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Detection of Plasmid Mediated Qnr Genes in Clinical Isolates of ESBL Producing *Enterobacteriaceae* from Tertiary Care Hospital in Rawalpindi, Pakistan

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Abstract

ESBL producing *Enterobacteriacae* harbouring multidrug-resistant plasmids is an emerging worldwide threat. One of the major concerns is the prevalence of plasmid-mediated quinolone resistance (PMQR) to Quinolones which has hindered the viability of these antibiotics. To date, very little data is available regarding the co-existence of ESBL and PMQR in Pakistan. This study aimed to detect PMQR genes in clinical isolates of ESBL producing *Enterobacteriaceae* in the city of Rawalpindi. A total of 60 clinical ESBL positive *Enterobacteriaceae* isolates were obtained from hospitalized patients at Holy family Hospital, Rawalpindi. It included strains of *E. coli* (n=24), *K. pneumoniae* (n=25), and *Enterobacter* spp (n=11). Antimicrobial susceptibility was determined by the standard disk diffusion method. Genes encoding quinolone resistance (qnrA, qnrB and qnrS) were detected using PCR. Out of 60 tested strains, 38 (63%) yielded PMQR determinants. QnrB was the most prevalent occurring alone and in combination with other qnr genes in about 89% (34/38) of the ESBL producing isolates. It was followed by qnrS (22/38 i.e 58%) and qnrA (12/38 i.e 32%). *E.coli* was the predominant qnrA (43%) and qnrS (69%) harbouring species.

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Introduction

Amid the most recent five decades, the use and abuse of antimicrobials in both human and veterinary medicine has led to the emergence of bacterial strains that are irresponsive to antimicrobial therapy. Among the most commonly used antibiotics for the treatment of bacterial infections are quinolones and beta-lactams and their wide usage has triggered increased bacterial resistance (Ambrozic Avgustin *et al.*, 2007). One of the major contributing factors for such resistance is the incidence of plasmid-mediated resistance genes (Briales *et al.* 2012; Alikhani *et al.* 2013).

Associated with the poor prognosis of the bacterial infection are the coexistence of extended-spectrum beta-lactamase (ESBL) and plasmid-mediated quinolone resistance (PMQR) in bacterial species (Hooper, 1999; Ewers *et al.*, 2012; Okubo *et al.*, 2014). PMQR resistance comprises the production of qnr proteins protecting the targets against the effects of quinolones (Martinez-Martinez *et al.*, 1998).

These genes encode pentapeptide repeat proteins that block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV (Tran and Jacoby, 2002; Tran *et al.*, 2005a; Tran *et al.*, 2005b). Three important qnr determinants belonging to this pentapeptide repeat family of proteins are namely qnrA, qnrB and qnrS (Martinez-Martinez *et al.*, 1998; Kim *et al.*, 2009).

Enterobacteriaceae harbouring resistance plasmids have been responsible for numerous outbreaks of infection throughout the world. Multidrug resistance in *Enterobacteriaceae* is a serious threat to community health as it limits the selection of antibiotics for the empirical treatment of infections caused by this family. There are few studies regarding co-resistance of β-lactamas and quinolones in *Enterbacteriaecae* isolated from clinical specimens in Pakistan. This study aimed to detect PMQR in ESBL producing *Enterobacteriaceae* isolated from various clinical specimens from a tertiary care hospital in Rawalpindi.

Materials and methods

Sample collection

By Consecutive Sampling technique, 60 ESBL positive *Enterobacteriaceae* isolates were picked up from different samples (Urine culture, swab culture, endotracheal tube tip culture) from Holy Family Hospital, Rawalpindi. It included strains of *E. coli* (n=24) *K. pneumoniae* (n=25*)* and *Enterobacters* spp (n=12). Inclusion criteria for samples were Gram –ve rods, enterobacterial species, and clear patron of ESBL in presence of clavulanic acid. Samples showing mixed growths were excluded.

ESBL detection

Antimicrobial susceptibility testing and interpretation for all isolates were conducted on Mueller Hinton agar (HiMedia, Mumbai, India) by the standard disk diffusion method per CLSI guidelines using disks of standard concentration (CLSI, 2016). The antibiotics tested were (concentrations in µg) as follows: ceftazidime (30), cefotaxime (30), cefepime (30), cefoxitin (30),

All isolates showing reduced susceptibility to ceftazidime (zone diameter of ≤ 22 mm and/or MIC 2 mg/L) and cefotaxime (zone diameter of ≤ 27 mm and/or MIC 2 mg/L) were selected for ESBL production. Isolates were tested for ESBL by both the standard CLSI double-disk diffusion method and Etests using ceftazidime/ceftazidime-clavulanate, cefotaxime/cefotaxime-clavulanate and cefepime/cefepime-clavulanate gradients. The tests were quality controlled using standard strains *E. coli* ATCC 25922 (ESBL negative), *Pseudomonas aeruginosa* ATCC 27853 (ESBL negative) and *K. pneumoniae* 700603 (ESBL positive).

Plasmid isolation and PCR

The bacterial plasmid was isolated by the procedure adopted by Takahashi *et al* (Takahashi and Nagano 1984). In an optimal PCR reaction master mix of 10X Taq buffer (Fermentas), 25Mm $MgCl_2$ (Fermentas), 2mM dNTPs (Fermentas), 10 pmol of each forward and reverse primer, 40U of Taq polymerase (Fermentas) and NF water to raise the volume up to

20ul was used. The primers utilized are listed in Table 1. The optimized PCR profile for qnrA, qnrS, and qnrB included: initial denaturation at 95°C (5minutes), followed by 35 cycles of denaturation at 94°C (40sec), annealing at 62°C (30 sec), extension at 72°C (30 sec) and final extension at 72°C for 7 minutes. Then PCR products were run on 2% agarose gel in 1X TAE buffer.

Results

To obtain an overview of multidrug resistance in *Enterobacteriaceae* from Rawalpindi region, clinical isolates were obtained from hospitalized patients at Holy family Hospital, Rawalpindi. A total of 60 ESBL producing *Enterobacteriaceae* isolates were screened for PMQR genes; qnrA, qnrB and qnrS.

The qnrA, qnrB and qnrS gene were detected in the majority of ESBL producing *Enterobacteriaceae* isolates. Overall 63% (38/60) of the samples harboured at least one qnr genes (Table 2). Eight samples (mostly *E.coli*) showed the presence of all three qnr genes (Table 3). Qnr genes were found in isolates from all age groups (neonates to the elderly) and various specimens and were not associated with a particular clinical diagnosis.

Table 1. Primer sequences for Qnr detection.

S.No	Primer code	Sequence (5′---3)	Product size (bp)	Reference
1	QnrA-F	ATTTCTCACGCCAGGATTTG	516	(Kim *et al.* 2009)
	QnrA-R	GATCGGCAAAGGTTAGGTCA		
2	QnrB-F	GATCGTGAAAGCCAGAAAGG	469	(Kim *et al.* 2009)
	QnrB-R	ACGATGCCTGGTAGTTGTCC		
3	QnrS-F	ACGACATTCGTCAACTGCAA	417	(Jacoby *et al.* 2009)
	QnrS-R	TAAATTGGCACCCTGTAGGC		

F: Forward; R: Reverse.

Among the 3 types of resistance genes that were detected, qnrB was the most prevalent, occurring alone and in combination with other qnr genes in about 89% (34/38) of the ESBL producing isolates. QnrB was followed by qnrS (22/38 i.e 58%) and qnrA (12/38 i.e 32%). The predominant qnrA and qnrS, harbouring species was *E.coli* with the positive occurrence of the gene in 43% and 69% of the qnr positive samples. For qnrB, a high rate of detection was observed in both *E.coli* (81%) and *K. pneumoniae* (80%) (Table 4 and Fig. 1).

Table 2. Percentage prevalence of Qnr genes in *Enterobacteriaceae* isolates.

S.No	Qnr Genes (QnrA, QnrB, QnrS)	*Enterobacteriaceae* (n=60)
1	Positive	38 (63%)
2	Negative	22 (37%)

Discussion

Multidrug-resistant (MDR) bacteria are a major health concern in South-Asian countries. In Pakistan, unfortunately, the lack of proper surveillance and documentation of MDR pathogens has led to an increase in the incidence of MDR associated diseases. In the past few decades an alarming increase in the coexistence of extended-spectrum β-lactamase (ESBL) and plasmid-mediated quinolone resistance (PMQR) has been observed in *Enterobacteriaceae* with serious consequences for treatment outcomes (Slama, 2008). PMQR genes are often on the same plasmid as the ESBL genes (Garcia-Fulgueiras *et al.*, 2011) and the resistance plasmids can be transferred by conjugation among different *Enterobacteriaceae* species (Rodriguez-Martinez *et al.*, 2011). The infections caused by these MDR isolates are associated with high public health

costs, therapeutic failures, restriction of the antibacterial agents choice, increased duration of hospitalization, rising morbidity, and mortality. Various studies have reported a high prevalence of ESBL producing fluoroquinolone-resistant strains in Asia (Dalhoff, 2012) however little is known about the frequency of their coexistence in Pakistan. To the best of our knowledge, this is the first study reporting the co-prevalence of ESBL and PMQR from Rawalpindi.

Table 3. Frequency distribution of PMQR genes alone and in combination with other genes in different *Enterobacteriaceae* isolates.

S.No	Gene	Qnr Positive Isolates (n=38)			Total no. of species
		E. coli (n=16)	*K. pneumoniae* (n=15)	*Enterobacter* (n=7)	
1.	qnrA+qnrB+qnrS	6	2	0	8 (21%)
2.	qnrA+qnrB	0	1	1	2 (5%)
3.	qnrA+qnrS	0	0	0	0 (0%)
4.	qnrB+qnrS	3	6	3	12 (32%)
5.	qnrA only	1	1	0	2 (5%)
6.	qnrB only	4	5	3	12 (32%)
7.	qnrS only	2	0	0	2 (5%)

We found a high prevalence (38/60 i.e. 63%) of PMQR in ESBLs producing *Enterobacteriaceae* with *E. coli* isolates (16/38 i.e. 42%) accounting for the majority of it. Previous studies suggested that the quinolone resistance rates in uropathogenic E. coli (UPEC) were high (84.2% antimicrobial resistance against nalidixic acid) because quinolones are the first choice of urinary tract infection (Muhammad *et al.*, 2011). We observed the prevalence of the qnr genes with varying frequency among the isolates.

The presence of qnrA gene was confirmed in 32% of the *Enterobacteriacae* isolates which is similar to the rate of occurrence for qnrA in some neighbouring countries (Pakzad *et al.*, 2011; Tripathi *et al.*, 2012; Harifi Mood *et al.*, 2015; Izadi *et al.*, 2017). However, the prevalence of qnrB and qnrS was observed to be much higher as compared to other studies from India, Iran and China (Wang *et al.*, 2008; Pakzad *et al.*, 2011; Tripathi *et al.*, 2012; Azargun *et al.*, 2018; Izadi *et al.*, 2017). In comparison to qnrA and qnrS, QnrB (as the sole determinant) was detected in a majority (32%) of the isolates. Interestingly, a large number of isolates harboured the qnrS gene, contrary to some studies reporting its complete absence (Pakzad *et al.*, 2011; Tripathi *et al.*, 2012).

Table 4. Total Prevalence of Qnr genes in *Enterobacteriaceae* isolates.

S.No	Gene	No of Qnr Positive Isolates			Total (Percentage)
		E. coli (n=16)	*K. pneumoniae* (n=15)	*Enterobacter* (n=7)	
1.	qnrA	7	4	1	12 (32%)
2.	qnrB	13	14	7	34 (89%)
3.	qnrS	11	8	3	22 (58%)

The findings of the present study confirm the coexistence of ESBL and PMQR in *Enterobacteriaceae* in the Rawalpindi region. The findings of the present study highlight the emergence of multidrug resistance in clinically relevant species of *Enterobacteriacae*.

The ESBL-producing *Enterobacteriacae* harbouring multidrug-resistant plasmids are of great concern due to the severely limited therapeutic options and increased risk of treatment failure in patients infected with such strains.

It seems that excessive and widespread administration of these antimicrobial agents is the most important predisposing factor that ultimately leads to the appearance of resistant bacteria in hospital settings.

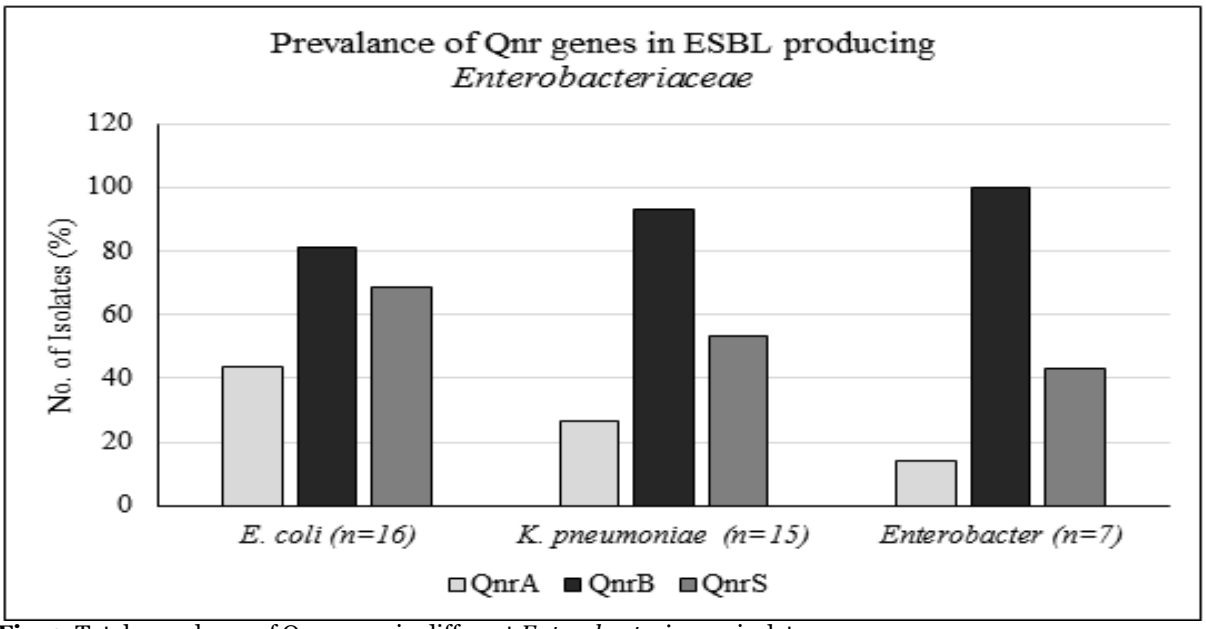

Fig. 1. Total prevalence of Qnr genes in different *Enterobacteriaceae* isolates.

Moreover, the resistance rate found in this study emphasizes the need for the formulation of local and national antimicrobial resistance surveillance policies and systems in hospital and healthcare settings.

Conclusion

Quinolones are commonly used empirically as a treatment of choice for gram-negative bacterial infections but emerging plasmid-mediated resistance is going to further complicate the treatment options for such diseases. The high prevalence of qnr genes in our study samples indicate the rising of multi-drug resistance in Pakistan and future complications of treatment strategies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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