231 isolates (184 STEC and 47 non-STEC), which were phenotypically resistant to streptomycin by disk diffusion method

were examined further by using PCR for the presence of *strA* and *strB* genes (Fig. 4 and 5). The frequencies of the major resistance genes for streptomycin in the *E. coli* isolates of different sources, buffalo meat, chevon, mutton, chicken, fish, humans, water and RTE meat foods are reported in Table 3.

Of the 184 STEC isolates showing phenotypic resistance to streptomycin were investigated for *strA* and *strB* resistance genes, 86 (47%) of isolates harboured both *strA* and *strB* genes. Sixty (33%) and 18 (10%) isolates carried *strA* and *strB* gene, respectively and 18 (10%) of isolates did not carry any. Seventy one per cent of isolates from humans and RTE meat foods, 63% of water, 57% of mutton, 46% of pork, 45% of fish, 41% of chevon and 27% from buffalo meat isolates harboured both *strA* and *strB* resistance genes. All 14 chicken isolates were negative for both *strA* and *strB* genes in combination. Eighty six per cent of chicken, 46% of buffalo meat, 42% of pork, 41% of chevon, 33% fish, 25% of water, 19% of mutton and RTE meat food isolates carried only *strA* gene. Twenty seven per cent of buffalo meat,

Source	Buffalo meat	Chevon	Mutton	Chicken	Fish	Pork	Water	Humans	RTE meat foods	Total		
Tetracycline resistant STEC(n)	17	33	20	11	19	38	16	7	17	178		
Tetracycline resistant non-STECC (n)	4	1	4	2	4	13	1	7	9	45		
<i>tetA</i>	<i>tetB</i>	<i>tetC</i>										
-	-	-	0(0)	0(0)	0(25)	0(0)	5(0)	0(0)	0(0)	0(0)	0.5(2)	
+	-	-	62.5(100)	67(100)	80(75)	86(0)	47(25)	63(62)	31(100)	100(43)	36(56)	60(58)
-	+	-	12.5(0)	27(0)	15(0)	14(50)	37(50)	32(23)	50(0)	0(0)	41(22)	27(18)
+	+	-	25(0)	6(0)	5(0)	0(50)	11(25)	5(15)	19(0)	0(57)	18(22)	12(22)
+	+	+	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5(0)	0.5(0)
Streptomycin Resistant STEC (n)	15	34	21	14	20	41	16	7	16	184		
Streptomycin Resistant non-STECC (n)	7	1	4	2	4	14	1	7	7	47		
<i>strA</i>	<i>strB</i>											
-	-	0(29)	15(0)	14(25)	0(0)	0(25)	0(28)	6(0)	29(28)	10(12)	10(22)	
+	-	46(14)	41(0)	19(25)	86(0)	33(0)	42(36)	25(0)	0(0)	19(25)	33(19)	
-	+	27(14)	3(0)	10(0)	14(0)	22(0)	7(0)	6(0)	0(0)	0(0)	10(2)	
+	+	27(43)	41(100)	57(50)	0(100)	45(75)	46(36)	63(100)	71(72)	71(63)	47(57)	

22% of fish, 10% of mutton, 14% of chicken, 7% of pork, 6% water and 3% of chevon isolates carried *strB* resistance gene.

Of 47 non-STECC isolates analyzed for *strA* and *strB* genes, 57% isolates carried both *strA* and *strB* genes, 19% and 2% isolates carried *strA* and *strB* resistance gene respectively and 22% of the isolates did not carry any gene.

## Nucleotide sequencing and accession numbers

The nucleotide sequencing of tetracycline resistance genes *tetA* and *tetB* of O69 serotype of STECC buffalo meat isolate determined in this study were submitted to the GenBank and have been given accession numbers AF497970 and FJ7917423. The nucleotide sequences of *tetA* and *tetB* of O69 serotype of STECC buffalo meat isolates were 99-100% similar to the other published sequences in GenBank.

## Discussion

Of the 253 *E.coli* isolates (203 STECC and 50 non-STECC) in this study, approximately 90% displayed resistance to one or more antimicrobials including penicillin G, sulphonamides, tetracyclines, cephalosporins and aminoglycosides. The results in the present study are more or less similar to the earlier findings, where along with multiple resistance of *E. coli* to several antibiotics, a varying degree of sensitivity was reported for norfloxacin, ciprofloxacin, ofloxacin, gentamycin, chloramphenicol, amikacin, cefaclor, cephotaxime and polymyxin-B [10,12-14].

The results from this study show alarming resistant frequencies in *E.coli* (both STECC and non-STECC) from humans, drinking water, raw meats and RTE meat foods. Antibiotic resistance patterns in this study also revealed that clear variations among resistance patterns between human isolates and foods. These differences could be related to the different antibiotic regimens used for the different antimicrobial agents in livestock species and humans [15]. In the present investigation high antimicrobi-

al resistance pattern is possibly as a consequence of extensive usage of these antibiotics in the treatment of livestock or in other sources e.g.:- contact with animal faeces, feeding, water sources, agriculture which may be a cause of the transmission of resistant genes from various sources to food producing animals. Some strains are resistant to more than 10 antimicrobial agents tested, is an alert for the consumers who eat improperly cooked meat and unhygienic handle of meat before and after preparation. Epidemiological studies are required to find out the incidence of STEC in different raw as well as RTE meat foods and major possible contamination sources including abattoir hygiene, transportation of carcass, butcher's hygiene, utensils used in butchers shop and storage conditions of meat and meat products should be checked regularly. No study so far has been conducted to understand the effects of antibiotic use in animals and agriculture in India. The unregulated use of antibiotics in such systems in India contributes significantly to the antibiotic residues in meat and meat products. The antibiotic resistance patterns of different meats and meat products strains of both STEC and non-STEC observed in this study suggests a greater risk in the form of transfer of resistance to other pathogenic bacteria. The antibiotic resistance in non-STEC cannot be ruled out as insignificant since the transfer of resistance genes can take place between closely related bacteria such as members of *Enterobacteriaceae* family.

The correlation between genotype (absence or presence of a resistance gene) and phenotype (sensitive or resistant) was high for tetracycline (98% agreement). In contrast to studies from other countries, where *tetB* and *tetC* have shown to be dominant [16-19], *tetA* was the frequent tetracycline determinant in the present study, where *tetC* was very infrequent. However, this observation is consistent with the studies of Lanz *et al* [14] and Boerlin *et al* [10], who reported high frequency of *tetA* than *tetB* and *tetC* in *E.coli* isolated from pigs with oedema and diarrhoea. The results of present study also showed that 22% of non-STEC isolates and 12% of STEC isolates contained both *tetA* and *tetB* genes. This is in contrast to results from previous findings, in which only 3.5% [20] and 5.4% [21] of isolates of pigs, cattle and chicken had two genes. However results of the present study are in agreement with the findings of Bryan *et al* 2004 [16] who reported 22.2% *E. coli* isolates of pigs, chickens, turkeys, sheep, cow, fish, goat, humans, dogs, cats, horses and goose, duck and deer contained two genes. Out of 223 isolates phenotypically resistance to tetracycline, only one STEC isolate from fish and one non-STEC isolate from mutton lacked all three tetracycline resistance genes. The reason for absence of all three resistance genes may be that in isolates point mutations may be causing phenotypic tetracycline resistance [22] or phenotypic resistance may be due to other plasmid mediated resistance determinants like *tetG* or chromosomal mediated *tetM* genes, which are not tested in the present study.

In this study 47% of STEC isolates and 57% of non-STEC isolates contained both *strA* and *strB* resistance genes; this finding is in agreement with the results of other researchers showing that both genes have to be present in order to obtain a functional streptomycin resistance [23]. Nineteen per cent of STEC and

33% of non-STEC isolates carried *strA* gene and 10% of STEC and 2% of non-STEC isolates carried *strB* gene only, in these isolates an additional *aadA* gene (The *aadA* gene did not tested in the present study) could be responsible for observed resistance phenotype. Ten per cent of STEC and 22% of non-STEC were lacked *strA* or *strB* or in combination, one step resistance mutations is the most probable explanation for resistance in these isolates [22]. Due to paucity of similar data, cluster analysis results could not be compared.

In conclusion, our work identified and compared for the first time resistance phenotypes and genotypes of STEC and non-STEC isolated from different raw meat, RTE meat foods, drinking water and human sources. We could show, that significance differences with regard to multidrug resistance patterns can be observed between STEC and non-STEC isolates of different sources; raw meat, RTE meat products, drinking water and human diarrhoeic samples not only at the phenotypic level but also at the genotypic level for tetracycline and streptomycin. A comprehensive surveillance is required to determine the presence and distribution of antibiotic resistant *E. coli* in foods of animal origin and agriculture in India to monitor any emerging antimicrobial resistance problems that may arise in this important human pathogen.

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