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Determinants of quinolone resistance in *Escherichia coli* causing community-acquired urinary tract infection in Bejaia, Algeria

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ABSTRACT

Objective: To investigate the mechanisms of quinolone resistance and the association with other resistance markers among *Escherichia coli* (*E. coli*) strains isolated from outpatient with urinary tract infection in north of Algeria. **Methods:** A total of 30 nalidixic acid-resistant *E. coli* isolates from outpatient with urinary tract infections from January 2010 to April 2011 in north of Algeria (Bejaia) were studied. Antimicrobial susceptibility was determined by disc diffusion assay, minimal inhibitory concentrations (MIC) of quinolone were determined by microdilution. Mutations in the Quinolone Resistance-Determining Region (QRDR) of *gyr*A and *par*C genes and screening for qnr (A, B and S) and bla genes were done by PCR and DNA sequencing. **Results:** Most of the *E. coli* isolates (56.66%) were shown to carry mutations in *gyr*A and *par*C (*gyr*A: Ser83Leu + Asp87Asn and *par*C:Ser80Ile). While, 16.66% had only an alteration in *gyr*A: Ser83Leu. One isolate produced *qnr*B-like and two qnrS-like. Four isolates were CTX-M-15 producers associated with TEM-1 producing in one case. Co-expression of $bla_{CTX-M-15}$ and *qnr*B was determined in one *E. coli* isolate. **Conclusions:** Our findings suggested the community emergence of *gyr*A and *par*C alterations and Qnr determinants that contributed to the development and spread of fluoroquinolone resistance in Algerian *E. coli* isolates.

1. Introduction

Escherichia coli (*E. coli*) is a major cause of urinary tract infections (UTI). Quinolones are commonly used to treat UTI due to *E. coli*[1]. These synthetic antimicrobial agents include nalidixic acid and ciprofloxacin which is a fluoroquinolone (FQ) with a wide spectrum of antibacterial activity in vitro, particularly against gram negative bacteria[2]. The extensive use of FQ has led to an increasing resistance in *E. coli*[3]. Resistance to quinolone occurs as a result of chromosomic and plasmidic mechanisms. Chromosomic-mediated quinolone resistance concerns an accumulation of mutations in the Quinolone Resistance Determining Region (QRDR) primarily in DNA gyrase (*gyr*A), then in topoisomerase IV (*par*C). It can be associated with decreased outer membrane permeability and/or with an overexpression of the efflux pump systems[4]. Plasmid-mediated quinolone resistance (PMQR) was first described in 1998[5]. Since then, five major groups of *qnr* determinants (*qnr*A, *qnr*S, *qnr*B, *qnr*C and *qnr*D) have been identified[5-9]. Two additional PMQR determinants, the AAC(6')Ib-cr enzyme, which acetylates not only aminoglycosides but also ciprofloxacin and norfloxacin[10] and quinolone extrusion by QepA or OqxAB[11, 12] have been also described. The association between PMQR and multidrug resistant isolates producing Extended Spectrum Beta-Lactamas (ESBL) has been reported in urinary *Enterobacteriaceae* isolates in Algeria[13, 14] and in different countries[15].

Fluoroquinolone resistance in *E. coli* is an important issue all over the world. To date there is no publication from Algeria evaluating the genetic determinants of resistance to this class of antimicrobials in *E. coli* causing community acquired urinary tract infections (no results on PubMed). The aim of our study was to investigate the mechanisms

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of quinolone resistance and the association with other resistance markers among *E. coli* strains isolated from outpatient with UTI in north of Algeria.

2. Materials and methods

2.1. Bacterial strains

Thirty non-repetitive nalidixic acid-resistant *E. coli* isolates from community-acquired UTI in private laboratories of medical analysis in the region of Bejaia (North Algeria) from January 2010 to April 2011 were included in this study. These isolates were identified by API 20E identification system (BioMérieux, France). *E. coli* ATCC25922 was used for susceptibility testing control. The following β-lactamase-producing isolates were used as control isolates: *Enterobacter aerogenes* CF 2403 (*E. aerogenes*) for TEM, *Klebsiella pneumoniae* (*K. pneumoniae*) KpS12 for SHV; and *K. pneumoniae* Bhe CD13 for CTX-M.

Qnr-positive strains provided by Pr. J Vila and Dr. A. Fàbrega were used as positive controls: *Enterobacter cloacae* (*E. cloacae*) for *qnr*A1, *K. pneumoniae* positive for qnrB1 and *Salmonella enterica* (*S. enterica*) serovar Saintpaul positive for *qnr*S1.

2.2. Antibiotic susceptibility testing

Susceptibility to different antimicrobials was performed by disc diffusion method on Mueller-Hinton agar and was interpreted according to Clinical and Laboratory Standards Institute recommendations[16]. Tested antibiotics included norfloxacin, ofloxacin, ciprofloxacin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, cefoxitin, aztreonam, imipenem, gentamycin, kanamycin, tobramycin, amikacin, and cotrimoxazole (Oxoid Ltd., Basingstoke, UK).

The nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin and levofloxacin minimum inhibitory concentration (MIC) were determined by broth microdilution method with cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) according to Clinical and Laboratory Standards Institute recommendations[16].

2.3. Phenotypic ESBL detection

Extended-Spectrum beta-Lactamase production was detected by a double-disk synergy test (DDST) and was performed by placing disks of ceftazidime, cefotaxime and aztreonam at a distance of 20 mm (centre to centre) from a disk with amoxicillin/clavulanic acid (20/10 μg). Enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime, ceftazidime or aztreonam indicated the ESBL production[17].

2.4. Analysis of quinolone resistance-determining regions (QRDRs) of gyrA and parC genes

The QRDR of *gyr*A and *par*C were amplified using the primers showed in Table 1. PCR conditions were as follows: initial denaturation step of 5 min at 94 ℃, 1 min at 94 ℃, 1 min at the annealing temperature (54 ℃ for *gyr*A and 55 ℃ for *par*C) and 1 min at 72 ℃ for 30 cycles, final extension step was 10 min at 72 ℃[18]. Reaction mixes without a DNA template served as negative controls. Amplified fragments were purified (Qiagen kit, Hilden, Germany).

Table 1
Primers used in this study.

Gene		Primers
*gyr*A	*gyr*A1	ACGTACTAGGCAATGACTGG
	*gyr*A2	AGAAGTCGCCGTCGATAGAAC
*par*C	*par*C1	AGTATGCGATGTCTGAACTG
	*par*C2	CTCAATAGCAGCTCGGAATA
*qnr*A	*qnr*A1	ATTTCTCACGCCAGGATTTG
	*qnr*A2	GATCGGCAAAGGTTAGGTCA
*qnr*B	*qnr*B1	GATCGTGAAAGCCAGAAAGG
	*qnr*B2	ACGATGCCTGGTAGTTGTCC
*qnr*S	*qnr*S1	ACGACATTCGTCAACTGCAA
	*qnr*S2	TAAATTGGCACCCTGTAGGC
bla_{CTX-M}	$bla_{CTX-M-A2}$	CTTCCAGAATAAGGAATC
	628R	CCTTTCATCCATGTCACCA
	405F	GTGGCGATGAATAAGCTGA
	$bla_{CTX-M-B2}$	CCGTTTCCGCTATTACAA
bla_{TEM}	bla_{TEM-A}	TAAAATTCTTGAAGACG
	bla_{TEM-B}	TTACCAATGCTTAATCA

Nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The gyrA and parC nucleotide sequences and the deduced amino acids were compared with that of *E. coli* K12 using ClustalW alignment program.

2.5. Multiplex PCR detection of the qnr genes

Screening for the *qnr*A, *qnr*B and *qnr*S genes was carried out by a multiplex PCR amplification using specific primers (Table 1) according to Robicsek *et al* 2006[19].

2.6. Detection and characterization of bla genes

Isolates positive for the DDST were screened for the presence of bla_{CTX-M}, bla_{TEM} and bla_{SHV} by PCR as previously described[20]. PCR products were sequenced and the DNA alignments and the deduced amino acid sequences were examined using the BLAST program.

3. Results

3.1. Antibiotic susceptibility testing

Eighty percent (24/30) of the 30 nalidixic acid-resistant isolates showed a decrease in susceptibility to fluoroquinolones (norfloxacin, ofloxacin, ciprofloxacin and levofloxacin) (Table 2).

Among these isolates, 40% (12/30) were resistant to cotrimoxazole, 20% (6/30) were resistant to amoxicillin/clavulanic acid, 13.33% (4/30) were resistant to kanamycin, cefotaxime, ceftazidime, aztreonam and gentamycin and 6.66% (2/30) were resistant to cefoxitin and tobramycin, while, all isolates remained susceptible to imipenem and amikacin (Table 2).

3.2. Analysis of quinolone resistance-determining regions (QRDRs)

Most of the *E. coli* isolates (22/30) presented point mutations in QRDRs at the codon 83 (Ser→Leu/Val) and 87 (Asp→Asn/Tyr/Gly) in *gyr*A gene and at the codon 80 (Ser80→Ile) and/or 84 (Glu84→Val/Lys) in *par*C gene. However, No amino acid change in QRDRs of *gyr*A and *par*C was detected in one isolate (KB426) (Table2).

Table 2
MICs values (mg/L) and chromosomic and plasmid mediated resistance in *E. coli* quinolone resistant isolates.

Isolate	Mutations		NAL	NOR	OFX	CIP	LEV	QNR	ESBL	Resistance phenotype
	GyrA	ParC								
KB426	WT	WT	32	1	1	0,5	0,25	*qnr*S	–	–
KB302	Ser83Leu	WT	64	1	0.5	0,25	0,5	–	–	SXT
AB364	Ser83Leu	WT	>256	2	2	2	2	–	–	AMC-CN-TOB-SXT
MB409	Ser83Leu	WT	32	1	0,5	0,25	0,25	–	–	–
LB415	Ser83Leu	WT	256	0,5	0,5	<0,25	0,25	–	–	–
LB423	Ser83Leu	WT	64	2	1	0,25	0,25	–	–	–
DB336	Ser83Leu/Asp87Asn	WT	>256	>256	128	64	64	–	–	–
KB308	Ser83Leu/Asp87Asn	WT	>256	128	64	64	32	–	–	–
AB305	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	32	64	8	–	–	–
KB312	Ser83Leu/Asp87Asn	Ser80Ile	>256	>256	>256	>256	>256	–	–	–
KB338	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	128	64	32	–	–	SXT
DB339	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	32	16	8	–	–	SXT
MB348	Ser83Leu/Asp87Asn	Ser80Ile	>256	>256	64	128	32	–	–	–
DB370	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	128	32	64	–	–	–
KB408	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	64	32	16	–	–	–
KB412	Ser83Leu/Asp87Asn	Ser80Ile	>256	128	128	32	32	–	–	FOX-SXT
DB413	Ser83Leu/Asp87Asn	Ser80Ile	>256	128	16	16	8	–	CTX-M-15	CTX-CAZ-ATM
MB418	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	32	16	16	–	–	–
KB420	Ser83Leu/Asp87Asn	Ser80Ile	>256	128	32	16	8	–	–	K-SXT
LB437	Ser83Leu/Asp87Asn	Ser80Ile	>256	128	32	64	64	–	–	–
LB435	Ser83Leu/Asp87Asn	Ser80Ile	>256	64	16	16	8	–	–	AMC-SXT
MB446	Ser83Leu/Asp87Asn	Ser80Ile	>256	256	32	64	16	–	–	–
KB452	Ser83Leu/Asp87Asn	Ser80Ile	>256	>256	128	128	32	–	–	–
KB460	Ser83Leu/Asp87Asn	Ser80Ile	>256	>256	64	64	16	–	–	–
LB443	Ser83Leu/Asp87Asn	Ser80Ile	>256	>256	128	128	64	*qnr*B	CTX-M-15	AMC-CTX-CAZ-ATM-CN-K-TOB-SXT
DB462	Ser83Leu/Asp87Asn	Glu84Lys	>256	32	32	16	32	*qnr*S	–	AMC
AB341	Ser83Leu/Asp87Tyr	Ser80Ile	>256	256	128	64	64	–	–	AMC-FOX-K-SXT
LB403	Ser83Val/Asp87Gly	Ser80Ile	>256	32	16	2	4	–	–	K-SXT
MB337	Ser83Leu/Asp87Asn	Ser80Ile/Glu84Val	>256	>256	64	32	32	–	CTX-M-15 /TEM-1	CTX-CAZ-ATM-CN-SXT
KB402	Ser83Leu/Asp87Asn	Ser80Ile/Glu84Val	>256	256	32	64	8	–	CTX-M-15	AMC-CTX-CAZ-ATM-CN-K-SXT

NAL: nalidixic acid; NOR: norfloxacin; OFX: ofloxacin; CIP: ciprofloxacin; LEV: levofloxacin; WT: wild type; AMC: amoxicillin-clavulanic acid; CTX: cefotaxime; CAZ: ceftazidime; FOX: cefoxitin; ATM: aztreonam; CN: gentamycin; K: kanamycin; TOB: tobramycin; SXT: cotrimoxazole.

3.3. Detection of qnr genes

Among 30 non-repetitive nalidixic acid-resistant *E. coli* isolates, three (10%) were qnr-positive: including one qnrB-like positive (LB443) and two *qnr*S-like positive (KB426 and DB462) (Table 2). However, *qnr*A was not identified in any of the tested nalidixic acid resistant isolates.

3.4. Characterization of ESBLs

The disk diffusion method showed synergy between ceftazidime, cefotaxime, aztreonam, and amoxicillin clavulanic acid in four isolates (DB413, LB402, MB337 and LB443) suggesting the production of extended-spectrum beta-lactamase (ESBL). PCR and sequence analysis of the deduced amino acid showed the presence of $bla_{CTX-M-15}$ in all tested isolates associated with bla_{TEM-1} in one case.

The co-expression of $bla_{CTX-M-15}$ and *qnr*B was determined in one *E. coli* isolate (LB443) (Table 2).

4. Discussion

E. coli is a main cause of urinary tract infections in the community. The extensive use of fluoroquinolones to treat these infections has led to an increasing resistance in *E. coli*[3]. To date there is no publications from Algeria evaluating the genetic determinants of resistance to this class of antimicrobials in *E. coli* causing community acquired urinary tract infections. In this study, 30 uropathogenic *E. coli* isolates were analyzed in terms of quinolone susceptibility and chromosomic/plasmidic quinolone resistance mechanisms.

Analysis of QRDR showed that no amino acid changes was detected in gyrA or parC proteins in 1/30 isolate (KB426) for which MIC of nalidixic acid was 32 mg/L and remained susceptible to fluoroquinolones (MIC of ciprofloxacin 0.5 mg/L), whereas at least one amino acid substitution in the *gyr*A protein at the codon 83 (Ser83Leu/Val) was detected in 29/30 *E. coli* isolates. This alteration in *gyr*A alone leads to high levels of resistance to nalidixic acid (MIC ranging from 32 mg/L to 256 mg/L) and a reduced susceptibility to fluoroquinolones (MIC of ciprofloxacin $\leqslant$ 2 mg/L). An additional mutation in the Asp87 codon of *gyr*A is associated with a greater increase in fluoroquinolone resistance (MIC of ciprofloxacin 64 mg/L).

Moreover, *par*C gene is a secondary target for quinolone resistance[21]. Point mutations at codon 80 in *par*C QRDRs (Ser80 Ile) and at the position 84 (Glu84 Val/Lys) were found in 70% (21/30) and 10% (10/30) of *E. coli* isolates respectively. Previous studies also demonstrated that the most prevalent amino acid mutations were Ser83Leu/Asp87Asn in *gyr*A and Ser80Ile/Glu84Val in *par*C and contribute to MIC change in clinical and community *E. coli* isolates[22, 23]. Thus, expression of high- level fluoroquinolone resistance in *Enterobacteriaceae* requires the presence of multiple mutations in *gyr*A and/or *par*C genes.

Among the seventeen *E. coli* isolates with the same types of mutation (Ser83Leu/Asp87Asn in *gyr*A and Ser80Ile in *par*C) differences in MICs values for fluoroquinolones were observed (MICs of ciprofloxacine ranging from 16 mg/L to 256 mg/L). This can be explained by other mechanisms implicated in fluoroquinolone resistance. Changes in permeability and active efflux are mechanisms that cannot be excluded and may enhance development of resistance and contribute to the selection of fluoroquinolone-resistant isolates in the course of treatments with these antibiotics[4].

The multiplex PCR showed that the qnr genes was present in 10% (3/30) of nalidixic acid resistant uropathogenic *E. coli* isolates. Among these three isolates, one was *qnr*B-like positive and two were qnrS-like positive. The presence of *qnr*B and *qnr*S are usually reported among qnr positive isolates from different part of the world[24]. It has been shown that in Algeria, the most prevalent qnr determinant is qnrB followed by *qnr*S determinant at least in *Klebsiella pneumoniae*[15] and *Enterobacter cloacae*[25].

Previous reports have pointed out that qnr determinants confer resistance to quinolones (nalidixic acid) and reduced the susceptibility to fluoroquinolones[26]. Furthermore, it has been described that the expression of qnr genes increases the mutant prevention concentration compared to strains without this genes and thus favors selection of mutations in *gyr*A and *par*C[27]. In our case, a *qnr*S positive isolate (KB426) which carried none mutation in QRDR region of *gyr*A and *par*C showed only an increased in nalidixic acid resistance with MIC of 32 mg/L for nalidixic acid and 0.5 mg/L for fluoroquinolone. While, the *qnr*B (LB443) and the *qnr*S (DB462) positive isolates which carried two mutations in *gyr*A (Ser83Leu and Asp87Ala) and one mutation in *par*C (Ser80Ile and Glu84Lys respectively) had a high level of both quinolone and fluoroquinolone resistance (MIC of nalidixic acid of 256 mg/L and MIC of ciprofloxacin of 128 mg/L and 16 mg/L respectively). Previous works also suggested that PMQR and chromosomal resistance mechanisms are additive and can enhance the quinolone resistance of clinical isolates[28].

Some of the community-acquired quinolone-resistant *E. coli* isolates presented a multiresistance phenotype. Thus,

40% were cotrimoxazole resistant. Among of these isolates, eight (26, 66%) were also resistant to cephalosporins and/or aminoglycosides. As it was previously reported[29], the high rate of resistance to sulfonamides is related with the commonly prescription of these molecules for urinary tract infections.

Moreover, four of these multidrug resistant *E. coli* isolates were positive for ESBL production (DB413, LB443, MB337 and KB402) for CTX-M-15 type associated with TEM-1 type in one isolate (MB337). CTX-M type ESBLs have been extensively reported for the past 15 years in both community and nosocomial isolates and a strong linkage between their production and quinolone resistance has been reported in *Enterobacteriaceae*[30]. In our study, we reported in a one isolate (LB443) the co-expression of *qnr*B and $bla_{\text{CTX-M-15}}$ genes. The association between *qnr*B and CTX-M-15 and CTX-M-28 was detected in clinical Enterobacterial isolates from Algeria[13, 14]. As it was also described in France[31], Singapore[32], Sweden[33] and Morocco[34].

The plasmid mediated-quinolone resistance (PMQR) genes are usually associated with the same mobile genetic elements as those of ESBL genes. The presence of mechanisms for broad spectrum resistance on the same plasmid, highlights the clinical importance of these genes and the potential for selection and dissemination of resistance to various antimicrobials through improper quinolone use.

Our findings suggested the community emergence of *gyr*A and *par*C alterations and *qnr* determinants that contributed to the development and spread of fluoroquinolone resistance in *E. coli* isolates in our region. Taking into account that such antimicrobials are in most cases the choice for community acquired Urinary Tract Infections (UTI), such increase is a main cause of concern for doctors and health authorities. Especially noteworthy is the dissemination of the plasmid-mediated quinolone resistance (PMQR) like the Qnr determinants. The presence of these determinants in the outpatient is worrisome, because of the potential spread of plasmids in a scenario of uncontrolled oral quinolone usage. Furthermore, plasmids usually harbor other resistance genes which can compromise therapeutic options against species that were formerly truly susceptible. Further research should be done to track the evolution of these determinants in our population.

Conflict of interest statement

We declare that we have no conflict of interest.

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