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Prevalence of Extended-spectrum β-lactamases in *Escherichia coli* Isolated from Chicken, Water and the Poultry Farm Workers

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ABSTRACT

The *Enterobacteria* harboring the extended-spectrum-β-lactamase (ESBL) are a serious threat to public health particularly in developing countries. The study was aimed to investigate the prevalence and genomic characterization of ESBL producing *E. coli* from poultry, environment and poultry farm workers in Islamabad and Rawalpindi Pakistan. A total of 250 poultry samples, 92 poultry environmental samples, and 50 poultry farm worker's urine samples were screened for ESBL producing *E. coli* using ChromeID^{TM} ESBL agar. Biochemical confirmation of the isolates was carried out by API 20E. The antimicrobial susceptibility profiling and phenotypic confirmation of ESBL producers were performed as per CLSI 2018 guidelines. The minimum inhibitory concentration of ESBL producing isolates was determined by the broth dilution method. Phenotypically confirmed ESBL producing strains were further subjected to molecular characterization for the presence of ESBL and carbapenemase-producing genes using PCR. Of 392 samples, 219 ESBL positive *E. coli* were recovered and among these 156/213 (73.2%), 42/63 (66.6%) and 21/27 (77.7%) were from poultry, environmental water, and urine samples, respectively. The PCR results revealed that 71.2% of bla_{CTX-M}, 67.5% $bla_{CTX-M-1}$ and 62.2% bla_{TEM} producing isolates were recovered from poultry, 19.1% bla_{CTX-M}, 21.6% $bla_{CTX-M-1}$, 36.8% bla_{TEM} from environment and 9.6% bla_{CTX-M}, 10.8% $bla_{CTX-M-1}$ 0.8% bla_{TEM} from urine samples. Moreover, $bla_{CTX-M-2}$ (n=19), $bla_{CTX-M-8}$ group (n=19) and $bla_{CTX-M-9}$ group (n=27) were only observed in poultry samples. In addition, carbapenemase encoding genes as 9.6% (19/219) bla_{IMP}, 21.4% (47/219) bla_{VIM} and 15.5% (34/219) bla_{NDM} were also detected. ESBL producing *E. coli* exhibited resistance against cephalosporins, β-lactamase inhibitors, monobactam, folate-pathway inhibitors, fluoroquinolones, and aminoglycosides. This study investigates the high prevalence of ESBL producing *E. coli* in chicken, farmworkers, and water that is alarming and could lead to serious threats to both livestock and public health.

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Key words
ESBL-*Escherichia coli*, Carbapenemases, Environmental water, Poultry, Poultry farm workers

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INTRODUCTION

Extended-spectrum-β-lactamase (ESBL) producing Enterobacteriaceae particularly *Escherichia coli* are becoming a serious threat to the public health sector globally, mainly in developing countries like Pakistan (Aslam *et al*., 2018). The use of antimicrobial agents in animals, poultry and agriculture have recognized benefits, but overuse has potentially serious implications for human health such as the development of antimicrobial resistance (AMR). In 2010, livestock consumed about 63,200 tons of antibiotics, which could increase to 105,600 tons by 2030 globally (Van Boeckel *et al*., 2015). According to the United Nation, by 2050, one person will die every three seconds and more than 10 million people die annually with an economic burden of above $100 trillion if AMR is not properly managed now. There are around 700,000 people die each year globally due to drug-resistant infections (Brogan and Mossialos, 2016). Two Pakistani studies also documented that AMR pathogens are responsible for 4/9 children death and 57% of children died due to the septicemia caused by AMR pathogens (Khan *et al*., 2016; Khurshid *et al*., 2017; Qamar *et al*., 2015).

In Pakistan, there is indiscriminate and extensive use of antibiotics in livestock, particularly in the poultry industry. Moreover, there is the same antibiotic regime (cephalosporins, macrolides, aminoglycosides, and fluoroquinolones) is being used in both animals and humans (Rehman *et al*., 2017). This overuse/misuse of antibiotics leads to the selection of resistant bacteria among animals and poultry. Hence, these resistant bacteria are transmitted to humans through various routes or may allow susceptible human pathogens to acquire resistance through genetic transfer, which leads to the emergence of new resistant

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strains (Landers *et al*., 2012). ESBL producing *E. coli* is frequently involved in various infections such as sepsis, pneumonia, gastroenteritis, and urinary tract infections (Abraham *et al*., 2012). ESBL producing *E. coli* showed resistant against a wide range of β-lactam drugs and β-lactam inhibitors except for carbapenems which are considered the last resort to treat infections caused by such pathogens (Canton *et al*., 2012). There are 224 bla_{CTX-M}, 228 bla_{SHV} and 237 bla_{TEM} ESBL genotypes that have been reported so far (Naas *et al*., 2017). Recently, a Pakistani study reported the prevalence of ESBL producing *E. coli* (bla_{CTX-M}, bla_{SHV}, bla_{OXA-48}) from both poultry meat and poultry farm environment (Rahman *et al*., 2018). Similarly, another study from Pakistan also revealed the presence of carbapenemases and ESBL producing *E. coli* from poultry (Ahmad *et al*., 2018). One Health approach has been proposed as the key to study the transmission dynamics of AMR. As per our knowledge, there are limited data available on the "Pakistan One Health issue" so far. Hence, we have planned this study to investigate the presence and spread of ESBL producing *E. coli* from poultry, environmental water, and poultry workers from twin cities, Pakistan.

MATERIALS AND METHODS

Ethical consideration

Before starting the research work, ethical approval was obtained from the Ethical Review Committee, Government College University, Faisalabad, Pakistan.

Collection of samples

A total of 250 poultry samples (chicken ceca) from live bird market, 92 water samples (23 poultry drinking water, 38 poultry wastewaters, 31 community sewage) and 50 urine samples from poultry farm workers (Table II) were collected aseptically during February 2018 to July 2019 from different areas of Islamabad and Rawalpindi, Pakistan. The geographic location of the sample area is given in Figure 1. The chicken ceca samples were kept in the sterile plastic bags and water samples (50ml) were collected in 100ml sterilized bottles (Beninati *et al*., 2015). The poultry workers were given the sterile container and requested to provide a 10-20 ml urine sample for examination. Samples were immediately transported to the bacteriology research laboratory under refrigerated conditions (5°C ± 3) for further analysis.

Isolation and ESBL confirmation of Escherichia coli

For the separation of cecal sacs and interstitial material, sterilized scissors were used to open 1-2 cm incision in the wall of the cecum. For each cecal sample, 1g of cecal content was placed into 9 ml of buffered peptone water and tubes were incubated at 37°C for 22h aerobically. Further, 10µl of each sample was cultured on MacConkey agar containing cefotaxime (4mg/L) (MAC-CEF) and plates were incubated at 37°C for 22h aerobically. Similarly, 10µl of urine sample was streaked on MacConkey and MAC-CEF agar and plates were incubated for 22 hours at 37°C. Moreover, 1ml of poultry water sample was diluted 10-folds in 9ml of phosphate buffer saline and 100µl of the sample from each dilution was inoculated on MacConkey and MAC-CEF agar and plates were incubated at 37°C for 22h aerobically. The presumptive pink-colored *E. coli* colonies were further sub-cultured on ChromeID™ ESBL agar for the screening of ESBL (BioMérieux, France) and plates were incubated at 37°C for 22 h. The preliminary identification was done based on their colonial morphology, culture characteristics and biochemically confirmed by API 20E (BioMérieux, France). Confirmed ESBL-producing *E. coli* strains were stored in Tryptic Soy Broth (TSB) containing glycerol in duplicate at -20°C and -80°C.

Presumptive confirmation of ESBL producing *E. coli* was determined by a combination disc diffusion method as per CLSI 2018 guidelines. ESBL positive strain *Klebsiella pneumoniae* ATCC 700603 was used for positive control.

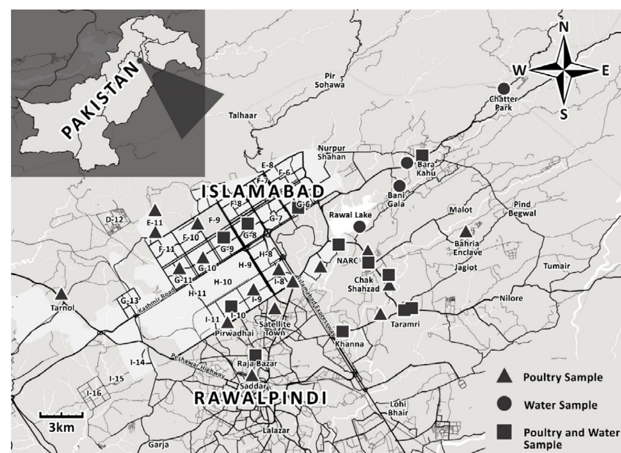

Fig. 1. Poultry and water sampling areas from Islamabad and Rawalpindi, Pakistan.

Molecular detection of ESBL and carbapenemase encoding genes

DNA extraction of ESBL positive *E. coli* was done using commercially available GeneJET Genomic Kit (Thermo Scientific, UK). ESBL encoding genes; bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and Carbapenemase encoding genes bla_{IMP}, bla_{VIM} and bla_{NDM} were identified through PCR using specific primers (Table I). Thermocycler conditions for

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Table I. Primers for the detection of ESBL genes.

Gene	Primers	Sequence (5´ to 3´)	Annealing temperature	Amplicon size (bp)	References
bla_{SHV}	SHV-F SHV-R	CTTTATCGGCCCTCACTCAA AGGTGCTCATCATGGGAAAG	62°C	237	(Fang *et al*., 2004)
bla_{TEM}	TEM-F TEM-R	CGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	62°C	445	(Monstein *et al*., 2007)
bla_{CTX-M}	CTX-M-F CTX-M-R	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	62°C	593	(Boyd *et al*., 2004)
bla_{OXA}	OXA-F OXA-R	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	62°C	813	(Ouellette *et al*., 1987)
bla_{CTX-M} group 1	CTX-M1-F CTX-M1-R	AAAAATCACTGCGCCAGTTC AGCTTATTCATCGCCACGTT	52°C	415	(Woodford *et al*., 2006)
bla_{CTX-M} group 2	CTX-M2-F CTX-M2-R	CGACGCTACCCCTGCTATT CCAGCGTCAGATTTTTCAGG	52°C	552	
bla_{CTX-M} group 9	CTX-M9-F CTX-M9-R	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCACC	52°C	205	
bla_{CTX-M} group 8	CTX-M8-F CTX-M8-R	TCGCGTTAAGCGGATGATGC AACCCACGATGTGGGTAGC	52°C	666	
bla_{CTX-M} group25	CTX-M25-F CTX-M25-R	GCACGATGACATTCGGG AACCCACGATGTGGGTAGC	52°C	327	
bla_{IMP}	IMP-F IMP-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCAC	52°C	232	(Poirel *et al*., 2011)
bla_{VIM}	VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	52°C	390	
bla_{NDM}	NDM-F NDM-R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	52°C	621	

Table II. Prevalence of *E. coli* and phenotypic confirmation of ESBL *E. coli* isolates from different sources.

Sample Sources	Number of samples	*E. coli* n (%)	ESBL producing-*E. coli* isolates n (%)
Environmental samples Water (poultry drinking water)	23	9 (39.1 %)	4 (44.4 %)
Wastewater	38	30 (78.9 %)	23 (76.6 %)
Community sewage	31	24 (77.4 %)	15 (62.5 %)
Subtotal	92	63 (68.4 %)	42 (66.6 %)
Poultry (Chicken ceca)	250	213 (85.2 %)	156 (73.2 %)
Urine (poultry farm workers)	50	27 (54 %)	21 (77.7 %)
Grand total	392	303 (77.2 %)	219 (72.2 %)

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ESBL encoding genes were; an initial denaturation for 5 min at 95°C; followed by 36 cycles at 95°C for the 30sec, 62°C for 90sec, and 72°C for 60sec; with a final extension for 10 min at 72°C and for carbapenemase encoding genes were; an initial denaturation for 10 minutes at 94°C and 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 52°C, and 50 s at 72°C, with 5 minutes at 72°C for the final extension. Gel electrophoresis of the amplified product was determined by 1.5% agarose gel and bands were visualized under UV light in the Gel Documentation instrument (Bio-Rad, UK) (Woodford *et al*., 2006).

Multiplex PCR for genotypic profiling of the bla$_{CTX-M}$ *group*

Genotypic detection of the bla_{CTX-M} group (1, 2, 9, 8 and 25) was performed through multiplex PCR using specific primers (Table I). Thermocycler conditions were as follows; an initial denaturation for 5 min at 94°C; followed by 30 cycles for 25sec at 94°C, for the 40sec at 52°C and for 50sec at 72°C; with a final extension for 6 min at 72°C. Gel electrophoresis of the amplified product was determined by 1.5% agarose gel and bands were visualized under UV light in the Gel Documentation instrument (Bio-Rad, UK) (Woodford *et al*., 2006).

Antimicrobial susceptibility testing

Antibiogram was performed by Kirby-Bauer disc diffusion method using Mueller-Hinton agar (MHA) (Oxoid, UK) as per CLSI 2018 guidelines. The tested antibiotics were as followed; cefotaxime (30µg), ceftazidime (30µg), cefixime (5µg), ceftriaxone (30µg), cefepime (30µg), cefotaxime/clavulanic acid (30/10µg), ceftazidime/clavulanic acid (30/10µg), amoxicillin/clavulanic acid (30/10µg), piperacillin/tazobactam (110µg) ciprofloxacin (5µg), aztreonam (30µg), imipenem (10µg), meropenem (10µg), gentamycin (10µg), amikacin (30µg), trimethoprim/sulfamethoxazole (25 µg) and colistin (10µg). Interpretation of the zone of inhibition was carried out according to CLSI 2018 guidelines. (Weinstein *et al*., 2018) ESBL-negative (*E. coli* ATCC 25922) and ESBL-positive (*K. pneumonia* ATCC 700603) strains were used as quality control for all the research processes. The minimum inhibitory concentration of the selected antimicrobial agents was also determined by the broth dilution method. The MIC of the ceftriaxone, cefotaxime, ceftazidime, cefepime, imipenem, ciprofloxacin, and colistin, was evaluated and the results were interpreted according to CLSI 2018 guidelines.

RESULTS

E. coli *and ESBL producing* E. coli *isolates*

A total of 213 *E. coli* isolates were recovered from chicken ceca, 63 from the environment and 27 were recovered from urine samples. Of 213 chicken *E. coli*, 156 (73.2%) were confirmed as ESBL producers, 63 environmental *E. coli*, 42 (66.6%) and 27 urinary *E. coli*, 21 (77.7%) were confirmed as ESBL producing isolates determined by combination disc diffusion method. Overall, 219 (72.2%) ESBL-producing *E. coli* isolates were phenotypically confirmed from 303 *E. coli* isolates. Prevalence of ESBL producing *E. coli* isolates from different water samples varied as 44% from poultry drinking water, 76.6 % from poultry wastewater and 62.5 % from community sewage as shown in Table II.

Prevalence of ESBL and carbapenemase encoding genes

The ESBL producing *E. coli* isolates were further examined for the presence of ESBL-encoding genes as well as for the detection of $bla_{CTX\text{-}M}$ groups and the results are shown in Table III. Our results showed that, of 219 $bla_{CTX\text{-}M,}$ 156 (71.2%) were isolated from poultry samples, 42 (19.1%) from water samples and 21 (9.6%) from urine samples. However, of 194 $bla_{CTX\text{-}M\text{-}1,}$ 131 (67.5 %), 42 (21.6 %) and 21 (10.8%) were isolated from poultry water and urine samples, respectively followed by 19 (100%) of $bla_{CTX\text{-}M\text{-}2,}$ $bla_{CTX\text{-}M\text{-}8}$ and 27 (100%) of $bla_{CTX\text{-}M\text{-}9}$ were only isolated from poultry samples. Most of these isolates were identified from a different region of Islamabad. The combination of $bla_{CTX\text{-}M\text{-}2}$/ $bla_{CTX\text{-}M\text{-}8}$/ $bla_{CTX\text{-}M\text{-}9}$ producing *E. coli* (n=16; 100%) was only isolated from poultry samples. Of 114 bla_{TEM} producing *E. coli*, 71 (62.2%), 42 (36.8%) and 1 (0.8%) were recovered from poultry, water, and urine samples respectively. However, 28 bla_{SHV} producing *E. coli* were only isolated from poultry samples and of 12 bla_{OXA}, 9 (75%) were isolated from poultry, 2 (16.7%) from urine and 1 (8.3%) from the water samples. Co-existence of $bla_{CTX\text{-}M}$/ bla_{TEM}/ bla_{SHV} was identified in the *E. coli* recovered from poultry samples (n=3; 100%). Of 4 $bla_{CTX\text{-}M}$/ bla_{TEM}/ bla_{OXA} combination, 2 (50%) were isolated from poultry 1 (25%) from water and 1 (25%) from urine samples. In addition, carbapenemase encoding genes were also detected in *E. coli* isolates as represented in Table III. Of 21 bla_{IMP} 16 (76.1%) were detected from poultry, 3 (14.2%) from both water and urine samples. While 47 bla_{VIM}, 36 (76.5%) were recovered from poultry, 8 (17%) and 3 (6.3%) from urine and water samples, respectively. However, of 34 bla_{NDM} producing *E. coli*, 31 (91.1%) and 3 (8.8%) were only isolated from poultry and urine samples, respectively. The co-existence of carbapenemase encoding genes was also observed in *E. coli* isolates as bla_{IMP}/ bla_{VIM} (n=4) were recovered from poultry and water samples, bla_{VIM}/ bla_{NDM} (n=21) were recovered from chicken and urine samples and bla_{IMP}/ bla_{VIM}/ bla_{NDM} (n=14) was also observed in isolates from chicken and urine samples.

Antimicrobial susceptibility testing

All these 219 isolates were further evaluated against various antimicrobials as shown in Table IV. The isolates displayed 100% resistance to cephalosporins, amoxicillin/clavulanic acid and aztreonam followed by 86.7% to trimethoprim/ sulfamethoxazole, 75.3% to ciprofloxacin, 58.4% to piperacillin/tazobactam, 42.9% to gentamycin and amikacin, 23.7% to meropenem and 23.2% to imipenem. Colistin was found as the most susceptible drug by displaying the lowest resistance (17.8%) against ESBL-producing *E. coli* isolates. Further, the minimum inhibitory concentration for cefotaxime against ESBL *E. coli* isolates was 256 µg/ml (12.3%) ≥ 256 µg/ml (87.6%), for ceftriaxone was 256 µg/ml (10.9%) ≥ 256 µg/ml (89%), for ceftazidime was 128 µg/ml (9.1%) ≥ 256 µg/ml (87%) and for cefepime was 128 µg/ml (9.4%) ≥ 256 µg/ml (83%). The MIC range was 0.25 µg/ml (10%) ≥ 256 µg/ml (5 %) for imipenem. The 50 (22.8%) out of 219 isolates were sensitive to ciprofloxacin with a MIC < 0.25 µg/ml. 4 (1.8%) of the isolates were intermediate having a MIC of 0.5 µg/ml. The majority of ESBL *E. coli* strains i.e. 111 (50.6%) exhibit a MIC of 64 µg/ml for ciprofloxacin. The MIC range of the ESBL *E. coli* isolates for colistin was 0.25 µg/ml (34.7%) – 8 µg/ml (2.2%) as shown in Table V.

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Table III. Prevalence of ESBL- producing genes among *E. coli* isolates recovered from poultry and environmental (water) samples.

ESBL Ggenotype	Total No. of ESBL genes	Poultry	Water	Poultry farmworkers
$bla_{\text{CTX-M}}$	219	156 (71.2%)	42 (19.1%)	21 (9.6%)
$bla_{\text{CTX-M-1}}$	194	131 (67.5%)	42 (21.6%)	21 (10.8%)
$bla_{\text{CTX-M-2}}$	19	19 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-8}}$	19	19 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-9}}$	27	27 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-1}}+ bla_{\text{CTX-M-2}}$	1	1 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-1}}+ bla_{\text{CTX-M-8}}$	1	1 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-1}}+ bla_{\text{CTX-M-9}}$	2	2 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-2}}+bla_{\text{CTX-M-8}}$	16	16 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-2}}+ bla_{\text{CTX-M-9}}$	18	18 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-8}}+ bla_{\text{CTX-M-9}}$	18	18 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M-2}}+ bla_{\text{CTX-M-8}}+ bla_{\text{CTX-M-9}}$	16	16 (100%)	0 (0%)	0 (0%)
bla_{TEM}	114	71 (62.2%)	42 (36.8%)	1 (0.8%)
bla_{SHV}	28	28 (100%)	0 (0%)	0 (0%)
bla_{OXA}	12	9 (75%)	1 (8.3%)	2 (16.7%)
$bla_{\text{CTX-M +}} bla_{\text{TEM+}} bla_{\text{SHV}}$	3	3 (100%)	0 (0%)	0 (0%)
$bla_{\text{CTX-M +}} bla_{\text{TEM+}} bla_{\text{OXA}}$	4	2 (50%)	1 (25%)	1 (25%)
bla_{IMP}	21	16 (76.1%)	3 (14.2%)	3 (14.2%)
bla_{VIM}	47	36 (76.5%)	3 (6.3%)	8 (17%)
bla_{NDM}	34	31 (91.1%)	0 (0%)	3 (8.8%)
$bla_{\text{IMP}}+ bla_{\text{VIM}}$	4	2 (50%)	2 (50%)	0 (0 %)
$bla_{\text{VIM}} + bla_{\text{NDM}}$	21	18 (85.7%)	0 (0%)	3 (14.2%)
$bla_{\text{IMP}}+ bla_{\text{VIM}}+ bla_{\text{NDM}}$	14	13 (92.8%)	0 (0%)	1 (7.1%)

Table IV. Antimicrobial susceptibility pattern of ESBL *E. coli* isolates.

Antimicrobial classes	Antimicrobials	Abb.	Concentration (µg)	Sensitive %	Intermediate %	Resistance %
1st generation cephalosporin	Cephradine	CE	30	0	0	100
3rd generation cephalosporin	Cefotaxime	CTX	30	0	0	100
	Ceftazidime	CAZ	30	0	13.2	86.7
	Ceftriaxone	CRO	30	0	0	100
	Cefixime	CFM	5	1.3	2.7	95.8
4th generation cephalosporin	Cefepime	FEP	30	0	7.7	92.2
β- lactamase inhibitors combination with cephalosporin	Cefotaxime/ Clavulanic acid	CTL	30/10	2.2	7.3	90.4
	Ceftazidime/ Clavulanic acid	CAL	30/10	24.6	0.4	74.8
β- lactamase inhibitors combination with Penicillin	Amoxicillin/ Clavulanic acid	AMC	30/10	0	0	100
	Piperacillin/ tazobactam	TZP	110	34.7	6.8	58.4
Carbapenems	Imipenem	IPM	10	67.1	9.6	23.2
	Meropenem	MEM	10	66.6	9.6	23.7
Monobactam	Aztreonam	ATM	30	0	1.8	98.1
Fluoroquinolones	Ciprofloxacin	CIP	5	22.8	1.8	75.3
Aminoglycosides	Gentamycin	CN	10	50.2	6.8	42.9
	Amikacin	AK	30	50.2	6.8	42.9
Folate pathway inhibitors	Trimethoprim/Sulfamethoxazole	SXT	25	13.2	0	86.7
Polymyxins	Colistin	CT	10	83.8	0	17.8

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Table V. Minimum inhibitory concentration of ESBL *E. coli* isolates.

MIC (µg)	Cefotaxime (CTX)	Ceftazidime (CAZ)	Ceftriaxone (CRO)	Cefepime (FEP)	Imipenem (IPM)	Ciprofloxacin (CIP)	Colistin (CT)
0.25	0 (0%)	0 (0%)	0 (0%)	0 (0%)	22 (10%)	50 (22.8%)	76 (34.7%)
0.5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	78 (36%)	4 (1.8%)	80 (36.5%)
1	0 (0%)	0 (0%)	0 (0%)	0 (0%)	47 (21%)	0 (0%)	24 (10.9%)
2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	21 (10%)	0 (0%)	2 (0.9%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	32 (14.6%)
8	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1.3%)	12 (5.4%)	5 (2.2%)
16	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (2.2%)	6 (2.7%)	0 (0%)
32	0 (0%)	0 (0%)	0 (0%)	0 (0%)	8 (3.6%)	21 (9.5%)	0 (0%)
64	0 (0%)	0 (0%)	0 (0%)	0 (0%)	8 (3.6%)	111 (50.6%)	0 (0%)
128	0 (0%)	20 (9.1%)	0 (0%)	17 (9.4%)	7 (3.1%)	0 (0%)	0 (0%)
256	27 (12.3%)	9 (4.1%)	24 (10.9%)	21 (9.5%)	9 (4.1%)	7 (3.1%)	0 (0%)
>256	192 (87.6%)	190 (87%)	195 (89%)	181 (83%)	11 (5%)	8 (3.6%)	0 (0%)
Total	219	219	219	219	219	219	219

DISCUSSION

The emergence of resistance in *E. coli* against cephalosporins is of major concern in human as well as veterinary medicine because cephalosporins are widely used in human healthcare settings (Dierikx *et al.*, 2010). In Pakistan, the urge to optimize animal production has led to the unselective use of antibiotics. The irrational use of antibiotics is evaluated as an imperative predisposing factor for the attainment of ESBL producing bacteria consequently increasing resistance to frequently used drugs such as erythromycin, ampicillin, gentamicin, tetracycline, cotrimoxazole, and third-generation cephalosporins (Reich *et al.*, 2013). Our study has shown an overall prevalence of 73.2%, 77.7 % and 66.6% of ESBL producing *E. coli* from chicken ceca, urine from poultry farmworkers and environmental water respectively. These findings agree with the previous study published in Egypt with a 65% prevalence of ESBL producing *E. coli* from poultry (Abdallah *et al.*, 2015; Hiroi *et al.*, 2011). However, a study from Pakistan revealed a low prevalence of ESBL producing *E. coli* from poultry meat (47.6%) and poultry water (38.8%) (Rahman *et al.*, 2018). In another study published from Korea in which 44 and 68 *E. coli* isolates from poultry and swine, farmworkers were recovered, respectively (Cho *et al.*, 2012). The high prevalence in our study might be due to recurrent administration of antibiotics to poultry which in turn increases the risk of highly resistant *E. coli* strains in the normal flora of intestine as reported in a study which evaluated that food animal was likely reservoir for antimicrobial-resistant fecal flora particularly *E. coli* (Carattoli, 2008).

The present study showed that bla_{CTXM} remained a predominant genotype among all the ESBL-producing *E. coli* isolated from poultry, environment, and urine. These findings are in accordance with other previous studies from Pakistan (Abrar *et al.*, 2017; Khan *et al.*, 2010) and India (Upadhyay *et al.*, 2015). They strongly suggested that bla_{CTX-M} is the most dominant genotype of ESBL in Asia. Analysis of β-lactamase genes in our study revealed that all the isolates from poultry carried bla_{CTX-M} followed by bla_{TEM}, $bla_{SHV,}$ and bla_{OXA} (Table III). These findings are in line with the previous studies from Japan (Kawamura *et al.*, 2014) and Brazil (Nogueira *et al.*, 2015). The high prevalence of bla_{CTX-M} producing *E. coli* might be attributable to the increased growth of indigenous strains harboring bla_{CTX-M} and there is a possibility for the emergence of bla_{CTX-M} producing strains by horizontal transfer on different farms. Furthermore, the ESBL positive *E. coli* in the existing study also carried multiple types of ESBL genotypes and a combination of bla_{CTX-M}/bla_{TEM} was predominant, followed by bla_{CTX-M}/bla_{SHV}, bla_{CTX-M}/bla_{OXA}. This type of co-existence of ESBL genes was also observed in a study from Turkey (Tekiner and Ozpinar, 2016). These findings consequently confirmed that ESBL-mediated plasmids can carry multiple β-lactamase genes and these determinants are easily transportable to other bacteria of the same genre or of different genres within *Enterobacteriaceae* (Giedraitiene *et al.*, 2011). In the current study, the $bla_{CTX-M-1}$ was the most prevalent in poultry, environment and urine accounted for 67.5 %, 21.6 %, and 10.8% respectively. The $bla_{CTX-M-2}$ (100%), $bla_{CTX-M-8}$ (100%), and $bla_{CTX-M-9}$ (100%) were only observed in poultry. A study from Austria has reported that

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$bla_{CTX-M-1}$ were the predominant genes in ESBL producing *E. coli* recovered from chicken meat (Zarfel *et al*., 2014). In contrast, a study from Japan has established that the most prevalent genes were $bla_{CTX-M-9}$ (67%), $bla_{CTX-M-1}$ (19%) and $bla_{CTX-M-2}$ (5.8%) among ESBL producing *E. coli* (Chong *et al*., 2013).

These findings suggested a serious threat to public health and an important food safety concern because the retail meat could be a source for drug-resistant bacterial strains that ultimately can be transmitted to humans. In addition, carbapenemase-producing genes as 9.6% (19/219) bla_{IMP} and 21.4% (47/219) bla_{VIM} were also detected from poultry, urine, and environmental samples, whereas 15.5% (34/219) bla_{NDM} was recovered only from chicken and urine samples. Also, the co-existence of multiple carbapenemase encoding genes was observed in *E. coli* isolates as the combination of bla_{IMP}/bla_{VIM} (2%), bla_{VIM}/bla_{NDM} (9.5%) and $bla_{IMP}/bla_{VIM}/bla_{NDM}$ (6.4%). These results are in agreement with a recent study from Pakistan as they isolated bla_{VIM} and bla_{NDM} producing *E. coli* strain from poultry meat (Younas *et al*., 2019). Whereas, some previous studies reported from Pakistan suggested that bla_{NDM} was a prominent gene in the community (Sartor *et al*., 2014) and clinical settings (Hussain, 2015; Perry *et al*., 2011; Sattar *et al*., 2016). Identification of carbapenemase encoding genes, particularly NDM in *E. coli* isolates recovered from poultry is alarming representing that NDM has been widespread distribution among both animals as well as humans. In the present study, ESBL producing *E. coli* showed a high level of resistance to β-lactam and β-lactam inhibitors, trimethoprim/sulfamethoxazole, ciprofloxacin, and aminoglycosides. These findings are in agreement with the previously published studies from Tanzania (Mshana *et al*., 2009), Zambia (Chishimba *et al*., 2016), and Thailand (Runcharoen *et al*., 2017) that reported a high level of drug resistance. In our settings, the high level of resistance is mainly due to the irrational use of antibiotics in the poultry industry, misuse of antibiotics, broad-spectrum antibiotics and availability of antibiotics over the counter (Qamar *et al*., 2014; Rahman *et al*., 2018).

CONCLUSION

It was concluded that a high prevalence of ESBL producing *E. coli* isolates in poultry, poultry farmworkers and environmental water was found in twin cities, Pakistan. Moreover, all the isolates were resistant to commonly used antibiotics confirming that poultry might be one of the significant and possible reservoirs for the multidrug and ESBL resistant genes which could spread into the food chain. The risk of zoonotic transmission from poultry to persons with close contact is still largely unknown, however, the data obtained imply that occupational exposure to ESBL *E. coli* strains from animal contact in the poultry industry might be a significant route of transmission of resistant *E. coli* into the community. As per our knowledge, there is no data available on ESBL encoding genes especially among the bacterial isolates from poultry workers in Pakistan. Therefore, there is an urgent need for continuous surveillance to obtain data that will help formulate appropriate antibiotic policies to manage the growing drug resistance problem.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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