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

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Key words
Multidrug resistance, Integrons, Efflux pump, Real time PCR, Avian *E. coli*

ABSTRACT

The contribution and diversity of integrons 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%). Integrons were detected in 37.09% of clinical isolates and carried cassettes conferring resistance mainly to aminoglycosides and trimethoprim. Class 2 integrons 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 integrons 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.

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) integrons 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, integrons 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

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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 50µl consisting of 5µl template DNA, 25µl PCR Mix (Best Bio, China), 19µl H_2O and 1µl COL-1 primer (3-AAGAGC CCGT-5) (Kilic *et al*., 2009). The samples were amplified through 45 cycles of 94 ºC for 30s, 36 ºC for 15s and 72 ºC 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 *Sul1* 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 25µl reaction mixtures (TaKaRa Bio, China) according to the manufacturer's instructions. PCR products (6µl) 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 intI1 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 contaning 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
16srDNAF	AACGCGAAGAACCTTAC	433bp	Oliver *et al*., 2008
16srDNA R	CGGTGTGTACAAGACCC		
TEM-F	ATAAAATTCTTGAAGACGAAA	1080bp	Weill *et al*., 2004
TEM-R	GACAGTTACCAATGCTTAATC		
OXA-F	TCAACTTTCAAGATCGCA	591bp	Ahmed *et al*., 2008
OXA-R	GTGTGTTTAGAATGGTGA		
SHV-F	TTATCTCCCTGTTAGCCACC	795bp	Weill *et al*., 2004
SHV-R	GATTTGCTGATTTCGCTCGG		
CTX-M-F	CGCTTTGCGATGTGCAG	550bp	Bonnet *et al*., 2000
CTX-.M-R	ACCGCGATATCGTTGGT		
Sul1-F	CTTCGATGAGAGCCGGCGGC	435bp	Gebreyes *et al*., 2005
Sul1-R	GCAAGGCGGAAACCCGCGCC		
Int1-F1	CCTCCCGCACGATGATC	280bp	Bass *et al*., 1999
Int1-R1	TCCACGCATCGTCAGGC		
Int2-F2	TTATTGCTGGGATTAGGC	233bp	Goldstein *et al*., 2001
Int2-R2	ACGGCTACCCTCTGTTATC		
Int3-F3	AGTGGGTGGCGAATGAGTG	600bp	Goldstein *et al*., 2001
Int3-F3	TGTTCTTGTATCGGCAGGTG		
5-CS	GGCATCCAAGCAGCAAG	Variable	Ahmed *et al*., 2008
3-CS	AAGCAGACTTGACCTGA		
Hep-51	CGGGATCCCGGACGGCATGCACGATTTGTA	Variable	White *et al*., 2001
Hep-74	GATGCCATCGCAAGTACGAG		
rmtA F	AGCTTTGACGATGCCCTAGC	716bp	Chen *et al*., 2007
rmtA R	CCAATGGTCTTGGTATCCTC		
rmtB F	ACATCAACGATGCCCTCAC	725bp	Chen *et al*., 2007
rmtB R	AAGTTCTGTTCCGATGGTC		
armA F	CAATCAGGGGCAGTTATCA	529bp	Chen *et al*., 2007
armA R	CCCTATAACCTTCGAATC		
tetM F	GTGGACAAAGGTACAACGAG	406bp	Warsa *et al*., 1996
tetM R	CGGTAAAGTTCGTCACACAC		
tetO F	AACTTAGGCATTCTGGCTCAC	515bp	Ng *et al*., 2001
tetO R	TCCCACTGTTCCATATCGTCA		
tetA F	GTAATTCTGAGCACTGTCGC	957bp	Nawaz *et al*., 2009
tetA R	CTGCCTGGACAACATTGCTT		
tetB F	CTCAGTATTCCAAGCCTTTG	436bp	Nawaz *et al*., 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).

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

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' ACCTTTCGCACTGTCGTATGTC 3'	
acrB-RTF	5' GGTACTGGTAGCGTTGATCCTG 3'	This study
acrB-RTR	5' GTGTAGTGGTGCGTGCTCTTCT 3'	
TolC-RTF	5' AAGCACGCCTTAGTAACCCG 3'	This study
TolC-RTR	5' GCGTTAGAGTTGATGCCGTTC 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.

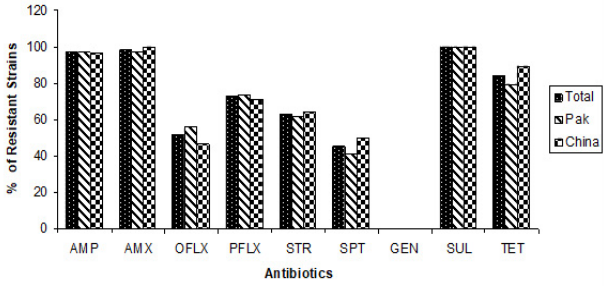

Fig. 1. Contribution and diversity of phenotypic resistance in *E. coli* strains isolated from Pakistan and China. AMP, Ampicillin; AMX, Amoxicillin; GEN, Gentamycin; OFLX, Ofloxacin; PFLOX, Pefloxacin; SPT, Spectinomycin; STR, Streptomycin; SUL, Sulfonamide; TET, Tetracycline.

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 $bla_{CTX\text{-}M}$ 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 *Sul1* gene.

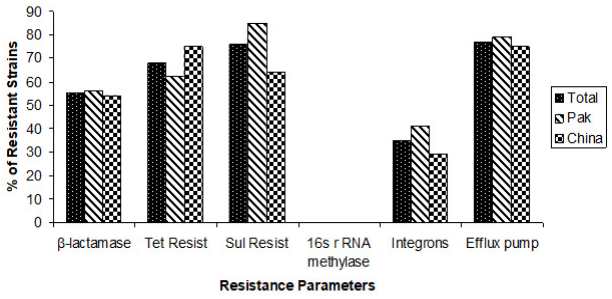

Fig. 2. Contribution and diversity of genotypic resistance in *E. coli* strains isolated from Pakistan and China.

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 class1 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 1 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	*dfrA1-sat1-aadA1*(2), *dfrA7(1), aadA22*(1)
Sul, Amx, Amp, Spt	1	
Sul, Amx, Amp, Tet, Pflx	13	*dfrA12-orfF-aadA2*(3), *dfrA1-aadA1*(2)
Sul, Amx, Amp, Oflx, Pflx	1	
Sul, Amx, Amp, Spt, Str	1	
Sul, Amx, Amp, Tet, Pflx, Str	8	*dfrA7*(1), *dfrA1-aadA1*(3)
Sul, Amx, Amp, Oflx, Pflx, Spt	1	
Sul, Amx, Amp, Tet, Pflx, Str, Oflx	7	*dfrA1-aadA1*(1), *dfrA7*(5)
Sul, Amx, Amp, Oflx, Pflx, Spt, Str	5	
Sul, Amx, Amp, Tet, Pflx, Str, Oflx, Spt	10	*aadA1*(1), *dfrA12-orfF-aadA2*(1), *dfrA1-aadA1*(2)

a, Abbreviation for antimicrobial agents: Amp, Ampicillin; Amx, Amoxicillin; Oflx, Ofloxacin; Pflox, Pefloxacin; Spt, Spectinomicin; Str, Streptomycin; Sul, Sulfonamide; Tet, Tetracycline.

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

		Mean MIC (S.D.), µg/ml							
	No (%)	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*.

	No	Mean MIC (S.D.), µg/ml							
		AMP	AMX	OFLX	PFLX	STR	SPT	SUL	TET
Over expression of *acrAB*									
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)
P value		0.083	0.33	0.003*	0.001*	0.128	0.0003*	0.230	0.006*
Over expression of *TolC*									
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)
P 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 this16S 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 class1 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 (class1 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 clinicaly 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 flouroqunolones (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*.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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