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Original Article

Plasmid-mediated quinolone resistance in expanded spectrum beta lactamase producing *enterobacteriaceae* in Morocco

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Abstract

Introduction: Although independently acquired, plasmid-mediated quinolone resistance appears to be linked with extended-spectrum or AmpC-type beta-lactamases. Since no data are available in African countries, the prevalence of *qnr* genes at the University Hospital Ibn Rochd, Casablanca, Morocco, was investigated.

Methodology: Between October 2006 and March 2007, the following 39 randomly selected non-duplicate *Enterobacteriaceae* producing an extended-spectrum beta-lactamase (ESBL), representing 20% of ESBL strains with respect to species and ward origin, were collected: *Escherichia coli* (n = 16); *Klebsiella spp* (n = 14); *Enterobacter cloacae* (n = 8); *Proteus mirabilis* (n = 1). Antibiotic susceptibility testing was performed according to CLSI guidelines. ESBL detection was performed by the double disc diffusion test. A multiplex PCR was conducted to detect *qnrA*, *qnrB* and *qnrS* genes that were confirmed by sequencing of the PCR product.

Results: The estimated overall prevalence of *qnr* reached 36% (n = 14; *qnrA*, 10.25%; *qnrB*, 23.07%; *qnrS*, 2.56%). Genes were identified in *E. coli*, *Klebsiella* and *Enterobacter* with a respective prevalence of 18.7%, 50% and 62.5%. The *qnr* genes were detected in nine wards and *qnrA1*, *qnrB1-B2-B4* and *qnrS1* variants were identified. Three genes were identified among nalidixic acid susceptible strains (n = 6); three of those were also susceptible to ciprofloxacin. Among nalidixic acid and ciprofloxacin resistant strains, all strains had *qnrB*.

Conclusions: This study highlights the high prevalence of *qnr* genes among ESBL strains in the Ibn Rochd CHU, Casablanca. Moreover, *qnr* were present in quinolone-susceptible strains which could lead to *in vivo* selection of ciprofloxacin-resistant strains.

Key words: *Enterobacteriaceae*, qnr, ESBL, Morocco

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Introduction

Quinolones are a class of molecules that are used extensively in the treatment of many infections [1]. Their availability and use have increased in recent years, especially in developing countries.

For more than 30 years, the only known mechanisms of resistance to quinolones were chromosome borne.

The two main mechanisms known to account for quinolone resistance are alteration of drug permeation (*i.e.*, decreased uptake mediated by mutations in the structural or regulating gene of porins [2], active efflux mediated by mutations in active expulsion pumps [2] and target alteration (*i.e.*, mutation in the quinolone-resistance determining regions (QRDRs) of *gyrA*-*gyrB* or *parC*-*parE* encoding topoisomerase II [3]. Recently, plasmid-mediated resistance mechanisms have been described. The first plasmid-mediated resistance to quinolones was discovered in 1998 [4], in a clinical isolate of *Klebsiella pneumoniae* that could transfer low-level resistance to quinolone to *Escherichia coli* or other Gram-negative bacteria. The plasmid-mediated quinolone resistance gene was named "*qnr*". This gene encoded a 218 amino-acid protein Qnr (later named QnrA), belonging to the pentapeptide-repeat family. More recently, four other markers (QnrB and QnrS, QnrC and QnrD) have been identified in several enterobacterial species [5-8]. These markers interact with quinolones, the topoisomerases, and DNA, thus limiting the binding of the quinolones to their target.

By itself the *qnr* gene confers resistance to quinolones at a low level. The clinical importance is linked to its ability to allow the selection of

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chromosomal mutations of quinolones at concentrations that could have been lethal in the absence of this gene.

The main distinction of *qnr* genes is carried on several integrons [9]. These determinants can be easily transferred, accelerating the spread of quinolone resistance through gene transfer mechanisms. In addition, the described integrons can carry genes which encode for resistance to third-generation cephalosporins (ESBL or ESC or derepressed cephalosporinase).

In 2005, a second plasmid-borne mechanism, which independently contributes to quinolone resistance by modification of the antibiotic molecule, was described [10]. This protein, AAC (6')-Ib-cr, is a variant of the 6'acetyl transferase, which is known to modify the chemical structure of aminoglycosides, and presents an enlarged enzymatic spectrum toward ciprofloxacin and norfloxacin [10].

A third mechanism of plasmid mediated quinolone resistance has recently been described in *E. coli* isolates from Japan. This work identified the gene *qepA* which encodes the protein QepA and acts as a quinolone efflux pump [11]. Levels of resistance to fluroquinolones were found to be significantly elevated in *E. coli* transformants harbouring *qepA*

Plasmid-mediated quinolone resistance has been subsequently isolated in various parts of the world [12-14]. In most cases, strains also had an expanded spectrum beta-lactamase.

The present study aimed to determine the prevalence of *qnr* genes in a collection of expanded spectrum beta-lactamase producing *Enterobacteriaceae* isolates recovered at the Ibn Rochd hospital of Casablanca and characterize the ESBL genes in *qnr* positives strains.

Methodology

Bacterial strains

Thirty-nine unrelated ESBL-producing strains were randomly selected from 188 *Enterobacteriaceae* isolated between October 2006 and March 2007 at the University Hospital of Casablanca. Each isolate was from a single patient: *16 E. coli* (14 isolated from urine, one from pus, and one from bronchial sampling); 14 *K. pneumoniae* (eight isolated from urine, three from blood culture, and three from pus); 8 *Enterobacter cloacae* (three from urine, three from blood culture, one from bronchial sampling, and one from pus) and one *Proteus mirabilis* (isolated from urine). All species were identified both by conventional techniques [15] and by using the API 20E Gallery (BioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility

Susceptibility to antimicrobials was determined and interpreted by disc diffusion and agar dilution methods following CLSI recommendations [16]. ESBL production was determined by a synergetic test between amoxicillin/clavulanic acid and at least one of the following antibiotics: cefotaxime, ceftazidime, aztreonam and cefepime. *Escherichia coli* ATCC 25922 was used as quality controls for antimicrobial susceptibility and ESBL screening tests [16].

The MICs were performed by the E-test method (AES, AB Biodisk, Solna,Sweden).

Beta-lactamase typing

PCR detection *of* bla_{SHV}, bla_{TEM}, *bla* genes and their subtypes $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, bla_{CMY-1}, bla_{CMY-2} and bla_{DHA-1} was performed with primers and methods described previously [17].

Screening for qnr gene and DNA sequencing

The strains were screened for the presence of the *qnr* gene by multiplex PCR. The reaction mixture contained DNA (1 µL), 2,5 mM $MgCl_2$, 100 µM dNTP, 20 pmol of each primer, 1x PCR buffer, and 3U of Taq DNA polymerase (Eurobio, Courtaboeuf, France) in a total volume of 100 µl.

The primers used are listed in Table 1. The amplification reaction consisted of 30 cycles of one minute of denaturation at 94°C, 45 seconds of hybridization at 60ºC and one minute of extension at 72°C, with a final extension cycle of 7 minutes at 72°C. Sequencing was performed using the BigDye terminator cycle sequencing kit and ABI Prism 3130 DNA Analyzer (both from Applied Biosystems, Courtabboeuf, France).

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Table 1. Primers used

Genes	Primers[a]	Sequence	Position[b]	Size of PCR Product (bp)	Accession n°
qnrA1 to 5	*qnrA*(+) *qnrA*(-)	TTCTCACGCCAGGATTTGAG TGCCAGGCACAGATCTTGAC	339-358 910-891	571	AY070235
qnrB 1 to 9	*qnrB*(+) *qnrB*(-)	TGGCGAAAAAATT(GA)ACAGAA GAGCAACGA(TC)GCCTGGTAG	54-73 648-630	594	DQ351241
qnrS 1 to 2	*qnrS*(+) *qnrS*(-)	GACGTGCTAACTTGCGTGAT AACACCTCGACTTAAGTCTGA	101-120 489-469	388	DQ485529

a (+), sense primer; (-): antisense primer
b Nucleotide numbering begins at the initiation codon of *qnr*

Table 2. Characteristics of the 14 qnr positive strains: qnr variant, susceptibility testing and ß-lactamase types

Strain no.	Isolation date day/mo/year	*qnr* Variant	Nalidixic acid		Ciprofloxacin		Cefepime		Cefotaxime		Ceftazidime		Cefoxitine		ß.lactamases types			
			MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC	bla_{CTX}	Bla_{SHV}	Bla_{TEM}	Bla_{DHA}
E.coli																		
54	18/10/2006	B4	512	R	64	R	1	S	4	S	32	I	512	R	negative	SHV12	TEM1	DHA1
35	07/112006	S1	8	S	4	R	32	R	512	R	256	R	8	S	CTXM15	negative	TEM1	negative
62	19/03/2007	A1	4	S	0.5	S	32	R	128	R	64	R	16	I	CTXM28	negative	TEM1	negative
E.cloacae																		
52	10/10/2006	B4	4	S	0.5	S	2	S	16	I	128	R	512	R	negative	SHV12	TEM1	DHA1
50	16/10/2006	A1	32	R	4	R	32	R	512	R	32	I	512	R	CTXM28	negative	TEM1	negative
31	07/02/2007	B4	16	I	1	I	1	S	4	S	64	R	512	R	negative	SHV12	TEM1	DHA1
46	24/02/2007	B4	4	S	0.13	S	1	S	4	S	32	I	512	R	negative	SHV12	TEM1	DHA1
64	19/03/2007	B4	512	R	64	R	4	S	8	I	512	R	512	R	negative	SHV12	TEM1	DHA1
K.pneumoniae																		
39	22/11/2006	A1	8	S	1	I	16	I	128	R	16	I	4	S	CTXM28	negative	negative	negative
23	21/12/2006	A1	8	S	1	I	16	I	512	R	32	I	4	S	CTXM15	SHV1	TEM1	negative
28	02/01/2007	B1	16	I	8	R	32	R	512	R	64	R	8	S	CTXM15	negative	TEM1	negative
26	08/01/2007	B4	512	R	64	R	4	S	4	S	512	R	512	R	negative	SHV1	TEM1	DHA1
25	26/01/2007	B4	512	R	64	R	32	R	512	R	512	R	16	I	CTXM28	SHV1	TEM1	DHA1
60	20/03/2007	B2	16	I	2	R	32	R	128	R	128	R	8	S	CTXM28	negative	negative	negative

Results

Among the 39 isolates studied (*E. coli* (n = 16), *K. pneumoniae* (n = 14), *E. Cloacae* (n = 8) and one *P. Mirabilis*), 14 (~36%) were positive for *qnr* (*qnrA* 10%; *qnrB* 23% and *qnrS* 3%).

The prevalence of *qnr* determinant was highest among *E. cloacae* (62.5%) followed by *K. pneumoniae* (50%) and *E. coli* (18.7%). The *qnr* genes were detected in nine wards of the Ibn Rochd CHU, Casablanca. *The qnrA* gene was detected in 14.2%of *K. pneumoniae* isolates (n = 2), 6.2% of *E. coli* (n = 1) and 12.5% of *E. Cloacae* (n = 1). The *qnrB* gene was detected in 28.5% of *K. pneumoniae* isolates (n = 4), 7.1% of *E. coli* and 50% of *E. cloacae*. The *qnrS* gene was found in one *E. coli* isolate (6.2 %). None of the isolates had more than one *qnr* gene (Table 2).

The *qnr* genes were sequenced in all positive isolates studied. All four sequenced *qnrA* genes were of the *qnrA1* allele. Most *qnrB* genes (n = 7/9) were the *qnrB4* variant. Two other alleles, *qnrB1* and *qnrB2*, were found in two strains of *K. pneumoniae* and one variant *S1* was identified in one isolate of *E. coli* (Table 2).

Of the 14 *qnr*-positive isolates, 57% (n = 8) and 78% (n = 11) were resistant (or intermediately

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resistant) to nalidixic-acid and ciprofloxacin respectively; 100% (n = 14) were resistant to ceftazidime; 71% (n = 10) were resistant to cefotaxime; 64% (n = 9) were resistant to cefoxitin; and 57% (n = 8) were resistant to cefepime. All the *qnr* positive strains were resistant to gentamicin and to aztreonam, and 78% and 100% were susceptible to amikacin and imipenem respectively.

The *qnr* genes (qnrA and B) were identified among ciprofloxacin-susceptible strains (n = 3), and these isolates were also susceptible to nalidixic-acid. The majority of isolates that were resistant to ciprofloxacin and nalidixic acid encoded *qnrB* (4/5 isolates).

Within the isolates encoding *qnr*, the *CTX-M-1* group was the major ESBL type, as it was detected in almost 80% of the ESBL-producing isolates (n=?). The $bla_{CTX\text{-}M}$ gene was detected in eight of 14 *qnr*-positive isolates, most of which carried the $bla_{CTX\text{-}M\text{-}1}$ group genes, with *CTX-M-28* (n=5) and *CTX-M-15 (n=3)* being the most prevalent variants.

Moreover, *TEM-1* type ß lactamase was detected in 12 *qnr*-positive isolates (85.7%) and *SHV-12* or *SHV-1* type ß lactamase was detected in eight *qnr*-positive isolates (57,1%) (Table 2).

Examination of the ESBL types associated with *qnr* subtypes showed that *qnrA1* type was most frequently associated with *CTXM-1*. On the other hand, *qnrB4* was associated with *SHV* (50%) and with *DHA1* (50%).

Discussion

Plasmid-mediated quinolone resistance may facilitate the spread and the increase of the prevalence of quinolone-resistant strains. Until now *qnr* genes have been widely detected in different parts of the world but not in Morocco. The present study reports the prevalence and diversity of *qnr* genes among ESBL-producing *Enterobacteriaceae* in Morocco, with the highest prevalence found among *E. Cloacae.*

Three *qnr* groups were detected and are described in this report. Among all the isolates detected, *qnrB* was the most prevalent, followed by *qnrA* and *qnrS*.

This study also shows that there is considerable genetic diversity within the identified *qnrB* gene; at least three variants (*qnr B1, B2* and *B4*) circulate among *Enterobacteriaceae* isolated at Ibn Rochd hospital. On the other hand, all *qnrA* identified were *qnrA1*.

Previous studies showed that *qnr*-positive strains frequently expressed ESBLs and/or ESC [6,11]. Among our *qnr*-positive strains, eight produce *CTXM 28* or 15, three produce *SHV12*, and seven produce *DHA1* (Table 2). The coexistence of *SHV 12* and *DHA1* was observed in five strains suggesting they could be carried by the same conjugative plasmid since the same association was observed in the transconjugants made from these strains (data not shown).

Five strains showed discordant clinical categorisation for quinolones (Nalidixic-acid S or I; ciprofloxacin I or R), suggesting the presence of another mechanism of resistance to ciprofloxacin. This phenotype could not be attributed to a specific chromosomal mutation to ciprofloxacin since the same phenotype was observed in the transconjugants made from these strains. However, this discordance (Nal.S Cip.R) could be attributed to the association of the *qnr* gene and aminoglycoside modifying enzyme AAC(6')1b-cr as recently described by Wang *et al*. [7]. Indeed, PCR with primers targeting this resistance determinant showed positive results in both strains and their transconjugants (data not shown).

The *qnr* genes were identified among isolates which were susceptible to all quinolones tested. This result has clinical implications since acquisition of the *qnr* genes by quinolone susceptible, ESBL-producing strains could lead to *in vivo* selection of ciprofloxacin and cephalosporin resistant strains.

The potential limitation of our study was the inclusion of only the ESBL strains; however, a recent study reports the presence of *qnr* genes in non ESBL *Enterobacteriaceae* [18]. The level of quinolone resistance among ESBL-producing strains in this study may be under-estimated due to the presence of currently unidentified *qnr* genes which cannot be characterised by the current PCR scheme. This work describes the prevalence of *qnr* genes among ESBL strains of *enterobacteriaceae* in the Ibn Rochd CHU, Casablanca and identifies the presence of *qnr* genes in quinolone-susceptible strains which could lead to *in vivo* selection of ciprofloxacin-resistant strains.

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