Antimicrobial resistance and molecular characterisation of E. coli *from poultry in Eastern India*

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Summary
In this study 252 poultry samples comprised of poultry meat (n = 228) and poultry eggs (n = 24) were screened for the isolation of *Escherichia coli* (*E. coli*). A total of 62 *E. coli* isolates were recovered from poultry meat. The *E. coli* isolates belonged to different serogroups based on 'O' serotyping of the isolates viz O29 (10.8%), O8 (7.7%), O40 (6.15%), O2 (4.61%), O60 (3.08%), O106 (3.08%), 42 (1.54%), O 87 (1.54%), and 01 serotypes of O1, O7, O30, O45, O59, O66, O105, O1116, O136, O141, O147, O148, O166, and O172. Sixteen (24.62%) of the isolates were UT (untypable) and 6 (9.23 %) were rough types. Molecular characterisation of the isolates was performed, targeting stx_1 and stx_2 virulence gene fragment. Out of 62 *E. coli* isolates, 10 (16.12%) were carrying virulence gene stx_2, whereas none of the isolate was carrying stx_1 gene. The *E. coli* isolates showed wide variation in resistance pattern against the antimicrobial agents that we used (9-90%). Among *E. coli* isolates, maximum resistance was observed against cefuroxime (89.1%) and penicillin (89.4%), followed by ampicillin (80.43%), vancomycin (74.1%), co-trimoxazole (73.1%), cephalothin (60.8%), ceftriaxone (28.2%), tetracycline (17.4%), gentamicin (13%), amikacin (13.04%), ofloxacin (13%), and ciprofloxacin (6.5%). A high degree of susceptibility was observed against amikacin (84.7%) and ciprofloxacin (76%) followed by gentamicin (71.73) and ofloxacin (60.86%). High multiple antibiotic resistances were observed and a total of 34 resistance patterns were identified.

Resistenza antimicrobica e caratterizzazione molecolare di Escherichia coli *nel pollame in India orientale*

Parole chiave
Pollame,
E. coli,
resistenza antimicrobica,
caratterizzazione molecolare,
stx_1 gene,
stx_2 gene.

Riassunto
In questo studio, condotto su 228 campioni di carne e 24 di uova, sono stati rilevati dalla carne 62 isolati di *Escherichia coli* appartenenti a differenti sierogruppi basati sul sierotipo O, ovvero O29 (10.8%), O8 (7.7%), O40 (6.15%), O2 (4.61%), O60 (3.08%), O106 (3.08%), O42 (1.54%), O87 (1.54%); per ciascun sierotipo O1, O7, O30, O45, O59, O66, O105, O1116, O136, O141, O147, O148, O166, O172, ne è stato individuato uno; 16 (24,62%) sono risultati non dimostrabili e 6 (9,23%) erano di tipi approssimativi. La caratterizzazione molecolare è stata eseguita mirando ai frammenti dei geni di virulenza stx_1 e stx_2; 10 (16,12%) trasportavano il gene stx_2 mentre nessuno lo stx_1. Gli isolati di *E. coli* hanno mostrato un'ampia variazione nel pattern di resistenza contro gli agenti antimicrobici usati (9-90%). La massima resistenza è stata osservata contro cefuroxima (89,1%) e penicillina (89,4%), seguita da ampicillina (80,43%), vancomicina (74,1%), cotrimossazolo (73,1%), cefalotina (60,8%) , ceftriaxone (28,2%), tetraciclina (17,4%), gentamicina (13,0%), amikacina (13,04%), ofloxacina (13%) e ciprofloxacina (6,5%). È stato invece notato un alto grado di suscettibilità nei confronti di amikacina (84,7%) e ciprofloxacina (76%), seguita da gentamicina (71,73) e ofloxacina (60,86%). Sono state riscontrate elevate resistenze antibiotiche multiple e identificato un totale di 34 pattern di resistenza.

Introduction

Escherichia coli (*E. coli*) is a commensal bacterium in humans and animals; however some strains are capable of causing intestinal diseases. This organism is commonly present in the environment and is considered an indicator of faecal contamination of food and water. On the basis of virulence factors, *E. coli* has been classified into different pathotypes: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic or shiga toxin-producing *E. coli* (EHEC/STEC). The latter is most commonly associated with outbreaks of foodborne diseases (Bandyopadhyay *et al.* 2011). The pathogenicity of EHEC/STEC is mediated mainly through Shiga toxins 1 and 2 encoded by stx_1 and stx_2 genes, respectively. Although bovines are considered to be the major carrier for EHEC/STEC, poultry may also harbour the organism and play an important role in transmission (Ferens and Hovde 2011). The stx_2 gene is associated with an increased risk of developing hemolytic-uremic syndrome (Boerlin *et al.* 1999). Although reports on the isolation, identification, and characterisation of EHEC/STEC in humans and animals are available in India, very few reports on the association of EHEC/STEC in poultry are available in the country (Chattopadhayay *et al.* 2001, Khan *et al.* 2002, Wani *et al.* 2004).

During the past decade, drug resistance to Enterobacteriaceae has increased worldwide. Since *E. coli* is present as gut commensal in humans and animals, it has become one of the microorganisms that are commonly resistant to antimicrobials due to the selective pressure imposed by the antimicrobial drugs used in the treatment of food animals and humans (Zhao *et al.* 2012). Moreover, in animals antimicrobial agents are not only used for therapy and the prevention of bacterial infection, but also for the promotion of growth (Van den Bogaard and Stobberingh 2000). In the poultry sector, maximum numbers of antimicrobial agents are continuously fed to animals in order to promote growth, which further imposes selective pressure on the organism leading to development of antimicrobial resistance. The emergence of antimicrobial resistance among *E. coli* of animal origin has important public health implications. Various authors report that animals are the source of drug-resistant *E. coli* in humans (Johnson and Nolan 2009, Hammerum and Heuer 2009, Overdevest *et al.* 2011). Szmolka and colleagues (Szmolka *et al.* 2012) report common multi-resistance patterns of *E. coli* from food animals and clinical cases, which suggests that the circulation of multi-drug resistant *E. coli* circulating in the food chain is significant. The purpose of this study is to determine the prevalence, antimicrobial resistance, and molecular characterisation of stx_1 and stx_2 genes in *E. coli* isolated from poultry meat.

Materials and methods

Sample Collection

A total of 252 samples constituting of fresh poultry meat (n = 228) and eggs (n = 24) were collected randomly from different shops and market of Patna, India during September 2010 to March 2013. The samples were transported under cold conditions to the laboratory in the Department of Veterinary Public Health, Bihar Veterinary College, Patna, India, and processed within 1 hour of collection.

Isolation and biochemical characterization

Ten grams of meat sample and egg white were inoculated in MacConkey broth (HiMedia, Mumbai, India) and incubated at 37 °C for 12 hours. Samples showing acid and gas production were further inoculated on MacConkey agar plates (HiMedia, Mumbai, India) and incubated at 37 °C for 18-24 hours. Pure and characteristic pink coloured single colonies selected from MacConkey agar were subcultured on Eosin Methylene Blue (EMB) agar (HiMedia, Mumbai, India) in order to observe the metallic sheen that is characteristic of *E. coli*. The pure colonies were picked up on Nutrient Agar slants and subjected to standard morphological and biochemical tests (Edwards and Ewing 1972).

Serotyping *E. coli*

All the biochemically confirmed isolates of *E. coli* were submitted to the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India for serotyping based on somatic (O) antigens.

Extracting *E. coli* DNA for the detection of virulence genes

The bacterial DNA was prepared as per Dutta and colleagues (Dutta *et al.* 2011), with slight modification. The isolates were inoculated into 5 ml Luria Bertani (L-B) broth and incubated at 37 °C for 18 hours. After incubation, 1 ml of the broth culture was centrifuged in a 1.5 ml microcentrifuge tube at 8,000 rpm for 10 minutes. The pellet was washed twice in sterile normal saline solution (NSS) (0.85% NaCl) then re-suspended in 400 µl of nuclease-free sterile distilled water and boiled for 10 minutes followed by immediate chilling. Cell debris was removed by centrifugation at 5,000 rpm for

5 minutes. The supernatant was used as a template DNA for polymerase chain reaction (PCR).

Detecting virulence genes by multiplex PCR

A multiplex PCR protocol was standardized using 2 sets of oligonucleotide primers targeting stx_1 and stx_2 genes (Table I). Amplification was carried out in a total volume of 25 µl containing 10 pmol each of primer, 50 µM each of dNTP, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase, 1X PCR of buffer, and 5 µl template DNA. A negative control containing the same reaction mixture except the DNA template was included in every experiment. The reaction condition was optimised with initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute. Finally, an additional extension was achieved for 5 minutes at 72 °C. Amplified products were separated by agarose gel (2% agarose in 1X Tris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 hours and stained with ethidium bromide (0.5 µg/ml) (Sambrook *et al.* 1989). A standard molecular-size marker (100 bp DNA ladder) was included in each gel. We observed and photographed DNA fragments in a gel documentation system (Biorad, USA).

Antimicrobial resistance

The antimicrobial susceptibility test of isolates was determined using the agar disc diffusion method (Wayne 2002). The antibiotic discs included ampicillin (10 µg), amikacin (30 µg), cefuroxime (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), gentamicin (10 µg), ofloxacin (5 µg), penicillin-G (10 units), tetracycline (30 µg), and vancomycin (30 µg). The isolates were grown on autoclaved Mueller Hinton broth (HiMedia, India) for 18 hours at 37 °C. About 100 µl of the inoculum was spread on Mueller Hinton agar using a sterile disposable L-shaped spreader. Antibiotic discs were placed onto the plate using sterile forceps. The plates were incubated at 37 °C for 24 hours and observed for zone of inhibition. The results were interpreted as susceptible, resistant, or moderately susceptible based on the diameter of the zone of inhibition that was produced by different isolates. We referenced the manufacturer (Hi Media) for the breakpoint diameter of the zone of inhibition for different antibiotics. Multiple-antibiotic resistance was defined as resistance to 2 or more antibiotic classes (Shekhar and Singh 2014).

Table I. *Oligonucleotide primers used in multiplex PCR reaction.*

Primer	Sequence	Product size
stx_1 F	5'-CAA CAC TGG ATG ATC TCA G-3'	349 bp
stx_1 R	5'-CCC CCT CAA CTG CTA ATA-3'	
stx_2 F	5'-ATC AGT CGT CAC TCA CTG GT-3'	110 bp
stx_2 R	5'-CTG CTG TCA CAG TGA CAA A-3'	

Results

Bacterial isolation and identification

A total of 62 *E. coli* out of 228 meat samples was isolated, whereas all egg samples were found negative for *E. coli*. The *E. coli* isolates belonged to 24 different serogroups: O29 (10.8%), O8 (7.7%), O40 (6.15%), O2 (4.61%), O60 (3.08%), O106 (3.08%), O42 (1.54%), O87 (1.54%), and 1 serotypes each of O1, O7, O30, O45, O59, O66, O105, O116, O136, O141, O147, O148, O166, and O172. Other results indicated 16 (24.62%) isolates as UT (untypable), and 6 (9.23 %) as rough types.

The molecular characterisation of *E. coli* by multiplex PCR

Multiplex PCR targeting stx_1 and stx_2 gene fragment of *E. coli* resulted in the amplification of 349 bp amplicon for stx_1 and 110 bp amplicon for stx_2, as we expected (Figure 1). Out of 62 *E. coli* isolates, 13 (21%) isolates that carried only the stx_2 gene were screened. These belonged to the serogroups O29, O8, O42, and O1.

Antimicrobial resistance

The *E. coli* isolates showed a wide variation in resistance patterns (9-90%) against the antimicrobial

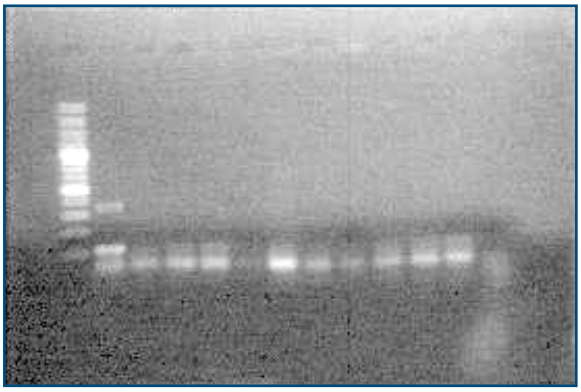

Figure 1. *Agarose gel electrophoresis showing amplified PCR product of* stx_1 *and* stx_2 *gene.* Lane M = 100 bp DNA marker, Lane1 = Positive control for stx_1 (349 bp amplicon) and stx_2 (110 bp amplicon) gene, Lane 2-11 = *E. coli* isolates from meat samples, Lane12 = Negative control.

agents that have been used. The maximum resistances among *E. coli* isolates were observed against cefuroxime (89.1%) and penicillin (89.4%), followed by ampicillin (80.43%), vancomycin (74.1%), co-trimoxazole (73.1%), cephalothin (60.8%), ceftriaxone (28.2%), tetracycline (17.4%), gentamicin (13%), amikacin (13.04%), ofloxacin (13%), and ciprofloxacin (6.5%). A high degree of susceptibility was observed against amikacin (84.7%) and ciprofloxacin (76%) followed by gentamicin (71.73%) and ofloxacin (60.86%) (Tables II and III). The number of multiple antibiotic resistances pattern of isolates we observed was high and a total of 34 resistance patterns were identified (Table IV).

Table II. *Antimicrobial susceptibility of* E. coli *isolates.*

E. coli serotypes	Cu	G	A	Ci	Ak	Ch	Cf	Co	P	Va	Of	T
UT	R	R	R	R	R	R	R	S	R	R	R	S
UT	R	R	R	R	R	R	I	S	R	I	S	I
UT	R	I	R	I	S	R	S	S	R	I	S	S
O106	I	I	R	I	I	R	S	R	R	R	S	I
O59	I	I	R	R	S	R	S	S	R	I	S	S
UT	R	R	R	I	I	I	R	S	R	I	S	S
O106	R	S	R	I	S	R	S	R	R	R	S	R
UT	R	S	R	S	S	R	S	R	R	I	S	R
Rough	I	R	R	R	R	R	S	R	R	I	S	R
Rough	R	R	R	R	S	R	S	R	R	R	R	I
UT	R	S	R	I	R	R	S	R	R	R	S	S
O136	R	S	R	S	S	R	S	R	R	S	S	S
O45	R	R	R	R	R	R	S	R	R	I	S	S
Rough	R	R	R	S	S	S	S	R	R	R	I	I
UT	R	R	R	R	S	S	S	R	R	R	R	I
O40	S	S	R	S	S	I	S	R	R	R	I	I
O40	R	S	R	S	S	R	I	S	R	R	R	I
O40	R	S	R	S	S	R	I	R	R	R	I	I
UT	S	S	R	S	S	S	S	R	R	R	S	I
Rough	R	S	R	S	S	R	I	R	R	R	R	I
UT	R	I	R	S	S	R	S	S	R	R	R	I
O147	R	S	R	S	S	R	S	R	R	I	I	S
UT	R	S	R	S	S	R	S	R	R	R	I	S
UT	R	R	R	S	I	R	S	S	I	R	S	I
UT	R	I	R	S	S	R	S	R	I	R	S	S
O141	R	S	R	I	S	S	S	R	R	S	S	S
O7	R	S	R	I	S	R	S	R	R	I	S	S
O40	R	S	R	S	S	S	S	R	R	R	I	I
O87	R	S	R	I	R	R	S	R	R	R	S	R
O29	R	S	R	I	S	R	I	R	I	R	S	I
O8	R	S	S	R	R	R	R	S	R	R	S	S
O29	R	I	S	R	S	R	I	R	R	R	R	I
O8	R	S	R	S	S	S	R	R	R	R	S	I
O60	R	S	R	S	S	I	I	R	R	R	R	S
O172	R	I	R	S	S	I	S	R	R	R	S	I
O42	R	I	R	I	R	R	S	R	R	I	S	S
O2	R	S	S	I	S	S	R	I	R	R	R	I
Rough	R	S	R	S	S	R	S	S	R	S	S	S
O148	R	S	R	R	S	I	S	R	R	R	I	I
O42	R	S	R	R	S	I	S	R	R	R	I	R
Rough	R	S	R	S	S	R	S	R	R	R	S	S
O1	R	S	R	I	S	I	S	S	R	R	I	I

Cu = Cefuroxime; G = Gentamicin; A = Ampicillin; Ci = Ceftriaxone; Ak = Amikacin; Ch = Cephalothin; Cf = Ciprofloxacin; Co = Co-trimoxazole; P = PenicillinG; Va = Vancomycin; Of = Ofloxacin; T, Tetracycline; S = Susceptible; I = Intermediate; R = Resistant; UT = Untypeable.

Table III. *Oligonucleotide primers used in multiplex PCR reaction.*

Antibiotic		Number of resistant strain (n = 46)	Percent
β lactums antibiotic	Ampicillin	37	80.4
	Penicillin	41	89.13
Cephalosporins	Cephalothin (First generation)	28	60.86
	Cefuroxime (Second generation)	41	89.13
	Ceftriaxone (Third generation)	13	28.26
Fluroquinolones	Ciprofloxacin	3	6.52
	Ofloxacin	6	13.04
Aminoglysides	Amikacin	6	13.04
	Gentamicin	6	13.14
Sulfonamides	Co-trimoxazole	34	73.9
Tetracyclines	Tetracycline	8	17.39
Glycopeptides	Vancomycin	34	74.1

Table IV. *Resistance pattern of* E. coli *serogroups isolated from poultry meat.*

Resistance patterns	*E. coli* serotypes
CU-CO-VA	2
A-CU-CH	1
G-A-P-CH-VA	1
CU-CO	1
G-P-CU-CH-CO-VA	1
P-CU-CH	1
A-P-CU-VA	2
P-CI-CU-CO-VA	1
A-P-CI-CU-CH-VA	1
A-P-CI-CU-CH-CO-VA-T	1
A-P-CU-CO-VA-T	1
A-P-CU-VA-T	1
A-P-CI-CU-CO-VA-T	1
A-P-CH-CO-VA	1
A-P-CI-CH	1
A-P-CU-CH-CO-VA-T	2
G-AK-A-P-CI-CH-CO-T	1
G-A-P-CI-CU-CH-CO-VA	2
A-P-CU-CH-CO	3
G-A-P-CU-CO-VA	2
G-A-P-CO-VA	1
A-P-CU-CH-OF-VA	1
A-P-CU-CH-CO-VA	3
A-P-CU-CH-OF-CO-VA	1
A-P-CU-CH-CO	4
A-P-CO-VA	1
A-CU-CH-CO-VA-T	1
A-CU-CH-CO-VA	1
P-CU-CH-CF-VA	1
P-CU-CH-OF-CO-VA	1
A-P-CU-CF-CO-VA	1
A-P-CU-OF-CO-VA	1
P-CU-CH-CF-OF-VA	1
A-P-CU-CH	1
Total	**46**

Antibiotics tested for multiple resistance were: Cu = Cefuroxime; G = Gentamicin; A = Ampicillin; Ci = Ceftriaxone; Ak = Amikacin; Ch = Cephalothin; Cf = Ciprofloxacin; Co = Co-trimoxazole; P = Penicillin G; Va = Vancomycin; Of = Ofloxacin; T = Tetracycline.

Discussion

Foodborne diseases are caused by a number of pathogens. Of these, *E. coli* is considered one of the most important. It is responsible for intestinal and extraintestinal diseases in humans (Vincent *et al.* 2010, Scallan *et al.* 2011). Food plays an important role in the dissemination of *E. coli*, causing community-acquired urinary tract infections (Vincent *et al.* 2010, Ferens and Hovde 2011, Jana and Mondal 2013). In the present study, poultry meat samples collected from different retail shops were contaminated with different serogroups of *E. coli*. Out of 228 poultry meat samples, 62 (27.19%) were positive for *E. coli*. This finding is both consistent with previous reports from different parts of India (Farooq *et al.* 2009, Bonyadian *et al.* 2011, Sahoo *et al.* 2012, Jana and Mondal 2013) but higher than the finding of Zende and colleagues (Zende *et al.* 2013), who reported the isolation of *E. coli* in 16% poultry meat from a small sample size of meat from Mumbai, India. Our results indicate the contamination of poultry meat with *E. coli*. This may be due to a lack of proper sanitation during slaughtering in the local markets or because of contaminated water is used to wash carcasses.

In the present study, *E. coli* isolates belonged to 24 different serogroups, namely O29, O8, O40, O2, O60, O106, O42, O87, O1, O7, O30, O45, O59, O66, O105, O116, O136, O141, O147, O148, O166, O172; UT (untypable) and R (rough types) were also recovered. Most of the serogroups identified have also been reported in other similar studies (Guth *et al.* 1989, Blanco *et al.* 2004, Fratamico *et al.* 2010, Bandyopadhyay *et al.* 2011). In the present study however 24 serogroups were detected. They were widely distributed in the eastern part of India. On the contrary to this finding many studies from India reported the isolation of a few numbers of serogroups from poultry (Malik *et al.* 2002, Wani *et al.* 2004, Dutta *et al.* 2011). The *E. coli* of serogroups O2, O87, and O172 reported in this study, are associated with Shiga toxin producing *E. coli* (STEC), as documented by Fratamico and other authors (Fratamico *et al.* 2010, Meng *et al.* 2014), whereas the

serogroups O8, O29, O111, and O60 are associated to enteropathogenic *E. coli* (Guth *et al.* 1989, Blanco *et al.* 2004, Wani *et al.* 2004, Bandyopadhyay *et al.* 2011). Thus our findings indicate that meat handlers and consumers are at risk of infection from STEC or enteropathogenic *E. coli*.

In this study, molecular characterisation for the presence of stx_1 and stx_2 genes showed the presence of stx_2 gene in 13 (21%) isolates of *E. coli*. This finding is consistent with other findings from Farooq and colleagues (Farooq *et al.* 2009) and Grossmann and colleagues (Grossmann *et al.* 2005), who reported the stx_2 gene in 11% and 9% of *E. coli* isolated from pigeons, respectively. Various authors reported the occurrence of the stx_2 gene in O45, O18, O75, O64, O89, O91, O17, and O78 serogroups (Schmidt *et al.* 2000, Morabito *et al.* 2001, Farooq *et al.* 2009, Dutta *et al.* 2011). However, our findings demonstrate the presence of the stx_2 gene in O29, O8, O42, and O1 serogroups. This indicates a variable distribution of virulent genes among different serogroups of *E. coli* in different geographical area in India.

Antimicrobial resistance was observed at a range of 9-90%, which shows extreme variation in the susceptibility of poultry meat to *E. coli*. Our findings show maximum resistance against cefuroxime (a cephalosporin) followed by penicillin, ampicillin, and vancomycin. The high level of susceptibility against amikacin and ciprofloxacin that has been observed is consistent with the findings of other studies (Mishra *et al.* 2002, Carraminana *et al.* 2004, Jana and Mondal 2013). These findings might be the result of the indiscriminate use of these antibiotics in poultry or because of sub-therapeutic doses of these antimicrobial agents being administered for the prevention or control of infection. Other explanations include transmissible or plasmid mediated drug resistance and mutational changes in the genes that are crucial to the development of resistance in *E. coli*. The coexistence of virulent and resistant genes in a species may result in the emergence of hybrid plasmids (both resistance and virulence) among *E. coli*. This, in turn, poses an increased public health risk (Szmolka and Nagy 2013). In the present study, all isolates of *E. coli* exhibited resistance against 2 or more antimicrobial agents, suggesting the presence of multi-drug resistant (MDR) *E. coli* (Joshi *et al.* 2012, Szmolka and Nagy 2013). The prevalence of such a large number of MDR *E. coli* indicates the widespread and extensive use of antibiotics in the area. Prevalence of such MDR *E. coli* has also been observed by Shakya and colleagues (2013) in human stool samples from India. The high rate of multiple antibiotic resistance against *E. coli* also indicates the injudicious use of several antimicrobial agents for preventive and therapeutic purposes. These all pose serious threats to animal and human health.

The present investigation highlights the prevalence of pathogenic *E. coli* (ETEC and EPEC) in poultry meat, which is of importance in the field of zoonosis. The occurrence of high levels of resistance against most of the antibiotics and prevalence of virulent genes call for further attention, and the maintenance of strict hygienic measures in retail meat shops. The present findings also provide information about the occurrence of STEC in poultry in eastern India.

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