

Contribution and Diversity of Integrations and *acrAB-TolC* Efflux Pump to Multidrug Resistance in Clinical Isolates of *Escherichia coli* Isolated from Pakistan and China

Muhammad Yaqoob, Li Ping Wang, Shaohui Wang, Javed Memon and Lu Cheng-Ping*

College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, PR China

Department of Veterinary Medicine, Faculty of Veterinary Sciences, PMAS University of Arid Agriculture, Muree Road, Rawalpindi

ABSTRACT

The contribution and diversity of integrations and efflux pump to multidrug resistance in avian *Escherichia coli*, isolated from Pakistan and China, was evaluated. Real time RT-PCR, RT-PCR and direct sequencing methods were used to detect the presence of potential mechanisms contributing to antimicrobial resistance in sixty-two clinical isolates. Antibiotic specific resistance was detected as β -lactamase genes (55%), tetracycline resistant genes (68%), sulfonamide resistant genes (76%) and 16S rRNA methylase enzyme (0%). Integrations were detected in 37.09% of clinical isolates and carried cassettes conferring resistance mainly to aminoglycosides and trimethoprim. Class 2 integrations in two isolates and class 3 were not found in all strains. Seventy-seven percent of the isolates expressed the *acrAB-TolC*, as compared with a control strain. The efflux pump was highly significant to ofloxacin, pefloxacin, spectinomycin, tetracycline and sulfonamide resistance of *E. coli*. Findings showed that contribution of efflux pump play important role in multidrug resistance than integrations and Pakistani isolates showed more resistance than China. Extrusion of ofloxacin was specific to the *acrAB-TolC* efflux pump. Contribution and diversity of resistance mechanisms reflects the genetic determinants responsible for multidrug resistance in avian *E. coli* between two countries.

Article Information

Received 23 March 2021

Revised 08 August 2022

Accepted 18 September 2022

Available online 22 January 2025

(early access)

Published 27 December 2025

Authors' Contribution

MY, LPW, SW, JM and LCP contributed by conducting the experiments, presented idea and concept, writing and editing the manuscript.

Key words

Multidrug resistance, Integrations, Efflux pump, Real time PCR, Avian *E. coli*

INTRODUCTION

The resistance of bacteria to antibiotics is increasing worldwide, which have concern about the public health. There is a need to minimize the spread of resistance genes, since these could be transferred to opportunistic and pathogenic bacteria (Blazquez *et al.*, 2002). The extensive use of antibiotics in human and veterinary medicine is contributing to the selection and dissemination of antibiotic-resistant microorganisms. In the last decades, the emergence of antibiotic resistance among pathogenic bacteria in clinical environments has become a serious problem worldwide (Henriques *et al.*, 2006).

The genetic flexibility and adaptability of *Escherichia coli* to constantly changing environments allows to acquire a great number of antimicrobial resistance mechanisms (Neu, 1992). One of these mechanisms is drug specific resistance, which resist by the inactivation of antibiotics such as β -lactamase, 16S rRNA methylase, tetracycline and sulfonamide. These mechanisms are all specific for a single drug or a single class of drugs (Nishino *et al.*, 2009).

However, there are general mechanisms of resistance like (acquired resistance) integrations and (intrinsic resistance) efflux pump that also contribute to the resistance of antibiotics in *E. coli* (Anadon *et al.*, 2005). In acquired resistance, mobile genetic elements transfer antimicrobial resistance genes among bacteria and transfer from animals to animals as well as to humans (Aarestrup and Wegener, 1999). For multidrug resistance in bacteria, integrations take part as acquired resistance mechanism by the acquisition of antibiotic resistance genes (Ammor *et al.*, 2008).

Intrinsic resistance is inherent to bacterial species by the presence of efflux mechanisms (Ammor *et al.*, 2008). Efflux pump proteins are responsible for resistance to a variety of unrelated antibacterial compounds in *E. coli* (Sander *et al.*, 2000). The *acrAB-TolC* efflux pump consists

* Corresponding author: lucp@njau.edu.cn
0030-9923/2026/0001-0245 \$ 9.00/0



Copyright 2026 by the authors. Licensee Zoological Society of Pakistan.

This article is an open access  article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

of three genes located on the chromosome (Gibreel *et al.*, 2007). The tripartite complex traverses both membranes and allows the extrusion of drugs directly into the extra cellular medium (Masi *et al.*, 2003). Previously these resistance mechanisms have studied separately (Ahmad *et al.*, 2008). However, little information is available about the comparison study of resistance mechanisms and no knowledge is available about the contribution and diversity of resistance mechanisms in avian *E. coli* between the neighbor countries (Pakistan-China).

The aim of the present study was to assess the contribution and diversity of integrons (acquired resistance) and efflux pump *acrAB-TolC* (intrinsic resistance) in *E. coli* isolated from Pakistan and China. There is a need to separate the susceptible and resistant bacteria, to assess the antibiotic resistance patterns of *E. coli* by distinguishing the intrinsic and acquired forms of resistance and to characterize the genetic determinants responsible for the resistances. To the best of our knowledge, this is the first report for contribution and diversity of integrons and efflux pump *acrAB-TolC* in *E. coli* isolated from Pakistan-China.

MATERIALS AND METHODS

Bacterial strains, isolation and identification

Sixty-two *E. coli* isolates were collected from poultry. Thirty-four isolates from veterinary diagnostic laboratories of Punjab province Pakistan and twenty-eight from veterinary diagnostic laboratories of Jiangsu province China. Isolation and identification was performed with on MacConkey agar (Aoboxing Biotech, China), *E. coli* special medium, chrome agar (Biocell Biotech, China) and 16S rDNA analysis (Henriques *et al.*, 2006).

Random amplified polymorphic DNA (RAPD)

In random amplified polymorphic DNA (RAPD) analysis of *E. coli* strains, the Polymerase Chain Reaction (PCR) mixture was prepared in a total volume of 50 µl consisting of 5 µl template DNA, 25 µl PCR Mix (Best Bio, China), 19 µl H₂O and 1 µl COL-1 primer (3-AAGAGC CCGT-5) (Kilic *et al.*, 2009). The samples were amplified through 45 cycles of 94 °C for 30s, 36 °C for 15s and 72 °C for 30s. In negative control reactions, the DNA template or the primer was replaced by sterile deionized water.

Antimicrobial susceptibility test

Antimicrobial minimum inhibitory concentrations (MICs) for *E. coli* strains were determined using the standard broth dilution method on Muller–Hinton medium (Oxide, UK) and interpreted according to Clinical Laboratory Standards Institute (CLSI) standards (Wayne, 2008). The following antibiotics: ampicillin, amoxicillin,

ofloxacin, pefloxacin, streptomycin, spectinomycin, gentamycin, tetracycline and sulfonamide (Sigma, USA) were used in this study. *E. coli* ATCC 25922 was used as quality control strains in MICs determinations.

PCR screening for antimicrobial resistance genes

All isolates were tested for β-lactamase genes TEM, OXA, SHV and CTX-M, tetracycline resistance genes *tetA*, *tetB*, *tetM* and *tetO*, sulfonamide resistance gene *SulI* and 16S rRNA methylase genes *armA*, *rmtA* and *rmtB* by polymerase chain reaction. Primers for β-lactamase genes, tetracycline resistance genes, sulfonamide resistance genes and 16S rRNA methylase genes are listed in Table I. The PCR mixtures used to detect the resistance genes contained 25 µl reaction mixtures (TaKaRa Bio, China) according to the manufacturer's instructions. PCR products (6 µl) were analyzed by electrophoresis on a 1% agarose gel and stained with Gold view. The PCR products were purified with gel purification kit (Geneaid Biotech, Taiwan) and cloned into pMD18-Tvector (TaKaRa Bio, China) according to the manufacturer's instructions for further sequencing.

Detection of class 1, 2, and 3 integrons

Detection of class 1, 2 and 3 integrons was performed by PCR as described previously (Celine *et al.*, 1995). The primers used for detection and characterization of integrons are shown in Table I. For class 1 integrons, two primer sets were used: Int1-F/Int1-R for amplifying the int1 gene and 5-CS/3-CS for amplifying the integron variable region containing gene cassettes. For class 2 integrons, the primers Int2-F/Int2-R were used for amplifying the int2 gene and hep51/hep74 for amplifying the integron variable region containing gene cassette. The PCR product of interest was excised from 1% agarose gel, purified with a purification kit (Geneaid Biotech, Taiwan) and cloned into pMD18-Tvector (Takara Bio, China) according to the manufacturer's instructions for further sequencing.

Real-time RT-PCR studies

The 62 clinical isolates were analyzed for the expression of *acrABC* efflux pump gene by real time RT-PCR. DNase-treated bacterial RNA was isolated from cultures grown to the late log phase in LB (Luria Bertani) broth by Bacterial RNA kit (OMEGA Bio-Tek, China), according to the manufacturers' protocol. These RNA samples were used as template for reverse transcription with the Revert Aid™ First Strand cDNA Synthesis Kit according to the protocol supplied by the manufacturer (MBI, Fermentas, Germany). Then, real time RT-PCR reactions were performed on an ABI Prism 7300 thermal cycler (Applied Biosystems, Foster, CA, USA).

Table I. Primers used for amplification of integrons and antibiotic resistant genes.

Gene name	Primer sequence 5'→3'	Primer size	References
16srDNA F	AACGCGAAGAACCTTAC	433bp	Oliver <i>et al.</i> , 2008
16srDNA R	CGGTGTGTACAAGACCC		
TEM-F	ATAAAATTCTTGAAGACGAAA	1080bp	Weill <i>et al.</i> , 2004
TEM-R	GACAGTTACCAATGCTTAATC		
OXA-F	TCAACTTTCAAGATCGCA	591bp	Ahmed <i>et al.</i> , 2008
OXA-R	GTGTGTTTAGAATGGTGA		
SHV-F	TTATCTCCCTGTTAGCCACC	795bp	Weill <i>et al.</i> , 2004
SHV-R	GATTTGCTGATTTGCTCGG		
CTX-M-F	CGCTTTGCGATGTGCAG	550bp	Bonnet <i>et al.</i> , 2000
CTX-M-R	ACCGCGATATCGTTGGT		
Sul1-F	CTTCGATGAGAGCCGGCGGC	435bp	Gebreyes <i>et al.</i> , 2005
Sul1-R	GCAAGGCGGAAACCCGCGCC		
Int1-F1	CCTCCCGCACGATGATC	280bp	Bass <i>et al.</i> , 1999
Int1-R1	TCCACGCATCGTCAGGC		
Int2-F2	TTATTGCTGGGATTAGGC	233bp	Goldstein <i>et al.</i> , 2001
Int2-R2	ACGGCTACCCTCTGTTATC		
Int3-F3	AGTGGGTGGCGAATGAGTG	600bp	Goldstein <i>et al.</i> , 2001
Int3-F3	TGTTCTTGATCGGCAGGTG		
5-CS	GGCATCCAAGCAGCAAG	Variable	Ahmed <i>et al.</i> , 2008
3-CS	AAGCAGACTTGACCTGA		
Hep-51	CGGGATCCCGGACGGCATGCACGATTGTA	Variable	White <i>et al.</i> , 2001
Hep-74	GATGCCATCGCAAGTACGAG		
rmtA F	AGCTTTGACGATGCCCTAGC	716bp	Chen <i>et al.</i> , 2007
rmtA R	CCAATGGTCTTGGTATCCTC		
rmtB F	ACATCAACGATGCCCTCAC	725bp	Chen <i>et al.</i> , 2007
rmtB R	AAGTTCTGTTCCGATGGTC		
armA F	CAATCAGGGGCAGTTATCA	529bp	Chen <i>et al.</i> , 2007
armA R	CCCTATAACCTTCGAATC		
tetM F	GTGGACAAAGGTACAACGAG	406bp	Warsa <i>et al.</i> , 1996
tetM R	CGGTAAAGTTCGTCACACAC		
tetO F	AACTTAGGCATTCTGGCTCAC	515bp	Ng <i>et al.</i> , 2001
tetO R	TCCCACTGTTCCATATCGTCA		
tetA F	GTAATTCTGAGCACTGTGCGC	957bp	Nawaz <i>et al.</i> , 2009
tetA R	CTGCCTGGACAACATTGCTT		
tetB F	CTCAGTATTCCAAGCCTTTG	436bp	Nawaz <i>et al.</i> , 2009
tetB R	ACTCCCCTGAGCTTGAGGGG		

Amplification mixtures 20µl contained 2µl template cDNA, 10µl SYBR Green Mix (Applied Biosystems), 0.4µl ROX Reference Dye, 0.4µl reverse and 0.4µl forward primers (Table II) and 6.8µl water. PCR was accomplished after a 10sec activation and denaturation step at 95 °C, followed by 40 cycles of 5sec at 95 °C, and 31s at 60 °C for annealing and extension. Each sample was repeated at least three times. The parameter Ct was defined as the threshold cycle number at which the fluorescence generated by the binding of SYBR Green dye to double-stranded DNA began to increase exponentially. The expression of each gene was normalized to that of a ribosomal gene. The relative expression of each target gene was then calibrated against the corresponding expression

by *E. coli* ATCC 25922 (whose expression was equal to 1.0), which served as the control. Final results, expressed as n-fold differences in expression of *acrA*, *acrB* and *tolC* genes, were determined as follows (Chang *et al.*, 2004).

$$n = \frac{\text{Ct } \textit{acrA} \text{ or } \textit{acrB} \text{ or } \textit{TolC} \text{ sample}}{\text{Ct rDNA sample}} \bigg/ \frac{\text{Ct } \textit{acrA} \text{ or } \textit{acrB} \text{ or } \textit{TolC} \text{ calibrator}}{\text{Ct rDNA calibrator}}$$

Values of $n < 1$ were considered to indicate expression of the *acrAB-TolC* efflux system.

Statistical analysis Student's *t*-tests (two-tailed) and ANOVA tests were used to determine the correlation between over expression of the *acrAB-TolC* efflux pumps and drug resistance in clinical isolates of *E. coli*.

Table II. Primer sequences for Real-Time RT PCR.

Primer name	Primer sequence	Reference
acrA-RTF	5' TCGCAGAAGTTCGTCCTCAAG 3'	This study
acrA-RTR	5' ACCTTTTCGCACTGTCGTATGTC 3'	
acrB-RTF	5' GGTACTGGTAGCGTTGATCCTG 3'	This study
acrB-RTR	5' GTGTAGTGGTGCGTGCTCTTCT 3'	
TolC-RTF	5' AAGCACGCCTTAGTAACCCG 3'	This study
TolC-RTR	5' GCGTTAGAGTTGATGCCGTTTC 3'	
16s rRNA-RTF	5'CTCCTACGGGAGGCAGCAG 3'	Lane (1991)
16s rRNA-RTR	5' GWATTACCGCGGCKGCTG 3'	

RESULTS

The genetic similarity for strain diversity of sixty-two isolates was evaluated by using the random amplified polymorphic DNA. All isolates were not completely identical.

MIC determination

Phenotypic resistance to ampicillin, amoxicillin, ofloxacin, pefloxacin, streptomycin, spectinomycin, sulfonamide and tetracycline in Pakistan and China are shown in [Figure 1](#). All isolates were susceptible to gentamycin.

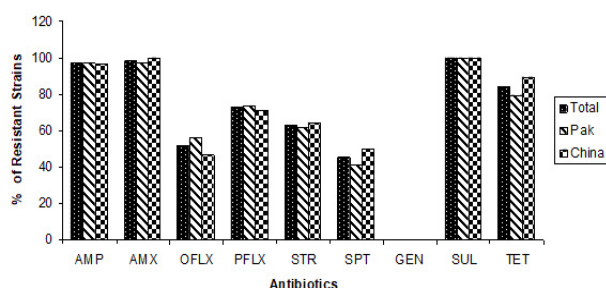


Fig. 1. Contribution and diversity of phenotypic resistance in *E. coli* strains isolated from Pakistan and China. AMP, Ampicillin; AMX, Amoxicillin; GEN, Gentamycin; OFLX, Ofloxacin; PFLX, Pefloxacin; SPT, Spectinomycin; STR, Streptomycin; SUL, Sulfonamide; TET, Tetracycline.

Detection of drug specific resistance genes

Drug specific resistance mechanisms between China and Pakistan are shown in [Figure 2](#). Thirty-four β -lactamase (55%). positive strains contained 28 *bla*_{TEM} genes, 24 *bla*_{CTX-M} genes and 3 *bla*_{OXA} genes. However, *bla*_{SHV} gene was not found in all strains. Tetracycline resistant genes were 68 %, which contained *tetA* 59 %, *tetB* 15 % and 5 % *tetA*. *B. TetM* and *tetO* were not found in all strains. Amplification of 16S rRNA methylase genes

(*armA*, *rmtA* and *rmtB*) was done but no gene was detected in all isolates. 77 % isolates were positive to *SulI* gene.

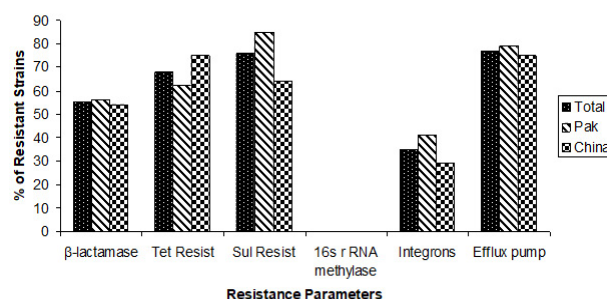


Fig. 2. Contribution and diversity of genotypic resistance in *E. coli* strains isolated from Pakistan and China.

Distribution of integrons in *E. coli* strains

As shown in [Figure 2](#), 37.09% ($n = 23/62$) of the isolates were positive for integrons. Consequently, the integron-borne gene cassettes were cloned and sequenced. The integrons were found to contain one to three gene cassettes and the combinations of these gene cassettes are shown in [Table III](#). Five distinct kinds of gene cassette arrays were characterized in class I integrons. These were *aadA1*, *aadA22*, *dfrA7*, *dfrA1-aadA1* and *dfrA12-orf-aadA2*, respectively. Two similar gene cassettes of class 2 integrons *dfrA1-sat1-aadA1* were found. Of them, *dfrA1-aadA1* (38.09%) was found most prevalent gene cassettes among class I integrons. These correlation analyses between antimicrobial resistant profile and occurrence of integrons are shown in [Table III](#). It can be seen that among the isolates whose resistant profile was relatively broad ($n \geq 5$), the percentage of positive-integron isolates was 41.30% (19/46). While, among the isolates whose resistant profile was relatively narrow ($n < 5$), positive-integron isolates were 25% (4/16). Statistically, these were significantly different from each other ($p < 0.05$). It can be seen that the *E. coli* strains, whose resistant

profile was relatively broad, tended to be easier to carry integrons. In this study, class 3 integron was not detected in all isolates.

The expression of acrAB-TolC

Real time RT-PCR methods were performed to assess the expression of *acrAB-TolC* efflux systems among *E. coli*. Our data indicated that *acrAB-TolC* was over expressed in 48 (77%) clinical isolates. MICs of ofloxacin and sulfonamide were significantly higher for isolates in which *acrAB-TolC* was highly expressed than those in which it was not expressed (Table IV). The expression levels of *acrAB-TolC* were classed into two categories, high level expression of *acrAB* and high level expression of *TolC* transporter. The correlation of antibiotic susceptibility with the expression of two genes (*acrAB*) and single gene (*TolC*) of *acrAB-TolC* efflux pump are shown in Table V.

The test would regard as significant only when $P < 0.01$. According to this relationship method *acrAB* efflux pump contain category was shown the significant result for

ofloxacin, pefloxacin spectinomycin and tetracycline. *TolC* gene contain category was shown the significant result for ofloxacin. However, other results were not reached this level of significance.

Contribution and diversity of integrons and acrAB-TolC efflux pump

The contribution and diversity of efflux pump and integrons among the multidrug resistant *E. coli* isolated from Pakistan and China were shown in Figure 2. The strains with class 2 integrons were isolated in Pakistan. Class 1 integrons gene cassette *dfrA1-aadA1* was more prevalent in Chinese strains while *dfrA7* was more in Pakistani strains. The expressions of efflux pump were significant for ofloxacin, pefloxacin, spectinomycin, tetracycline and sulfonamide resistant isolates. Both resistant mechanisms (acquired and intrinsic) were showed high level for multidrug resistance in Pakistan than China.

Table III. Antibiotic resistance patterns of *E. coli* strains in this study and its relationship with occurrence of the integrons.

Resistant patterns ^a	No. of resistant strains (n=62)	Inserted gene cassettes and occurrence rates in the resistant strains (n=62)
Sul, Amp, Tet	1	
Sul, Amx, Tet	2	
Sul, Amx, Amp	1	
Sul, Amx, Amp, Tet	11	<i>dfrA1-sat1-aadA1</i> (2), <i>dfrA7</i> (1), <i>aadA22</i> (1)
Sul, Amx, Amp, Spt	1	
Sul, Amx, Amp, Tet, Pflx	13	<i>dfrA12-orfF-aadA2</i> (3), <i>dfrA1-aadA1</i> (2)
Sul, Amx, Amp, Oflox, Pflx	1	
Sul, Amx, Amp, Spt, Str	1	
Sul, Amx, Amp, Tet, Pflx, Str	8	<i>dfrA7</i> (1), <i>dfrA1-aadA1</i> (3)
Sul, Amx, Amp, Oflox, Pflx, Spt	1	
Sul, Amx, Amp, Tet, Pflx, Str, Oflox	7	<i>dfrA1-aadA1</i> (1), <i>dfrA7</i> (5)
Sul, Amx, Amp, Oflox, Pflx, Spt, Str	5	
Sul, Amx, Amp, Tet, Pflx, Str, Oflox, Spt	10	<i>aadA1</i> (1), <i>dfrA12-orfF-aadA2</i> (1), <i>dfrA1-aadA1</i> (2)

a, Abbreviation for antimicrobial agents: Amp, Ampicillin; Amx, Amoxicillin; Oflox, Ofloxacin; Pflx, Pefloxacin; Spt, Spectinomycin; Str, Streptomycin; Sul, Sulfonamide; Tet, Tetracycline.

Table IV. Correlation of antibiotic susceptibility with expression of *acrAB-TolC* in *E. coli*.

	No (%)	Mean MIC (S.D.), µg/ml							
		AMP	AMX	OFLX	PFLX	STR	SPT	SUL	TET
AcrAB-TolC +	48(77%)	117.3(31.7)	217.5(81.4)	19.3(23.7)	176.8(214.3)	49.3(75.9)	75.7(123.7)	502.6(64.6)	106.7(109.2)
AcrAB-TolC _	14	100.5(45.0)	210.3(77.1)	5.6 (5.3)	94.7 (141.6)	40.7(66.5)	27.0(66.8)	403.7(68.41)	68.1(51.7)
P value		0.198	0.762	0.0004*	0.098	0.680	0.058	0.000010*	0.070

AMP, ampicillin; AMX, amoxicillin; OFLX, ofloxacin; PFLX, pefloxacin; STR, streptomycin; SPT, spectinomycin; TET, tetracycline; SUL, sulfonamide. P values were obtained by Student's t-test; * $P < 0.01$

Table V. Correlation of antibiotic susceptibility with over expression of *acrAB* and *TolC*.

		Mean MIC (S.D.), µg/ml							
	No	AMP	AMX	OFLX	PFLX	STR	SPT	SUL	TET
Over expression of <i>acrAB</i>									
acrAB +	52	118.2(30.6)	220.5(78.9)	24 (49.2)	179.3(211.9)	48.2(73.5)	75.4(122.5)	503.4(62.1)	104.7(106.1)
acrAB _	10	89.6 (49.6)	192 (85.3)	3.1 (2.8)	49.4 (83.5)	43.3 (77.2)	9.5 (12.7)	484.4 (81)	63.2 (50.3)
<i>P</i> value		0.083	0.33	0.003*	0.001*	0.128	0.0003*	0.230	0.006*
Over expression of <i>TolC</i>									
TolC gene +	50	117.8 (31.1)	219 (80.1)	18.9(23.5)	173 (211.1)	48.6 (74.4)	72.8 (122)	503 (63.4)	105 (107.3)
TolC gene _	12	96 (47.3)	202.7 (81.1)	5.7 (5.6)	97.2 (152.4)	42.1 (72.1)	31 (71.8)	469.3(99.6)	68.7 (56.2)
<i>P</i> value		0.126	0.533	0.0007*	0.160	0.781	0.126	0.271	0.108

AMP, ampicillin; AMX, amoxicillin; OFLX, ofloxacin; PFLX, pefloxacin; STR, streptomycin; SPT, spectinomycin; TET, tetracycline; SUL, sulfonamide. *P* values were obtained by Student's *t*-test; **P* < 0.01

DISCUSSION

E. coli is a leading cause of nosocomial infections. Eradication of the *E. coli* is difficult due to the multiple antibiotic resistance. Resistance mechanism is very complex. Multidrug resistance is involved in drug specific resistance, (acquired resistance) integron, target based mutation and over expression of efflux pumps (intrinsic resistance). It is useful to study the relationships between different mechanisms of resistance to a particular antibiotic that can coexist in the same bacterial cell. For contribution and diversity of integrons and efflux pump, we investigated the drug specific, integron-mediated and efflux-based resistance to the multiple antibiotics resistant *E. coli*.

All isolates were not clonally related to each other it may be due to the collection of strains from different area or there may be not a common source of infection (wang *et al.*, 2008).

In our results drug specific resistance by β -lactamase gene was in accordance to the previous report (Henriques *et al.*, 2006). Twenty-eight isolates had no β -lactamase gene, which might be due to lack of transcriptional activator in these isolate or might be other than these four β -lactamase were present (Bass *et al.*, 1999).

Genotypic resistance for tetracycline was agreed to the previous report, in which 71% tetracycline resistant genes were found in Enterobacteriaceae (Kobashi *et al.*, 2007). Tetracycline results approximately agreed to previous research in which 71 and 25% isolates were contained *tetA* and *tetB* genes and 5.4% were contained both genes (Sengelov *et al.*, 2003). Our results showed that tetracycline efflux genes contribute more than the ribosomal protection genes for tetracycline resistance.

Isolates were resistant to streptomycin and spectinomycin but susceptible to gentamycin, but no

isolate was positive for this 16S rRNA methylase genes, which was strongly supported by previous report in which none of the 16S rRNA methylase genes was detected in the strains susceptible to gentamycin (Wu *et al.*, 2009). Contribution of *Sul* genes for sulfonamide resistance was closely similar to the previous work in which they showed 86% in Enterobacteriaceae (Frank *et al.*, 2007).

The positive-integron incidence rate was 37.09%, out of these 33.87% were class 1 integrons and 3.22% were class 2 integrons, while class 3 integrons were not found, which was similar to that report which indicated that the positive-integron incidence rate was 40% and class 1 and class 2 were 37 and 3.3%, respectively and dissimilar to that report in which mentioned, integron incidence rates in *E. coli* isolates from chicken were 63 and 82%, which was higher than our study (Bass *et al.*, 1999). It has been indicated that the prevalence of integrons is related to the antimicrobial pressure in environment (Rosser and Yound, 1999). The investigation of resistance gene cassettes in this study revealed aminoglycosides resistance determinants (*aadA1*, *aadA2* and *aadA22*), trimethoprim resistance determinants (*dfrA1*, *dfrA7* and *dfrA12*) and unknown protein determinants (*orfF*) were prevalent among *E. coli* strains isolated from poultry. This might be due to the facts that aminoglycosides and trimethoprim were often widely used in the past years (Wang *et al.*, 2008). However, the integrons examined in this study did not account for the total resistance phenotype observed among the *E. coli* strains isolated from poultry. This was possibly attributed to the presence of other mobile genetic elements or might be other than these resistance genes (Bass *et al.*, 1999). In this study one isolate has both types of integrons (class 1 and class 2) and *dfrA1*–*aadA1* (38.09%) gene cassettes of class 1 integrons were more prevalent which was agreed to previous report that *dfrA1*–*aadA1* cassettes were found most frequently in *E. coli* isolates from Europe (Henriques

et al., 2006). These data seemed to suggest that the contribution of integrons might play a role in the acquired resistance mechanism (Wang *et al.*, 2008).

In the present study, real-time PCR was used to quantify the contribution of *acrAB-TolC* efflux pump in clinically isolated strains from two different countries. Efflux pump of (rinder nodulation division) RND family are now recognized as major players in the (multidrug resistance) MDR of many Gram-negative bacteria. The *acrAB-TolC* efflux pump is also the member of RND family. In this study, expression of *acrAB-TolC* efflux pump was found 77% in clinical isolate. We observed in this work a significant correlation between efflux pump expression and ofloxacin, pefloxacin, spectinomycin, tetracycline and sulfonamide resistance, it showed that the efflux pump plays a role in resistance of *E. coli* to ofloxacin, pefloxacin, spectinomycin, tetracycline and sulfonamide. These result were strongly supported by previous reports, in which they mentioned that the over expression of RND efflux pump genes in a constructed multidrug resistant strain induced resistance to several antibiotics including sulfamethaxazole and flouroquinolones (Chang *et al.*, 2004; Nikaido *et al.*, 2009). Here we did not observe a significant correlation between the expression of *acrAB-TolC* pump and ampicillin, amoxicillin and streptomycin phenotypic susceptibility. This finding may imply that exposure to the respective drugs during therapy may not significantly exert selective pressure leading to the expression of the pump observed in these isolates (Kumar *et al.*, 2008).

Pakistan and China are neighbour countries of Asia. Both countries have good relationship for the trading of veterinary products. Emergence of multidrug resistance in *E. coli* is increasing in both countries; it may be due to worldwide effect or due to the same boundaries of both countries. Integrons and efflux pump *acrAB-TolC* are different resistance mechanisms that play an important role in multidrug resistance of *E. coli*. Both have important role but *acrAB-TolC* efflux pump have the major contribution in multidrug resistance. Diversity of efflux pump and integrons showed the difference of resistance mechanism for multidrug resistance between two countries. According to these two resistance mechanisms, Pakistani isolates were found more resistant than China. With the best of our knowledge this is the first report for the contribution and diversity of integrons and *acrAB-TolC* efflux pump in avian *E. coli* isolated from Pakistan and China. This study is helpful to distinguish between acquired and intrinsic forms of resistance, and to explore the molecular mechanisms responsible for the spread of resistance among avian *E. coli*.

ACKNOWLEDGEMENT

This study was supported by Higher Education Commission of Pakistan in the form of fellowship through Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan

Funding

Higher Education of Pakistan (HEC, Pakistan) funded the study.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

- Ahmed, A.M., Younis, E.E.A., Osman, S.A., Ishida, Y., El-Khodery, S.A., and Shimamoto, T., 2008. Genetic analysis of antimicrobial resistance in *Escherichia coli* isolated from diarrhoeal neonatal calves. *J. Vet. Microbiol.*, **136**: 397-402. <https://doi.org/10.1016/j.vetmic.2008.11.021>
- Ammor, M.S., Florez, A.B., van Hoek, A.H.A.M., de los Reyes-Gavilan, C.G., Aarts, H.J.M., Margolles, A., and Mayo, B., 2008. Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and *Bifidobacteria*. *J. mol. Microbiol. Biotechnol.*, **14**: 6-15. <https://doi.org/10.1159/000106077>
- Anadon, A., Arzo, M.A., and Bories, G., 2005. Opinion of the FEEDAP Panel on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. *Euro. Fd. Safe. Authority J.*, **223**: 1-12. <https://doi.org/10.2903/j.efsa.2005.223>
- Bass, L., Liebert, C.A., Lee, M.D., Summers, A.O., White, D.G., Thayer, S.G., and Maurer, J.J., 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob. Agents Chemother.*, **43**: 2925-2929. <https://doi.org/10.1128/AAC.43.12.2925>
- Blazquez, J., Oliver, A., and Gomez-Gomez, J.M., 2002. Mutation and evolution of antibiotic resistance antibiotics as promoters of antibiotic resistance. *Curr. Drug. Targets*, **3**: 345-349. <https://doi.org/10.2174/1389450023347579>
- Bonnet, R., 2004. Growing group of extended-spectrum β -lactamases: The CTX-M enzymes. *Antimicrob. Agents Chemother.*, **48**: 1-14. <https://doi.org/10.1128/AAC.48.1.1-14.2004>
- Celine, L., Lyson, P., Chantal, L., and Roy, P.H., 1995.

- PCR mapping of integrons reveals several novel combination of resistance genes. *Antimicrob. Agents Chemother.*, **39**: 185-191. <https://doi.org/10.1128/AAC.39.1.185>
- Chang, L.L., Chen, H.F., Chang, C.Y., Lee, T.M., and Wu, W.J., 2004. Contribution of integrons, and SmeABC and SmeDEF efflux pumps to multidrug resistance in clinical isolates of *Stenotrophomonas maltophilia*. *J. Antimicrob. Chemother.*, **53**: 518–521. <https://doi.org/10.1093/jac/dkh094>
- Chen, L., Chen, Z.L., Liu, J.H., Zeng, Z.L., Ma, J.Y., and Jiang, H.X., 2007. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. *J. Antimicrob. Chemother.*, **59**: 880–885. <https://doi.org/10.1093/jac/dkm065>
- Clinical and Laboratory Standards Institute, 2008. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, approved standard M31-A3*. 3rd edition, Wayne, PA, USA: Clinical and Laboratory Standards Institute.
- Frank, T., Gautier, V., Talarmin, A., Bercion, R., and Arlet, G., 2007. Characterization of sulphonamide resistance genes and class I integron gene cassettes in Enterobacteriaceae, Central African Republic (CAR). *J. Antimicrob. Chemother.*, **59**: 742–745. <https://doi.org/10.1093/jac/dkl538>
- Gebreyes, W.A., and Thakur, S., 2005. Multidrug-resistant *Salmonella enterica* serovar Muenchen from pigs and humans and potential inter serovars transfer of antimicrobial resistance. *Antimicrob. Agents Chemother.*, **49**: 503–511. <https://doi.org/10.1128/AAC.49.2.503-511.2005>
- Gibreel, A., Wetsch, N.M., and Taylor, D.E., 2007. Contribution of the CmeABC efflux pump to macrolide and tetracycline resistance in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.*, **51**: 3212–3216. <https://doi.org/10.1128/AAC.01592-06>
- Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A.O., White, D.G., and Maurer, J.J., 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob. Agents Chemother.*, **45**: 723–736. <https://doi.org/10.1128/AAC.45.3.723-726.2001>
- Henriques, I.S., Fonseca, F., Alves, A., Saavedra, M.J., and Correia, A., 2006. Occurrence and diversity of integrons and β -lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.*, **157**: 938–947. <https://doi.org/10.1016/j.resmic.2006.09.003>
- Kilic, A., Muz, A., Ertas, H.B., and Ozbey, G., 2009. Random amplified polymorphic DNA (RAPD) analysis of *Escherichia coli* isolated from chickens. *FUS Bil. Vet. Derg.*, **23**: 1–4.
- Kobashi, Y., Hasebe, A., Nishio, M., and Uchiyama, H., 2007. Diversity of tetracycline resistance genes in bacteria isolated from various agricultural environment. *Microb. Environ.*, **22**: 44–51. <https://doi.org/10.1264/jsme2.22.44>
- Kumar, A., Mayo, M., Trunck, L.A., Cheng, A.C., Currie, B.J., and Schweizer, H.P., 2008. Expression of resistance-nodulation-cell-division efflux pumps in commonly used *Burkholderia pseudomallei* strains and clinical isolates from northern Australia. *Trans. R. Soc. Trop. Med. Hyg.*, **102**: 45–51. [https://doi.org/10.1016/S0035-9203\(08\)70032-4](https://doi.org/10.1016/S0035-9203(08)70032-4)
- Lane, D.J., 1991. 16S/23S rRNA sequencing. In: *Nucleic acid techniques in bacterial systematics* (eds. E. Stackebrandt, and M. Goodfellow). JW&S Publishers., New York, pp. 115–175.
- Masi, M., Pages, J.M., and Pradel, E., 2003. Over expression and purification of the three components of the *Enterobacter aerogenes* AcrA–AcrB–TolC multidrug efflux pump. *J. Chromatogr. B*, **786**: 197–205. [https://doi.org/10.1016/S1570-0232\(02\)00746-8](https://doi.org/10.1016/S1570-0232(02)00746-8)
- Nawaz, M., Khan, A.A., Khan, S., Sung, K., Kerdahi, K., and Steele, R., 2009. Molecular characterization of tetracycline-resistant genes and integrons from avirulent strains of *Escherichia coli* isolated from catfish. *Foodb. Pathog. Dis.*, **6**: 553–559. <https://doi.org/10.1089/fpd.2008.0204>
- Neu, H.C., 1992. The crisis in antibiotic resistance. *Science*, **257**: 1064–1073. <https://doi.org/10.1126/science.257.5073.1064>
- Ng, L.K., Martin, I., Alfa, M., and Mulvey, M., 2001. Multiplex PCR for the detection of tetracycline resistant genes. *Mol. Cell. Prob.*, **15**: 209–215. <https://doi.org/10.1006/mcpr.2001.0363>
- Nikaido, H., and Takatsuka, Y., 2009. Mechanisms of RND multidrug efflux pumps. *Biochim. biophys. Acta*, **1794**: 769–781. <https://doi.org/10.1016/j.bbapap.2008.10.004>
- Nishino, K., Nikaido, N.E., and Yamaguchi, A., 2009. Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*. *Biochim. biophys. Acta*, **1794**: 834–843. <https://doi.org/10.1016/j.bbapap.2009.02.002>
- Oliver, K.L., Hamelin, R.C., and Hintz, W.E., 2008. Effects of transgenic hybrid aspen overexpressing

- polyphenol oxidase on rhizosphere diversity. *Appl. environ. Microbiol.*, **74**: 5340–5348. <https://doi.org/10.1128/AEM.02836-07>
- Rosser, S.J., and Yound, H.K., 1999. Identification and characterization of class I integrons in bacteria from an aquatic environment. *J. Antimicrob. Chemother.*, **44**: 11–15. <https://doi.org/10.1093/jac/44.1.11>
- Sander, P., Rossi, E.D., Boddingtonhaus, B., Cantoni, R., Branzoni, M., Bottger, E.C., Takiff, H., Rodriguez, R., Lopez, G., and Riccardi, G., 2000. Contribution of the multidrug efflux pump LfrA to innate mycobacterial drug resistance. *FEMS Microbiol. Lett.*, **193**: 19–23. <https://doi.org/10.1111/j.1574-6968.2000.tb09396.x>
- Sengelov, G., Halling-Sroensen, B., and Aarestrup, F.M., 2003. Susceptibility of *Escherichia coli* and *Enterococcus faecium* isolated from pigs and broiler chickens to tetracycline degradation products and distribution of tetracycline resistance determinants in *E. coli* from food animals. *Vet. Microbiol.*, **95**: 91–101. [https://doi.org/10.1016/S0378-1135\(03\)00123-8](https://doi.org/10.1016/S0378-1135(03)00123-8)
- Wang, A., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., Ding, H., Deng, Q., Zhang, H., Wang, C., Liu, L., Xu, X., Wang, L., and Shen, X., 2008. Presence of qnr gene in *Escherichia coli* and *Klebsiella pneumoniae* resistant to ciprofloxacin isolated from pediatric patients in China. *BMC Infect. Dis.*, **8**: 68. <https://doi.org/10.1186/1471-2334-8-68>
- Wang, G.Q., Wu, C.M., Du, X.D., Shen, Z.Q., Song, L.H., Chen, X., and Shen, J.Z., 2008. Characterization of integrons-mediated antimicrobial resistance among *Escherichia coli* strains isolated from bovine mastitis. *Vet. Microbiol.*, **127**: 73–78. <https://doi.org/10.1016/j.vetmic.2007.08.003>
- Warsa, U.C., Nonoyama, M., Ida, T., Okamoto, R., Okubo, T., Shimauchi, C., Kuga, A., and Inoue, M., 1996. Detection of tet(K) and tet(M) in *Staphylococcus aureus* of asian countries by polymerase chain reaction. *J. Antibiot.*, **49**: 1127–1132. <https://doi.org/10.7164/antibiotics.49.1127>
- Weill, F.X., Demartin, M., Tande, D., Espie, E., Rakotoarivony, E., and Grimont, P.A.D., 2004. SHV-12 like extended spectrum β -lactamase producing strains of *Salmonella enterica* serotypes Babelsberg and Enteritidis isolated in France among infants adopted from Mali. *J. clin. Microbiol.*, **42**: 2432–2437. <https://doi.org/10.1128/JCM.42.6.2432-2437.2004>
- White, P.A., MacIver, C.J., and Rawlinson, W.D., 2001. Integrons and gene cassettes in the Enterobacteriaceae. *Antimicrob. Agents Chemother.*, **45**: 2658–2661. <https://doi.org/10.1128/AAC.45.9.2658-2661.2001>
- Wu, Q., Zhang, Y., Han, L., Sun, J., and Ni, Y., 2009. Plasmid-mediated 16S rRNA methylases in aminoglycoside resistant *Enterobacteriaceae* isolates in Shanghai, China. *Antimicrob. Agents Chemother.*, **53**: 271–272. <https://doi.org/10.1128/AAC.00748-08>
- Aarestrup, F.M., and Wegener, H.C., 1999. The effects of antibiotic usage in food animals on the development of antimicrobial resistance of importance for humans in *Campylobacter* and *Escherichia coli*. *Microb. Infect.*, **1**: 639–644. [https://doi.org/10.1016/S1286-4579\(99\)80064-1](https://doi.org/10.1016/S1286-4579(99)80064-1)