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Antibiotic Resistance in Diarrheagenic *Escherichia coli* Isolated from Broiler Chickens in Pakistan

M. Amir [1,2], M. Riaz [1*], Y.-F. Chang [2], A. Ismail [1], A. Hameed [1,3], M. Ahsin [4]

1. *Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan 60800, Pakistan*
2. *Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*
3. *Department of Food Science and Agricultural Chemistry, Macdonald Campus, McGill University, Ste Anne de Bellevue, Quebec H9X 3V9, Canada (UK)*
4. *National Institute of Food Science and Technology, Faculty of Food, Nutrition and Home Sciences, University of Agriculture, Faisalabad 38000, Pakistan*

HIGHLIGHTS

- Enteropathogenic *Escherichia coli* was the predominant strain in both feces (76%) and meat (90%) samples.
- The highest resistance (40-90%) was observed against penicillin, oxytetracycline, and nalidixic acid in fecal isolates.
- The broiler meat sold in open markets of Pakistan was contaminated with multi-drug resistant diarrheagenic *E. coli*.

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Acronyms and abbreviations
DEC=Diarrheagenic *E. coli*
EAEC=Enteroaggregative *E. coli*
EHEC=Enterohemorrhagic *E. coli*
EIEC=Enteroinvasive *E. coli*
EPEC=Enteropathogenic *E. coli*
ETEC=Enterotoxigenic *E. coli*
PCR=Polymerase Chain Reaction
STEC=Shiga toxin-producing *E. coli*
tEPEC=typical Enteropathogenic *E. coli*

ABSTRACT

Background: Diarrheagenic *Escherichia coli* (DEC) strains are predominant cause of gastrointestinal tract illnesses. The main objective of the study was to determine antibiotic resistance in various types of DEC isolated from chicken broilers farmed in Pakistan.

Methods: A total of 200 feces and 200 meat samples from broiler chickens were collected from the slaughtering shops in Southern Punjab, Pakistan. The confirmed fecal (n=150) and meat (n=150) *E. coli* isolates were investigated against 16 antibiotics. Fourteen virulence genes specific for Enteropathogenic (EPEC), Shiga Toxin-producing (STEC), Enteroinvasive (EIEC), Enteroaggregative (EAEC), and Enterotoxigenic (ETEC) *E. coli* were identified using Polymerase Chain Reaction.

Results: EPEC was the most detected pathotype in both feces (76%) and meat (90%) samples, followed by STEC, EIEC, and ETEC. The highest resistance (40-90%) was observed against penicillin, oxytetracycline, and nalidixic acid in fecal isolates. More than 50% EPEC and EAEC fecal isolates, and 60% EAEC meat isolates were simultaneously resistant to 6 or more antibiotics.

Conclusion: Conclusively, the broiler meat sold in open markets of Pakistan was considerably contaminated with multi-drug resistant DEC. To mitigate the issue, the government should regulate the use of antibiotics at poultry farms and monitor slaughtering practices in slaughterer houses.

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Introduction

Escherichia coli is a common inhabitant of the gut of mans and warm-blooded animals (Kaper et al., 2004). Only a few strains of *E. coli* cause diseases in humans. These include diarrheagenic *E. coli* (DEC) causing

* Corresponding author (M. Riaz)
E-mail: riaz@bzu.edu.pk
ORCID ID: https://orcid.org/0000-0003-3838-4348

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gastrointestinal tract syndromes ranging from self-limiting diarrhea to life-threatening Hemolytic Uremic Syndrome. The DEC strains are further classified into various pathotypes depending on their virulence traits. There are six most common DEC groups, including Shiga Toxin-producing *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), and Diffusely Adherent *E. coli* (DAEC).

Pathogenesis of DEC pathotypes depends upon the presence and expression of virulence traits (Gomes et al., 2016). A common phenomenon of STEC pathogenesis results in gastroenteritis and Hemolytic Uremic Syndrome ultimately cause kidney failure in infants (Bielaszewska et al., 2007). Globally, EPEC infects young children by attaching and effacing intestinal cells surfaces via the *eae* gene product. EPEC are further classified into two types named as typical EPEC (tEPEC) and atypical EPEC (aEPEC) depending upon the appearance of adhesion and bundle-forming pili (*bfp*) genes. The tEPEC is well recognized in developing countries while aEPEC is a common pathogen in both developing and developed countries (Ochoa et al., 2008). Pathogenesis of ETEC depends upon the presence of heat-stable (ST) and heat-labile (LT) toxins. Contrarily, presence of adhesion proteins in EIEC assist it to bind host intestinal cells that results in bacillary dysentery in humans, specifically more common in developing countries (Gomes et al., 2016; Lan et al., 2004). On the other hand, EAEC is well known for the secretory diarrhea through its considerable damaging effects on intestinal epithelium (Jensen et al., 2014).

In Pakistan, diarrheal outbreaks due to *E. coli* have already been reported time to time (Ahmed et al., 2009; Bokhari et al., 2013; Khalil et al., 2016; Mohsin et al., 2007). It seems imperative to find the source of food contamination for effective reduction in the spread of food-borne diseases. However, the role of animals and birds in the spread of diarrheal diseases could not be overlooked as they are a harbor of DEC strains (Kagambèga et al., 2012a). Evidence indicates the co-transfer of DEC strains between birds and human (Ewers et al., 2009). Recent studies indicated that slaughtering of animals and birds in unhygienic conditions may facilitate the cross contamination of meat (Amir et al., 2017; Islam et al., 2014; Kagambèga et al., 2012b). Fecal contamination during carcass removal may also affect the microbiological quality of the meat. Consequently, consumption of contaminated or undercooked meat and meat products results in diarrheal episodes as reported recently (Chokoshvili et al., 2014; Currie et al., 2007; Jay et al., 2004; Sartz et al., 2008).

Overuse of antibiotics may result in the development of antibiotic resistance. As a result, the spread of infections during organ transplantation, chemotherapy, and surgery constitutes a serious public health issue. Industrial scale antibiotic use for enhancing chicken growth rates has provided great selective pressure for the spread of resistance, rendering the current crop of antibiotics for human application increasingly useless. As a result, countries like Pakistan are running out of options for controlling infections caused by highly pathogenic zoonotic *E. coli* strains. Overuse of antibiotics has been implicated in the emergence and spread of multi-drug resistant diarrheal strains in developing countries. Moreover, these strains may easily spread to developed countries (Byarugaba, 2004; Guiral et al., 2011).

From slaughter shops in open markets of Pakistan, *E. coli* may spread from broiler carcasses to the environment. However, based on our knowledge, no data have been reported yet concerning the prevalence and antibiotic resistance of DEC strains from broiler meat sold in Pakistan. Therefore, the main objective of this study was to determine antibiotic resistance in various types of DEC (EPEC, STEC, EAEC, EIEC, and ETEC) isolated from chicken broilers farmed in Pakistan.

Materials and methods

Sampling

During March to June 2019, 200 feces and 200 meat samples from broiler chickens (*Gallus gallus domesticus*) were collected from the slaughtering shops situated in open markets of major cities (Khanewal, Multan, Dera Ghazi Khan and Bahawalpur) in Southern Punjab, Pakistan. The fresh samples of feces and meat from same bird, about 30 g each, were collected in sterile plastic bags using sterile scissors and tissue forceps. These plastic bags were placed in an ice box and transported to the institutional laboratory within 4 h.

Isolation of E. coli

Isolation of *E. coli* and biochemical tests were performed as described in a previous study (Amir et al., 2017). For isolation of *E. coli*, fecal and meat samples (25 g each) were separately homogenized in 225 ml Butterfield's phosphate-buffered water and homogenized in a stomacher (Seward 400 Circular, USA). The diluted blend about 10 µl was streaked on the Levine's Eosin Methylene Blue (L-EMB) agar. Two to three flat, dark centered colonies with or without metallic sheen were confirmed as *E. coli* by IMViC and sugar biochemical reactions. All culture media and chemicals were purchased from Thermo Fisher Scientific (UK). Confirmed fecal and meat isolates (150 each) of *E. coli* were further processed.

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Polymerase Chain Reaction (PCR)

At first, boiling method was adopted for DNA extraction. Briefly, confirmed isolates (almost five colonies of a single isolate) were suspended in 250 µl of sterile water in micro tubes. These tubes were boiled at 100 °C for 10 min and then centrifuged (Sigma, Germany) for one min at 13 000 rpm. The supernatant was used for detection of virulence genes of diarrheagenic strains STEC (*stx1* and/or *stx2*, possibly *eaeA*, *escV*, *ent*, and *EHEC-hly*), EPEC (*eae*, possibly *escv*, *ent* and *bfpB*), EAEC (*aggR* and/or *pic/astA*), ETEC (*elt* and/or *estIa* or *estIb*), and EIEC (*ipaH* and *invE*) by conventional PCR (Ingenius 3, UK) assay. For positive control, *E. coli* O127:H6 (E2348/69) for EPEC, ATCC 35401 for ETEC, ATCC 43895 for STEC, ATCC 23501 for EAEC and ATCC 23520 for EIEC were purchased from BEI Resources-ATCC Virginia, USA. While, *E. coli* strain ATCC 25922 was used as a negative control. Nucleotide sequences of primers (Thermo Fisher Scientific Waltham, MA, USA) used in this study present in Table 1. PCR reactions were carried out with 5 µl DNA (400 ng/µl) of the isolates, 2.5 µl PCR buffer (10x), 2 µl $MgCl_2$ (25 mM), 5 µl dNTP mix (5 mM), 0.25 µl of each primer (20 µM), and 0.2 µl Taq DNA polymerase (5 U/µl) making the total volume of 25 µl. PCR mixture was preheated at 95 °C for 5 min followed by denaturation at 94 °C for 60 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s (30 cycles), with further extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis (Bio-rad, UK) in 1.5% agarose gel stained with ethidium bromide. Protocol was followed according to Lorenz (2012) with little modification.

Antibiotic resistance in DEC

Identified strains of DEC were tested for antibiotic resistance through Kirby-Bauer disk diffusion method according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2014). Isolates were categorized as resistant according to protocols outlined by the National Antimicrobial Monitoring System. Sixteen antibiotics (Oxoid, UK), including penicillin (5 units), amoxicillin (30 µg), oxytetracycline (30 µg), gentamycin (30 µg), azithromycin (15 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), ciprofloxacin (30 µg), cephradine (30 µg), cephalothin (30 µg), cephalexin (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefoperazone (75 µg), cefotaxime (30 µg), and cefepime (30 µg) were selected for this study. Antibiotics were chosen due to routine use of these drugs in ailments of humans and animals. Briefly, the fresh culture of *E. coli* strains was inoculated in the nutrient broth and incubated at 37 °C for 20 h. Before swabbing on the Mueller Hinton agar (Oxoid, UK) plates, bacterial suspension turbidity was adjusted to equivalent to 0.5 McFarland standard. Antibiotic disks were placed on the inoculated plates and incubated at 37 °C for 20 h. Zone diameters were measured with the digital caliper. *E. coli* ATCC 25922 was used as negative control. Any strain resistant to more than three antibiotic class was considered as multi-drug resistant strain (Canizalez-Roman et al., 2013).

Results

DEC in broiler feces

Out of 150 confirmed fecal *E. coli*, 91 (60.6%) were characterized as DEC (Table 2). Among all identified isolates, 69 (about 76%) were identified as EPEC. Detected EPEC possessed *eae*, *escv*, *ent*, and *bfp* genes in 47 (68%) isolates depicting the presence of tEPEC. All the other EPECs were atypical and possessed *eae*, *escv*, and/or *ent*. Of the STEC positive isolates, 5 contained *stx1*, *stx2*, and *eae*, 7 possessed both *stx1* and *stx2*; 2 *stx1/stx2* with *hlyA* while 3 *stx1* alone and 2 *stx2* alone. *EHEC-hly* was detected in 7 isolates of confirmed STEC, however, absent in all EPEC confirmed isolates. It is necessary to mention that some isolates had genes of two or more DEC serotypes.

DEC in broiler meat

Out of 150 meat isolates, 53 (35.3%) samples were characterized as DECs (Table 2). EPEC was the most frequent category (48 isolates) followed by EAEC, EIEC, and STEC. Of 48 (90.6%) EPEC positive isolates, 38 (79%) were further categorized as tEPEC based on the presence of *eae*, *escv*, *ent*, and *bfp*. Of 4 STEC positive samples, 3 contained *stx1*, *stx2*, and *eae*. Again, it should be noted that some isolates had genes of two or more DEC serotypes.

Antibiotic resistance in fecal DEC

Antibiotic resistance patterns in the detected fecal DEC isolates varied against the examined antibiotics but generally remained higher against nalidixic acid and oxytetracycline (Table 3). All STEC isolates showed antibiotic resistance rates below 30% except against nalidixic acid (47%), ciprofloxacin (65%), and oxytetracycline (71%). Similarly, EIEC isolates showed less than 50% resistance to all the tested antibiotics except against nalidixic acid (80%) and oxytetracycline (90%). EPEC isolates expressed 71 to 83% resistance rates against penicillin, nalidixic acid, and also oxytetracycline. Similar to EPEC, EAEC isolates were maximally resistant to nalidixic acid (79%), penicillin (79%), and oxytetracycline (93%).

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Table 1: Primer pairs used for identification of virulence genes of diarrheagenic *Escherichia coli*

Primers	Sequence	Target gene	Sub-sp.	PCR product (bp)	Concentration (µM)	Reference
MP4-stx1A-F	CGATGTTACGGTTTGTTACTGTGACAGC	*stx1*	STEC	244	0.2	(Müller et al., 2007)
MP4-stx1A-R	AATGCCACGCTTCCCAGAATTG				0.2	
MP3-stx2A-F	GTTTTGACCATCTTCGTCTGATTATTGAG	*stx2*		324	0.4	
MP3-stx2A-R	AGCGTAAGGCTTCTGCTGTGAC				0.4	
MP2-aggR-F	ACGCAGAGTTGCCTGATAAAG	*aggR*	EAEC	400	0.2	(Müller et al., 2007)
MP2-aggR-R	AATACAGAATCGTCAGCATCAGC				0.2	
MP2-pic-F	AGCCGTTTCCGCAGAAGCC	*pic*		1111	0.2	
MP2-pic-R	AAATGTCAGTGAACCGACGATTGG				0.2	
MP2-astA-F	TGCCATCAACACAGTATATCCG	*astA*		102	0.4	
MP2-astA-R	ACGGCTTTGTAGTCCTTCCAT				0.4	
M5-F	GCAAATTTAGGTGCGGGTCAGCGTT	*eae*	EPEC, STEC	494	0.4	(Germani et al., 1997)
M5-R	GGCTCAATTTGCTGAGACCACGGTT				0.4	
MP3-escV-F	ATTCTGGCTCTCTTCTTCTTTATGGCTG	*escV*		544	0.4	(Müller et al., 2007)
MP3-escV-R	CGTCCCCTTTTACAAACTTCATCGC				0.4	
ent-F	TGGGCTAAAAGAAGACACACTG	*ent*		629	0.4	
ent-R	CAAGCATCCTGATTATCTCACC				0.4	
MP3-bfpB-F	GACACCTCATTGCTGAAGTCG	*bfpB*		910	0.1	
MP3-bfpB-R	CCAGAACACCTCCGTTATGC				0.1	
M4-F	CTCGGCACGTTTTAATAGTCTGG	*ipaH*	EIEC	933	0.1	(Vidal et al., 2005)
M4-R	GTGGAGAGCTGAAGTTTCTCTGC				0.1	
MP2-invE-F	CGATAGATGGCGAGAAATTATATCCCG	*invE*		766	0.2	(Müller et al., 2007)
MP2-invE-R	CGATCAAGAATCCCTAACAGAAGAATCAC				0.2	
MP2-LT-F	GAACAGGAGGTTTCTGCGTTAGGTG	*elt*	ETEC	655	0.1	(Müller et al., 2007)
MP2-LT-R	CTTTCAATGGCTTTTTTTTGGGAGTC				0.1	
MP4-STIa-F	CCTCTTTTAGYCAGACARCTGAATCASTTG	*estIa*		157	0.4	
MP4-STIa-R	CAGGCAGGATTACAACAAAGTTCACAG				0.4	
MP2-STI-F	TGTCTTTTTCACCTTTCGCTC	*estIb*		171	0.2	
MP2-STI-R	CGGTACAAGCAGGATTACAACAC				0.2	

-PCR: Polymerase Chain Reaction; STEC: Shiga toxin-producing *E. coli*; EAEC: Enteroaggregative *E. coli*; EPEC: Enteropathogenic *E. coli*; EIEC: Enteroinvasive *E. coli*; ETEC: Enterotoxigenic *E. coli*

Table 2: Prevalence of virulence genes (n) in diarrheagenic *Escherichia coli* (DEC) pathotypes isolated from poultry feces and meat

Virulence genes	Feces					Meat				
	STEC (n=17)	EIEC (n=10)	EPEC (n=69)	EAEC (n=14)	ETEC (n=4)	STEC (n=4)	EIEC (n=5)	EPEC (n=48)	EAEC (n=5)	ETEC (n=0)
aggR				9					5	
astA				5					2	
bfp			7					2		
eae	5		69			3		48		
EHEC-hly	7					1				
elt					4					
ent	5		64			3		47		
escv	5		67			3		47		
estIa					2					
estIb					4					
invE		10					5			
ipaH		10					5			
pic				9					4	
Stx-1	15					4				
Stx-2	14					4				

-A total of 144 DEC isolates (91 fecal and 53 meats) were identified in 300 confirmed isolates of *E. coli* from broiler samples (150 each of feces and meat).
- EIEC: Enteroinvasive *E. coli*; EAEC: Enteroaggregative *E. coli*; EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxigenic *E. coli*; STEC: Shiga Toxin producing *E. coli*

Table 3: Prevalence of antibiotic resistance [counts (percentage)] in diarrheagenic strains of *Escherichia coli* isolated from broiler feces

Antibiotic class	Antibiotics	STEC (n=17)	EIEC (n=10)	EPEC (n=69)	EAEC (n=14)	ETEC (n=4)
Aminoglycosides	Chloramphenicol	2 (12)	5 (50)	20 (29)	5 (36)	0 (0)
	Gentamicin	2 (12)	3 (30)	25 (36)	2 (40)	1 (25)
Quinolones	Nalidixic acid	8 (47)	8 (80)	52 (75)	11 (79)	3 (75)
	Ciprofloxacin	11 (65)	4 (40)	21 (30)	4 (29)	0 (0)
Beta lactams	Penicillin	5 (29)	4 (40)	49 (71)	11 (79)	0 (0)
	Amoxicillin	4 (23)	2 (20)	15 (22)	2 (14)	0 (0)
Macrolides	Azithromycin	5 (29)	2 (20)	36 (52)	4 (29)	0 (0)
Tetracycline	Oxytetracycline	12 (71)	9 (90)	57 (83)	13 (93)	3 (75)
Cephalosporin	Cephradine	4 (23)	1 (10)	29 (42)	8 (57)	1 (25)
	Cephalothin	3 (18)	3 (30)	24 (35)	2 (14)	0 (0)
	Cephalexin	1 (6)	3 (30)	10 (14)	3 (21)	0 (0)
	Ceftriaxone	3 (18)	1 (10)	20 (29)	3 (21)	0 (0)
	Ceftazidime	5 (29)	1 (10)	11 (16)	4 (29)	0 (0)
	Cefoperazone	0 (0)	0 (0)	17 (25)	0 (0)	0 (0)
	Cefotaxime	2 (12)	2 (20)	8 (12)	3 (21)	1 (25)
	Cefepime	0 (0)	0 (0)	5 (7)	1 (7)	0 (0)

-STEC: Shiga Toxin producing *E. coli*; EIEC: Enteroinvasive *E. coli*; EPEC: Enteropathogenic *E. coli*; EAEC: Enteroaggregative *E. coli*; ETEC: Enterotoxigenic *E. coli*

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Antibiotic resistance in meat DEC

In meat samples, all the detected DEC strains were completely sensitive to cefoperazone and cefepime except a few EPEC isolates (Table 4). Detected STEC isolates in meat samples showed higher antibiotic resistance rates against penicillin (75%) as compared to other antibiotics. Contrarily to other strains, EIEC isolates were completely sensitive to nalidixic acid and a lower resistance against penicillin (20%) was observed. However, EAEC showed comparatively higher resistance rates against all tested antibiotics except for cephalothin, cephalexin, ceftazidime, cefoperazone, cefotaxime, and cefepime. The EPEC isolates showed similar resistance patterns (44%) against nalidixic acid and penicillin but more isolates were resistant against oxytetracycline (58%). Moreover, STEC isolates had common resistance patterns for cephradine, amoxicillin, and azithromycin. Almost all detected strains of meat samples exhibited higher resistance against penicillin, oxytetracycline, and nalidixic acid.

Multi-drug resistance in DEC

Only one isolate from STEC and one from EAEC of meat samples showed sensitivity to all the tested antibiotics (Table 5). Surprisingly, 83% STEC, 90% EIEC, 95% EPEC, and 99% EAEC fecal isolates were resistant to more than two antibiotics. Resistant fecal isolates against more than five antibiotics were: 4 STEC, 3 EIEC, 44 EPEC, and 9 EAEC strains. In meat samples, 25% STEC, 20% EIEC, 69% EPEC, and 60% EAEC isolates were simultaneously resistant against more than two antibiotics. A smaller number of meat isolates, 1 STEC, 12 EPEC, and 3 EAEC expressed multi-drug resistance against more than five antibiotics.

Table 4: Prevalence of antibiotic resistance [counts (percentage)] in diarrheagenic strains of *Escherichia coli* isolated from broiler meat

Antibiotic Class	Antibiotics	STEC (n=4)	EIEC (n=5)	EPEC (n=48)	EAEC (n=5)
Aminoglycosides	Chloramphenicol	2 (50)	0 (0)	9 (19)	2 (40)
	Gentamicin	0 (0)	1 (20)	14 (29)	2 (40)
Quinolones	Nalidixic acid	2 (50)	0 (0)	21 (44)	3 (60)
	Ciprofloxacin	0 (0)	1 (20)	10 (21)	2 (40)
Beta lactams	Penicillin	3 (75)	1 (20)	21 (44)	3 (60)
	Amoxicillin	1 (25)	0 (0)	9 (19)	2 (40)
Macrolides	Azithromycin	1 (25)	0 (0)	7 (15)	1 (20)
Tetracycline	Oxytetracycline	1 (25)	2 (40)	28 (58)	3 (60)
Cephalosporin	Cephradine	1 (25)	0 (0)	15 (31)	2 (40)
	Cephalothin	0 (0)	2 (40)	11 (23)	0 (0)
	Cephalexin	1 (25)	0 (0)	8 (17)	0 (0)
	Ceftriaxone	0 (0)	0 (0)	9 (19)	1 (20)
	Ceftazidime	0 (0)	1 (20)	5 (10)	0 (0)
	Cefoperazone	0 (0)	0 (0)	9 (19)	0 (0)
	Cefotaxime	0 (0)	1 (20)	6 (13)	0 (0)
	Cefepime	0 (0)	0 (0)	1 (2)	0 (0)

- EIEC: Enteroinvasive *E. coli*; EAEC: Enteroaggregative *E. coli*; EPEC: Enteropathogenic *E. coli*; STEC: Shiga Toxin producing *E. coli*

Table 5: Multi-drug resistance [counts (percentage)] in diarrheagenic strains of *Escherichia coli* isolated from broiler feces and meat

DEC pathotype	Number of isolates	Number of drugs									
		0	1	2	3	4	5	6	7	8	9
Fecal isolates											
STEC	17		1 (6)	2 (12)	6 (35)	2 (12)	2 (12)	2 (12)	2 (12)		
EIEC	10			1 (10)	2 (20)	1 (10)	3 (30)	1 (10)	1 (10)	1 (10)	
EPEC	69			4 (6)	6 (9)	6 (9)	9 (13)	16 (23)	19 (28)	6 (9)	3 (4)
EAEC	14				1 (7)	3 (21)	1 (7)	7 (50)	2 (14)		
ETEC	4			3 (75)	1 (25)						
Meat isolates											
STEC	4	1 (25)				2 (50)		1 (25)			
EIEC	5		3 (60)	1 (20)		1 (20)					
EPEC	48		7 (15)	7 (15)	14 (29)	4 (8)	4 (8)	5 (10)	2 (4)	5 (10)	
EAEC	5	1 (20)	1 (20)					1 (20)	2 (40)		
ETEC	0										

-DEC: Diarrheagenic *Escherichia coli*; EIEC: Enteroinvasive *E. coli*; EAEC: Enteroaggregative *E. coli*; EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxigenic *E. coli*; STEC: Shiga Toxin producing *E. coli*

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Discussion

We identified *E. coli* isolates in all the collected samples of broiler. Due to similar conditions, the colony counts of *E. coli* in feces and meat samples (data not given) could be considered similar to our previous study (Amir et al., 2017). Of the total identified 144 DEC strains (48% of the total *E. coli* isolates), 91 were from fecal and 53 were from meat samples (Table 2). About 76% of the identified DEC fecal isolates were EPEC. A recent study in Japan found a 63% prevalence of EPEC in broiler feces (Wang et al., 2017). Similarly, the prevalence of EPEC in Burkina Faso was also highest among the other detected DEC strains from broiler feces (Kagambèga et al., 2012b). Based on the presence of *eae* gene, broiler fecal samples were EPEC about 23% in Korea (Oh et al., 2012), 28% in Argentina (Alonso et al., 2012), 37% in Burkina Faso (Kagambèga et al., 2012b), 40% in India (Farooq et al., 2009), 46% in Egypt (Byomi et al., 2017), and 63% in Japan (Wang et al., 2017). Presence of *bfpA* gene with *eae* reflects the presence of tEPEC which were 10% of total EPEC isolates in the current study. The tEPEC is the major cause of infant diarrhea in developing countries and also more often found in humans than animals (Farooq et al., 2009; Ochoa et al., 2008).

For fecal DEC strains, EPEC strains were the most common, followed by STEC in about 19% of the identified DEC strains (Table 2). Similar to a previous report from Burkina Faso (Kagambèga et al., 2012b) with few *stx2* negative but *EHEC-hly* positive samples of chicken feces, we also found an *EHEC-hly* positive but *stx2* negative fecal isolate (Table 2). In contrast to EPEC and STEC, virulence genes of ETEC and EIEC were lower in frequency when compared to Kagambèga et al. (2012b). However, *astA* of EAEC was detected in about 36% of DEC isolates that was little more than half of the identified *aggR* and *pic* (Table 2). The high prevalence of DEC strains in broiler feces may be related to the use of contaminated feed and water at poultry farms. Poultry farmers may also be a source of DEC spreading in the workplace. Once host colonization occurs, the DEC strains rapidly spread from diseased to healthy birds, as large number of birds are kept at the same production site (Wang et al., 2017).

With few exceptions, the DEC pathogroups found in fecal isolates were also present at a similar prevalence rate in raw meat samples (Table 2). Our meat and feces samples were collected from local poultry shops situated in open markets. These shops are located in a dusty environment of the roadsides. Slaughterers on these shops wear local dress and slaughter the birds have no gloves or sterilized knife. Slaughtering and evisceration are performed on-site at the shops as per the demand of consumers. About 1 to 3 kg broiler meat is kept ready for sale in utensils or on sheet in open environment. Cross-contamination of fecal *E. coli* to broiler meat has already been reported (Amir et al., 2017). Alonso et al. (2012) also suggested the possibility of cross-contamination of meat during evisceration as they found high EPEC in samples of carcass, cloacae, and giblets. Along with improper evisceration techniques, several factors are involved in cross-contamination of meat at slaughter shops. Wooden cutting boards used without replacement on time provide hiding places for the survival of microbes. Washing of the handling equipment with contaminated water may also increase microbial loads in meat (Amir et al., 2017; Müller et al., 2001).

In the present study, EPEC isolates were predominant (90.6% of identified DEC strains) in meat samples. To date, there are no studies reporting a higher prevalence of EPEC in meat, instead, Canizalez-Roman et al. (2013) detected EPEC in 83% of tested meat samples in Mexico. However, none of the meat samples were positive for *elt* and/or *estIa* or *estIb*, suggesting ETEC was not present in the sample set of the current study. Similarly, this pathotype has not been previously found in chicken, mutton, and beef meat as well as in the processed meat products (Canizalez-Roman et al., 2013; Kagambèga et al., 2012b).

DEC isolates were tested against 16 antibiotics (Tables 3 and 4). Against oxytetracycline, we found a maximum resistant rate of 93% in EAEC isolates followed by 90% in EIEC, 83% in EPEC, 71% in STEC, and 57% in ETEC isolates. The elevated level of antibiotic resistance strongly suggests the presence of antibiotic resistant bacteria in feed, water, litter, and environment of poultry farms (Braykov et al., 2016). Studies on the presence of resistant *E. coli* in poultry feed, water, and litter are reported in various countries (Amir et al., 2017; Islam et al., 2014). Dissemination of resistant plasmids from one animal to another animal also results in the development of antibiotic resistance (De Been et al., 2014; Mo et al., 2017).

Nalidixic acid has been ranked the second most ineffective drug, after oxytetracycline, which was unable to kill about half of the DEC strains. Studies in Canada (Diarrassouba et al., 2007) and West Indies (Miles et al., 2006) found tetracycline resistant genes in about 80% of *E. coli* isolated from poultry. Consistent higher resistance rates against tetracycline in this research (Table 3) and previous studies can be related to the common addition of this antibiotic in feed and water for poultry (Diarrassouba et al., 2007). Similarly, Da Costa et al. (2007) detected high tetracycline resistance rate in bacteria isolated from poultry feed and feed ingredients in Portugal.

It has also been revealed that resistant *E. coli* may remain the part of the microflora of chickens even after

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many days of absence of selective pressure of antibiotics (Chaslus-Dancla et al., 1987). Slaughtering exposes these drug resistant *E. coli* for spread into the environment via multiple routes (Amir et al., 2017). Meat may be cross-contaminated by drug resistant *E. coli* from the gut of the bird (Kagambèga et al., 2012b). Likewise, higher antibiotic resistance in EAEC and EPEC of meat samples was observed against oxytetracycline (Table 4). Canizalez-Roman et al. (2013) identified a high tetracycline resistance rate (83%) in DEC isolates of meat and meat products in Mexico. Moreover, cefoperazone was the drug for which only EPEC isolates shown resistance. The study conducted in India on DEC prevalence and resistance in diarrheal stools of children found more EPEC resistant isolates against cefoperazone (Chellapandi et al., 2017). Hence, EPEC resistance against cefoperazone and other cephalosporins is a serious health concern in this region. To our knowledge, this study is first to report about the cross-contamination of broiler meat with resistant DEC isolates from Pakistan.

Of the studied DEC strains, more than 80% of fecal isolates were simultaneously resistant to three or more antibiotics (Table 5). In a previous study, only 10% *E. coli* strains from wild birds in Spain were simultaneously resistant to four antibiotics (Sacristán et al., 2014). Such difference in the studies might be due to a difference in species of birds, their habitat, and type of feed they consume. No STEC isolate from meat samples was simultaneously resistant to two antibiotics; however, one isolate was resistant to six antibiotics. On the other hand, Canizalez-Roman et al. (2013) found 2 STEC isolates resistant to two antibiotics in food samples. An alarming rate of multi-drug resistance in the present study (Table 5) illustrates the unintended consequences of the large-scale agronomic usage of antibiotics in Pakistan. Irrational use of antibiotics either for therapeutic purposes or as a growth regulators serves to create strong selective pressure driving the rapid spread of antibiotic resistance determinants (Chopra and Roberts, 2001).

Conclusion

Out of confirmed *E. coli* isolates, the present study on broiler chickens in Pakistan found 91 fecal and 53 meat samples positive for DEC strains. Among these, the majority were of tEPEC category among both fecal and meat isolates. Elevated level of EPEC contamination in meat provided evidence of cross-contamination from feces, carcasses, and contaminated slaughterhouse and working surfaces. Almost all detected DEC strains were found resistant against oxytetracycline, nalidixic acid, and penicillin. Meat contamination with resistant DEC strains presents an obvious route for microbial spread into consumers households, creating a serious public health problem. To minimize the chances of cross-contamination, it is necessary to educate slaughterers so that they adopt hygienic practices regarding slaughtering and handling. The government should also initiate regulatory policies concerning usage of antibiotics at poultry farms along with the vigorous screening of broiler for resistant DEC at farm and market levels in Pakistan.

Author contributions

M.Am. and M.R. planned and designed the experiment; M.Am. conducted the experiment; M.Am., A.I., and A.H. prepared the first draft of the manuscript; Y.-F.C. interpreted the data; M.Ah. collected the samples. The final revised manuscript was approved by all the authors.

Conflicts of interest

The authors declared no conflict of interest in the study.

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