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Plasmid Mediated Quinolones Resistance ESBL-*Enterobactériaceae* in Moroccan

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Abstract

Multidrug Resistance (MDR) in *Enterobacteriaceae* including resistance to quinolones is rising worldwide. This study was conducted to assess the resistance level to antibiotics and to detect plasmid genes mediated quinolones resistance in Extended-Spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* collected from regional hospitals of Fes–Meknes in central Morocco. ESBL phenotype was determined according to the combination disc method recommended by the Clinical and Laboratory Standards Institute (CLSI) using double disc synergy test (DDST). The antimicrobial susceptibility patterns of isolates showed high resistance rate to most antibiotics except imipenem which showed 100% of susceptibility.

A sub-site of 27 isolates was screened for *qnr* genes by multiplex PCR. *qnrB* gene was detected in 8 ESBL isolates (*2 E. coli, 4 K. pneumoniae, 01 E. aerogenes* and *01 C. freundii*) while no *qnrA* neither *qnrS* could be detected. *aac(60)-Ib-cr* gene was detected in 15 strains, 13 of them were ESBL.

Our results are in agreement with the general rule that imipenem stays the drug of choice for the treatment of infections caused by ESBL producers. Moreover, the presence of *qnr* determinants is closely related to ESBL phenotype while *aac(60)-Ib-cr* gene could be detected in isolates with or without ESBL phenotype.

Keywords: *Enterobacteriaceae*; ESBL; Qquinolone; Plasmid genes

Introduction

Enterobacteriaceae represent the major class of Gram Negative Bacteria (GNB) responsible for the majority of infectious diseases. They acquired mechanisms of resistance to various families of antibiotics [1].

Although quinolone resistance in *Enterobacteriaceae* results mostly from chromosomal mutations, it may also be mediated by plasmid-encoded *Qnr* genes [2]. *Qnr* proteins have been identified worldwide with a frequent association with clavulanate inhibited expanded-spectrum β-lactamases and plasmid-mediated cephalosporinases. *Qnr* proteins protect DNA from the inhibitory activity of quinolones such as nalidixic acid.

The first transferable plasmid-encoded quinolone resistance gene (*qnrA*, *qnrB* and *qnrS*) was isolated from a clinical isolate of ciprofloxacin- resistant Klebsiella pneumonia in 1998 [3].

The isolated *qnrA, qnrB* and *qnrS* gene products protect DNA gyrase from inhibition by ciprofloxacin [4].

QnrB determinants are associated with the ESBL SHV-12 inch several isolates which may explain in part the frequent association between fluoroquinolone and expanded-spectrum cephalosporin resistance in *Enterobacteriaceae* [4,5].

Two other plasmid-mediated quinolone resistance mechanisms have been described. The *aac (60)-Ib-cr* determinant, a variant of aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, is widely prevalent and seems to be associated to *qnr* genes [6-9].

The other is the quinolone efflux pump gene, *qepA*, and is mediated by a probable transposable element flanked by two copies of IS26 [10,11].

The emergence of plasmid-mediated quinolone resistance determinants in *Enterobacteriaceae* may compromise further the efficacy of quinolones that are, together with β-lactams and aminosides, the most commonly prescribed antibiotics for treating human infections.

The aim of this study is to evaluate the efficiency of divers antibiotic classes in multidrug resistance and also to assess an eventual correlation between the presence of *qnr* and *aac(60)-Ib-cr* genes and the ESBL-production from *Enterobacteriaceae* in Morocco.

Material and Methods

Bacterial isolates

We examined 148 *Entrerobacteriaceae* (*E. coli* = 96; *Klebseilla spp.* = 40; others = 12) collected from hospital bacteriological laboratories in Fes-Maknes region during 2010 (from 1th July to 30th September). They were isolated from urinary samples (n=97), surgical wound sepsis (n=24), blood cultures (n=15), genital tract infections (n=05), catheter (04), endotracheal tube-associated pneumonias (n=03).

Identification of species was carried out by API 20E (Bio-Mérieux SA, France).

ESBL detection methods

ESBL screening and confirmation were performed according to the

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Clinical and Laboratory Standards Institute (CLSI, 2008) [12] criteria. All isolates were tested by double disc synergy test (DDST) described by Jarlier [13].

ESBL producer was detected by using a disc containing ceftazidime (CAZ, 30 µg) or cefotaxime (CTX, 30 µg) in combination with and without clavulanic acid (CLA, 10 µg). The presence of an ESBL was determined by a 05 mm increase in zone diameters for CAZ/CLA and CTX/CLA compared with those for CAZ and CTX, respectively (Figure 1).

Antibiotic susceptibility

Susceptibility and synergy-testing for ESBLs were simultaneously performed on Mueller- Hinton agar. The following antibiotic disks (Bio- Rad) were tested: amoxicillin (AMX), amoxicillin-clavulanic acid (AMC), cefalotin (CF), cefotaxime (CTX), ceftazidin (CAZ), cefuroxime (CRO), imipenem (IPM), Tobramycin (TB), ofloxacin (OFX), ciprofloxacin (CIP), norfloxacin (NOR), corticomoxasol (SXT), colistin (CL).

The reference strains were *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.

PCR Amplification and Molecular Detection of *qnr* and *aac(60)-Ib-cr* genes

A subset of 27 non-duplicates isolates *(16 E. coli, 09 Kleb. spp, 01 E. aerogenes* and *01 C. freundii*) was subjected to molecular analysis. The characteristics of those isolates are listed in Table 2.

DNA preparation was performed by a boiling technique including a heating step at 100C of a single colony in a total volume of 100 µL of distilled water followed by centrifugation of the cell suspension.

A multiplex PCR was used to detect simultaneously *qnrA*, *qnrB* and *qnrS* genes [14,15].

The amplified DNA products were examined as described previously; the expected sizes for *qnrA*, *qnrB* and *qnrS* were 580, 264 and 428 bp respectively.

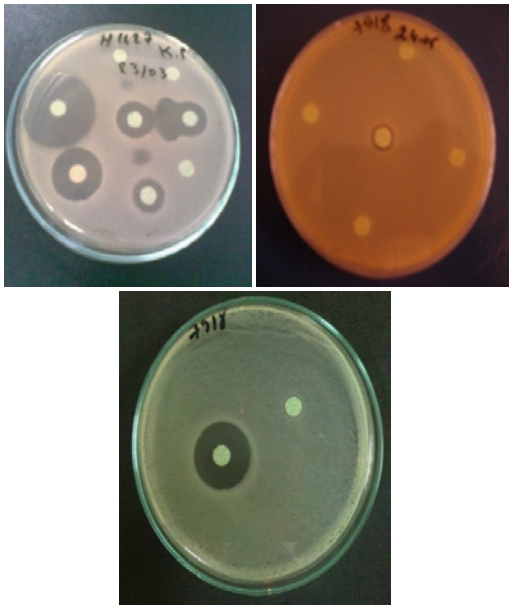

Figure 1: Appearance of ESBL-producing isolates. (a): *Klebsiella* pneumonia with clear zone synergy between amoxicillin.

A pair of degenerated primers was specifically choused to amplify variants of *qnrB* and another pair of primers was used to detect the *aac(60)-Ib-cr* in selected stains. The expected size was 473 bp (result none shown).

DNA amplification was carried out by using 2 µl of the extract in 500 mM, 2mM KCl, 10mM Tris HCl, 1.5 mM MgCl , containing 500 µM of each dNTP, 0.2 µM of each primer, 0.5U of Taq DNA polymerase (Roche Diagnostics, France) in a final volume of 25 µl.

PCR products were analyzed by electrophoresis in 1.2 % agarose gel in TBE buffer staining with ethidium bromide and visualized with UV light.

Result and Discussion

Phenotypic detection and antimicrobial susceptibility patterns

The antimicrobial susceptibility patterns of isolates were examined. *Enterobacteriaceae* showed high resistance rate to β-lactam in general with values surrounding 80% for amoxicillin, 60% for clavulanic acid, 40% for cephalosporin of first generation (C1G) and 30% for cephalosporin of third generation (C3G). 56% of isolates were resistant to sulfamethoxazole/ trimethoprim (SXT). Resistance to fluoroquinolones showed a rate of about 40%, whereas for the tested aminoglycosides (tobramycin) is only 14%. Colistin stills an efficient antibiotic against *Enterobactériaceae* with 96% of susceptibility while all examined isolates were susceptible to imipenem (Table 1).

The high ratio of co-resistance to quinolone, trimethoprim/ sulfamethoxazole and aminoglycosides among ESBL producers collected from this region has greatly limited the therapeutic role of those classes of antibiotics. However, our results are in agreement with the general rule that imipenem stays the drug of choice for the treatment of infections caused by ESBL producers.

Clavulanic acid and 3 surrounding cephalosporins CAZ, CTX and ATM;

ESBL-producer *E.coli* detected by DDST using CTX in combination without (b1) and with (b2) CLA showing increased zone inhibition diameter for CTX/CLA.

ESBL test revealed that 13 of 27 isolates were ESBL- producers (5 *E. coli*, 6 *K pneumonia*, *1 K. oxytoka* and *1 E. aerogenes*). 11 isolates presented resistance simultaneously to β-lactam, quinolones and aminosides (Table 2). This may be explaining by the association of antibiotic resistance genes between fluoroquinolone and expanded-spectrum cephalosporin in *Enterobacteriaceae*.

Prevalence of *qnr* and *aac(60)-Ib-cr* genes

Screening of *qnr* genes resulted in 8 positives cases all of them carried *qnrB-like* gene with the expected size (*2 E. coli, 4 K. pneumoniae, 01 E. aerogenes* and *01 C. freundii*), while no *qnrA* neither *qnrS* could be detected (result non shown. The rate of *qnr* carriage among *K. pneumonia* was higher than in *E. coli.* To date, *qnr* genes have been widely detected worldwide.

The 27 analyzed isolates showed the presence of *aac(60)-Ib-cr*

Antibiotic	*E.coli* n =96 (%)	*Klebseilla spp* n=40 (%)	Other n=12 (%)	Total N=148 (%)
AMX	67 (69,79)	40 (100)	10 (83,33)	117 (79,05)
AMC	51 (53,12)	30 (75)	10 (83,33)	91 (61,48)
CF	30 (31,25)	22 (55)	8 (66,66)	60 (40)
CTX	24 (25)	17 (42,5)	5 (41,66)	46 (31,08)
CAZ	24 (25)	18 (45)	5 (41,66)	47 (31,75)
CRO	24 (25)	18 (45)	5 (41,66)	47 (31,75)
IPM	0	0	0	0
TM	17 (17,7)	4 (10)	0	21 (14,18)
OFX	40 (41,66)	17 (42,5)	5 (41,66)	62 (41,89)
CIP	40 (41,66)	17 (42,5)	5 (41,66)	62 (41,89)
NOR	40 (41,66)	15 (37,5)	5 (41,66)	60 (40)
SXT	55 (57,29)	24 (60)	4 (33,33)	83 (56,08)
CL	4 (4,16)	0	2 (16,66)	6 (4,04)

Table 1: Antibiotic resistance profile of the bacterial isolates.

Isolate	Species	BLSE : 13	*qnr* : 8	*Aac* : 15	Resistance profile
E1	E .C	—	—	—	AMX, AMC, CF,CTX, CRO, CAZ, OFX,CIP,NOR, NET, SXT]
E2	E.C	+	—	+	AMX,AMC,CF,CTX,CRO,CAZ,OFX,CIP,NOR,TM,NET,SXT]NA=I
E3	E.C	+	—	+	AMX,AMC,CF,CRO,CAZ,CTX,OFX,CIP,NOR,NA,NET,AK,SXT
E4	E. aero	+	+	+	[AMX,AMC,CF, CRO,CAZ,CTX, OFX ,**CIP**,NOR,NET,SXT,CL]I=NA
E5	E.C	+	+	+	AMX, AMC,CF, CTX ,CRO,CAZ,OFX, **CIP**, NOR,SXT,NET,CL
E6	K.P	+	+	+	AMC, AMX, CF, CTX, CRO, CAZ**, CIP**,NOR, OFX, NET, SXT
E7	K.P	+	+	+	AMX, AMC, CF, CTX, CRO, CAZ, CIP, OFX, NOR,NET, SXT
E8	K .P	+	—	+	AMX, AMC ,CF, CRO, CTX, **CIP**, OFX,NOR,, NET,TOB, SXT
E9	E.C	+	—	+	AMX, AMC, CF, CTX, CRO, CAZ, **CIP**, NOR, OFX, NET, TOB, SXT
E10	E.C	—	—	—	AMX,AMC, CF, CAZ, NOR, OFX, CIP,
E11	K.P	+	+	+	AMX,AMC,CF,CTX,CAZ,CRO,**CIP**,OFX,NOR,TOB,NET,SXT] NA=I
E12	K.P	+	+	+	AMX, AMC,CF, CTX, CRO, CAZ**, CIP**,OFX, NOR, TOB,NET, SXT
E13	K.O	+	—	+	AMX, AMC, CF, CTX, CRO, CAZ, CIP/ NOR/ OFX, NET, TOB, SXT
E14	E.C	—	—	—	AMX, AMC, CIP,NOR, OFX, SXT
E15	E.C	—	—	—	AMX, AMC, CIP, OFX NOR, SXT,
E16	CITRO	—	+	—	AMX, AMC, **CIP**, NA, SXT
E17	K.O	—	—	—	AMX, AMC, CF, CRO, CAZ, TOB, SXT
E18	E.C	—	—	—	AMX,AMC ,NOR,OFX,CIP,,SXT, CL
E19	E.C	—	—	+	AMX, AMC, **CIP**,OFX, NOR, SXT
E20	E.C	—	—	—	AMX, AMC, CIP, OFX
E21	K.O	—	—	—	AMX, OFX, CIP, NOR, NET, SXT
E22	K .P	+	—	+	AMX, AMC, CF, CTX, CRO, CAZ, TOB
E23	E.C	—	—	+	AMX, AMC,CF,NA,NOR,OFX,CIP, SXT
E24	E.C	—	—	—	AMX, AMC, CF, CIP, OFX,NOR TOB, SXT
E25	E.C	—	—	—	AMX, CIP,NOR, OFX, SXT
E26	E.C	—	—	—	AMX, CF, CTX, CRO, CAZ, SXT
E27	E.C	+	+	+	AMX,AMC,CF,CRO,CAZ, CTX, NOR, OFX,**CIP**, NET, TOB, SXT, CL
E28	E.C	—	—	—	TEMOIN SENSIBLE

Table 2: Resistance profile for 27 analyzed isolates.

variant gene with the expected size in 15 strains (result non shown), 13 of them were ESBL. All *qnr* strains showed ESBL phenotype and possessed *aac (60)-Ib-cr* determinant except a *C. freundii* which carried only *QnrB* gene and showed relatively a lower resistance profile (Table 2). The rate of *aac (60)-Ib-cr* carriage was comparable between *E. coli* and *klebseilla*.

Plasmid-mediated genes, such as *qnr* and *aac(60)-Ib-cr*, may facilitate spread and increase the prevalence of quinolone-resistant strains.

The *aac (60)-Ib-cr* gene was detected in non-ESBL-producing *E. coli* isolates which were C3G and aminoglycosides susceptible.

Conclusion

Total results obtained for this work is summarized in Table 2 with phenotypic and genotypic patterns. Further study is required to precise *aac (60)-Ib-cr* functions and relationship with divers' classes of antibiotics resistance. Furthermore, plasmid's carriage of these genes (*qnr* and *aac(60)-Ib-cr*) is assessed by conjugation test while the identification of divers variants is running by sequencing.

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