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Short Communication

Analysis of plasmid-mediated quinolone resistance genes in clinical isolates of the tribe *Proteeae* from Argentina: First report of *qnrD* in the Americas

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ABSTRACT

To analyse the occurrence and prevalence of plasmid-mediated quinolone resistance (PMQR) genes in the tribe *Proteeae*, 81 isolates (65 *Proteus* spp., 12 *Morganella morganii* and 4 *Providencia stuartii*) consecutively collected in 66 hospitals belonging to the WHONET-Argentina Resistance Surveillance Network were studied. Of the 81 isolates, 50 (62%) were susceptible to quinolones [43/65 (66%) *Proteus* spp. and 7/12 (58%) *M. morganii*). The remaining 31 isolates (22 *Proteus* spp., 5 *M. morganii* and all *P. stuartii*) showed high-level resistance to nalidixic acid (NAL) and decreased susceptibility or resistance to ciprofloxacin. All NAL-resistant isolates harboured mutations associated with quinolone resistance (MAQRs) in both *gyrA* (S83I/R) and *parC* (S80I/R), and some also had MAQRs in *gyrB* (S464Y/F). The unique PMQR gene detected was *qnrD*, which was found in 2/81 isolates (*Proteus mirabilis* Q1084 and *Proteus vulgaris* Q5169), giving a prevalence of 2.5% in *Proteeae*. These two isolates were from different geographical regions and both harboured MAQRs in *gyrA* and *parC*. The *qnrD* genes were located on the related plasmids pEAD1-1 (2683 bp) and pEAD1-2 (2669 bp). Plasmid pEAD1-1 was 100% identical to pCGH15 and differed in only three nucleotides from pDIJ09-518a, which were previously found in clinical isolates of *P. mirabilis* (China) and *Providencia rettgeri* (France), respectively, whilst pEAD1-2 was not previously described. The extended-spectrum β-lactamase CTX-M-2 was found in 27% (22/81) of the isolates and was significantly associated with quinolone resistance but not with *qnrD* (only *P. mirabilis* Q1084 expressed CTX-M-2). This is the first report of *qnrD* in the Americas.

1. Introduction

Three kinds of plasmid-mediated quinolone resistance (PMQR) mechanisms have been described to date: protection of the quinolone targets mediated by proteins encoded by the *qnr* genes; acetylation of ciprofloxacin and norfloxacin by the bifunctional enzyme AAC(6′)-Ib-cr; and efflux pumps that either specifically (QepA) or unspecifically (OqxAB) decrease the intracellular concentration of quinolones [1]. The *qnr* genes have been found in almost all genera of the family Enterobacteriaceae, and five classes are currently known (*qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*), with *qnrA*, *qnrB* and *qnrS* including several alleles (http://www.lahey.org/qnrStudies). The most recently reported *qnr* gene (*qnrD*) was found in human clinical isolates from a few countries in Europe (The Netherlands, France, Italy and Poland), Africa (Algeria and Nigeria) and Asia (China) [2–9]. Unlike other *qnr* determinants, *qnrD* was always located on small plasmids that may be classified into two groups of very similar members. One of them comprises non-conjugative plasmids of ca. 2.7 kb and the other one includes mobilisable plasmids with an approximate size of 4.3 kb [2,5–7,9].

The *qnrD* gene was mostly found in bacterial species of the tribe *Proteeae* [4–9]. In this study, in order to analyse the occurrence of PMQR genes and to estimate their prevalence in this tribe, 81 unselected, non-duplicate clinical isolates from Argentina were analysed.

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2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

All of the 81 isolates of the tribe *Proteeae* included in a collection of 1059 consecutive (unselected), non-duplicate (one per patient) enterobacteria were analysed. These isolates were collected during a period of 5 consecutive days in 2007 in 66 hospitals of the WHONET-Argentina Resistance Surveillance Network that were located in Buenos Aires City and all 23 provinces of the country. The 81 isolates comprised 58 *Proteus mirabilis*, 7 *Proteus vulgaris*, 12 *Morganella morganii* and 4 *Providencia stuartii*.

Antimicrobial susceptibility tests for nalidixic acid (NAL), ciprofloxacin and levofloxacin were performed by disc diffusion and agar dilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (M100-S23, January 2013). Phenotypic detection of extended-spectrum β-lactamases (ESBLs) was carried out by assaying synergy between cefotaxime and ceftazidime with clavulanic acid by disc diffusion according to the CLSI.

2.2. Analysis of the quinolone resistance-determining regions (QRDRs) of gyrA, parC and gyrB

The QRDRs of *gyrA* and *parC* from the 31 isolates that showed resistance to NAL were analysed by PCR and DNA sequencing as previously described [10]. In the case of *P. stuartii* isolates, these regions were amplified using primers gyrA-Mmo-F and gyrA631-R or parC72-F and parCMS-R, respectively [10], and the obtained sequences were compared with those of the reference strain *P. stuartii* ATCC 29914 (GenBank accession nos. AF052259 and AF363612, respectively). The QRDR of *gyrB* was analysed using the primers gyrB-Fnew (5′-GGTGAYGATGCSCGTGAAGG) and gyrB-Rnew (5′-GGCATTTGACGRTAGAAGAAVGTCA), which were designed to target the *gyrB* sequences from *P. mirabilis* and the other species of the tribe *Proteeae* that were available in GenBank (*Proteus penneri*, *P. stuartii*, *Providencia rettgeri* and *M. morganii*). The *gyrB* sequences were compared with those of the reference strains *P. mirabilis* HI4320 and *P. stuartii* ATCC 25827 and with the sequence available in GenBank for *M. morganii* KT (accession nos. NC_010554, DS607671 and NC_020418, respectively).

2.3. Genetic analysis of the plasmid-mediated quinolone resistance mechanisms and extended-spectrum β-lactamases

Presence of the PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA* and *aac(6′)-Ib-cr* was determined by PCR as described previously [10] using DNA templates obtained through the boiling method [11]. Positive controls were implemented with previously characterised enterobacterial strains that harboured the corresponding PMQR gene [10]. All of the *qnrD* genes found to date were located on small plasmids of 2.7–4.3 kb [2,5–7,9]. Therefore, the divergent primers qnrDout-F (5′-AAATCGCTGGAATGGCACTGTGATAACTGG) and qnrDout-R (5′-AATCACACCCTTCCAAGCTATTCCTGTCG) were designed to analyse the genetic support of *qnrD*.

The presence of the most common ESBL-encoding genes in Argentina ($bla_{CTX-M-2}$, bla_{PER}, bla_{TEM} and bla_{SHV}) was investigated by PCR in those isolates that showed an ESBL phenotype using the primers and conditions already described [11]. The obtained amplicons were completely sequenced as described previously [10]. The CTX-M universal primers CTX-MU1 (5′-ATGTGCAGYAC-CAGTAARGT) and CTX-MU2 (5′-TGGGTRAARTARGTSACCAGA) were also used in order to search for CTX-M-type ESBLs belonging to groups other than CTX-M-2.

2.4. Nucleotide sequence accession nos

Sequences of the S464Y-harbouring *gyrB* QRDRs from *M. morganii* have been assigned GenBank accession nos. KF732711 and KF732712, and those of the *qnrD*-harbouring plasmids have been deposited under GenBank accession nos. KF498970 and KF498971.

3. Results and discussion

3.1. Quinolone susceptibility and quinolone resistance-determining region genotypes

Of the 81 isolates, 50 (62%) were susceptible to quinolones [ranges of minimum inhibitory concentration (MIC) and disc diffusion inhibition zone were: NAL, 1–4 μg/mL and 20–33 mm; ciprofloxacin, 0.008–0.06 μg/mL and 31–44 mm; and levofloxacin, 0.015–0.12 μg/mL and 30–42 mm]. This group included 66% (43/65) of the *Proteus* sp. isolates (37 *P. mirabilis* and 6 *P. vulgaris*) and 58% (7/12) of the *M. morganii* isolates. The remaining 31 isolates (22 *Proteus* spp., 5 *M. morganii* and all *P. stuartii*) showed high-level resistance to NAL (disc diffusion inhibition zone of 6 mm; MIC > 128 μg/mL) and decreased susceptibility or resistance to ciprofloxacin (MIC, 0.5 μg/mL to >64 μg/mL) (Table 1). These phenotypes suggested the presence of mutations associated with quinolone resistance (MAQRs) in the topoisomerase II-encoding genes [7,10,12]. It was reported that the occurrence of these mutations might be associated with the acquisition of PMQR genes and that the interaction of both mechanisms can result in higher

Table 1
Disc diffusion (DD) and agar dilution susceptibility to quinolones and the mutations associated with quinolone resistance (MAQRs) found in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *parC* and *gyrB* of the 31 isolates that showed high-level resistance to nalidixic acid.

Species	*N*	Ciprofloxacin		Levofloxacin		MAQRs in the QRDR		
		MIC (μg/mL)	DD (mm)	MIC (μg/mL)	DD (mm)	*gyrA*	*parC*	*gyrB*
Morganella morganii	3	0.5–1	24–28	1–2	21–26	S83I	S80I	None
	2	32, 64	6	8, 32	6, 12	S83R	S80I	S464Y
Providencia stuartii	2	1, 4	16, 24	2, 8	14, 19	S83R	S80I	None
	2	16, 64	6, 14	4, 16	7, 15	S83I	S80I	None
Proteus mirabilis	1	2	21	4	20	S83I	S80I	None
Proteus vulgaris Q5169[a]		**4**	**16**	**4**	**16**	**S83I**	**S80I**	**None**
P. mirabilis Q1084[a]		**4**	**12**	**8**	**9**	**S83I**	**S80I**	**None**
P. mirabilis	1	16	10	4	18	S83I	S80R	None
	1	32	10	8	12	S83I, E87G	S80I	None
	6	32–64	6	8–32	6–8	S83R	S80I	S464F
	1	>64	6	16	8	S83R	S80I	S464F
	10	>64	6	32 to >64	6	S83R	S80I	S464Y

MIC, minimum inhibitory concentration.
[a] The two *qnrD*-harbouring isolates are indicated in bold.

MICs of ciprofloxacin [1,13,14]. Therefore, the presence of MAQRs in the 31 NAL-resistant isolates was first investigated. Analysis of the QRDRs of *gyrA* and *parC* showed that all of these isolates harboured a MAQR in *gyrA* (S83I/R) and *parC* (S80I/R) and one isolate had an additional *gyrA* MAQR (E87G) (Table 1). In *P. mirabilis*, it was reported that the presence of MAQRs in both *gyrA* and *parC* did not correlate with the ciprofloxacin MIC and that additional MAQRs in *gyrB* were needed to reach high-level ciprofloxacin resistance [7,12]. In keeping with these observations, it was found that all isolates with similar *gyrA*/*parC* genotypes showed MICs of ciprofloxacin and levofloxacin in the range of decreased susceptibility to high-level resistance. Since we observed this fact not only in *P. mirabilis* but also in *P. vulgaris*, *M. morganii* and *P. stuartii*, the QRDR of *gyrB* was analysed in the 31 NAL-resistant isolates. Considering this subset, we found the MAQRs S464Y/F in 77% (17/22) of the *Proteus* sp. and 40% (2/5) of the *M. morganii* isolates. The presence of S464Y/F was significantly associated with an MIC $\geq$ 32 μg/mL or a disc diffusion inhibition zone of 6 mm for ciprofloxacin ($P < 0.0001$, Fisher's test) (Table 1). The association of *gyrB* MAQRs with high ciprofloxacin MICs was previously reported only in *P. mirabilis* [7,12].

3.2. Analysis of plasmid-mediated quinolone resistance genes and association with mutations associated with quinolone resistance

The unique PMQR gene detected was *qnrD*. It was found in 2 of the 81 analysed isolates (*P. mirabilis* Q1084 and *P. vulgaris* Q5169) (Table 1), which results in PMQR prevalence rates of 2.5% (2/81) in the tribe *Proteeae* and 3.1% (2/65) in *Proteus* spp. A *qnrD* prevalence of 1.0% (4/412) in human clinical isolates from China was reported but the sample set used included other enterobacterial species besides those of the tribe *Proteeae* [9]. *Proteus mirabilis* Q1084 was isolated from a urine specimen of a 2-year-old non-hospitalised ambulatory patient (Buenos Aires City), and *P. vulgaris* Q5169 was from a surgery wound specimen of a 65-year-old hospitalised patient (city of Rosario, province of Santa Fe). Of note, *qnrD* was found in isolates with MAQRs, which is consistent with a previous report where such mutations were present in 89% of *qnrA*-positive clinical enterobacteria [14]. In the *Proteus* spp. group, both *qnrD*-harbouring isolates displayed disc diffusion inhibition zones of ciprofloxacin (12 mm and 16 mm) and levofloxacin (9 mm and 16 mm) that were lower than those of the other isolate (21 mm and 20 mm, respectively) with the same *gyrA*/*parC*/*gyrB* genotype (S83I/S80I/wild type) (Table 1), which might be due to an additive effect of *qnrD* to MAQRs as has been reported for other *qnr* genes [1,13,14].

3.3. Genetic support of qnrD

The PCR products obtained with the divergent primers qnrDout-F and qnrDout-R from isolates Q5169 and Q1084 (ca. 2.4 kb) confirmed the location of *qnrD* on small plasmids, named pEAD1-1 and pEAD1-2, respectively, that were completely sequenced (Fig. 1). Plasmid pEAD1-1 (2683 bp) was 100% identical to pCGH15 and differed in only three nucleotides from pDIJ09-518a, which were previously found in clinical isolates of *P. mirabilis* from China [9] and *P. rettgeri* from France [5], respectively. In turn, pEAD1-2 (2669 bp) was not previously described and displayed 96.9% identity with pEAD1-1 (70 single nucleotide differences and three deletions, two of 1 nucleotide and one of 12 nucleotides; Fig. 1). Upstream of *qnrD*, both plasmids contained the same promoter and a LexA-binding site as found in p2007057, a representative of the 4.3-kb group of the *qnrD*-harbouring plasmids [2] (Fig. 1). This fact strongly suggests that the expression of *qnrD* in pEAD1-1 and pEAD1-2 is inducible by SOS activation as has been demonstrated for p2007057 [15].

Besides *qnrD*, pEAD1-1 includes three open reading frames (ORFs), *orf2*, *orf3* and *orf4*, as described previously for the nearly identical plasmid pDIJ09-518a [5]. It was proposed that *orf4* (384 bp) encodes the replication protein and that its upstream region has some features of the theta-type replicons: three DnaA boxes, two pairs of inverted repeats (IR1 and IR2) and four iterons [5]. However, in pEAD1-2, *orf4* is disrupted by a frameshift due to a 1-bp deletion at position 145, indicating that either it is not responsible for plasmid replication or there is a second functional replicon. In addition, the 12-bp deletion and several substitutions in pEAD1-2 with respect to pEAD1-1 located upstream of *orf4* disrupted a DnaA box and the dyad symmetries of IR1 and IR2 (Fig. 1). Therefore, we believe that the mechanism and replication

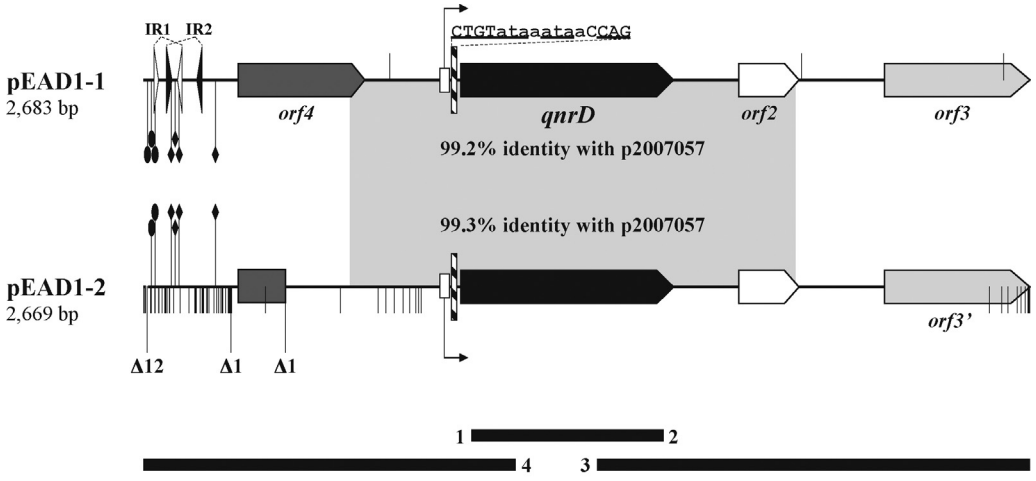

Fig. 1. Genetic platforms of the *qnrD* gene. The two *qnrD*-harbouring plasmids found in this work are shown. The upward vertical thin lines in pEAD1-1 represent its three nucleotidic differences compare with pDIJ09-518a (accession no. HQ834472); and the downward vertical thin lines in pEAD1-2 represent the 70 nucleotide differences between this plasmid and pEAD1-1 (the three deletions in the former are marked by the Δ symbols and numbers indicate the deleted nucleotides). Genes/open reading frames (ORFs) are represented by arrowed boxes (*orf3* and *orf3*′ differ only by three amino acids). In pEAD1-2, the remaining 5′-portion of the disrupted *orf4* is represented by a dark grey box. White and striped vertical bars show the *qnrD* promoter and the LexA box (the canonical sequence is underlined and the essential sequence is in upper case), respectively. The features of the theta-type replicons found in pDIJ09-518a [5] are indicated: inverted repeats (IR) 1 and 2 (white and black triangles, respectively); DnaA boxes (ovals); and putative iterons (diamonds). The grey shaded area highlights the region of pEAD1-1 or pEAD1-2 (1349 bp) with maximum nucleotide identity with p2007057 (accession no. FJ228229). Bottom: thick horizontal lines represent the two amplimers used for sequencing both pEAD1-1 and pEAD1-2, which were generated with primers qnrD-F (number 1) and qnrD-R (number 2) [10] or qnrDout-F (number 3) and qnrDout-R (number 4).

region of the 2.7-kb group of *qnrD*-harbouring plasmids remain unknown.

3.4. Extended-spectrum β-lactamase production and association with quinolone resistance

Of the 81 analysed isolates, 22 (18 *P. mirabilis*, 2 *M. morganii* and 2 *P. stuartii*) showed an ESBL phenotype. By PCR analysis and DNA sequencing, it was found that all these 22 isolates harboured both the $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-2}}$ genes, encoding a broad-spectrum β-lactamase and an ESBL, respectively (PCR assays for bla_{PER} and bla_{SHV} were negative). Therefore, the prevalence rates of ESBLs were 27% (22/81) in the tribe *Proteeae*, 31% (18/58) in *P. mirabilis*, 17% (2/12) in *M. morganii* and 50% (2/4) in *P. stuartii*.

We previously reported that the PMQR genes *qnrB10* and *aac(6′)-Ib-cr* were significantly associated with ESBLs, whilst *qnrB19* was not [10]. Here we found that quinolone resistance was significantly associated with CTX-M-2: 65% (20/31) of the NAL-resistant isolates, but only 4% (2/50) of the NAL-susceptible isolates expressed this ESBL ($P < 0.0001$, Fisher's test). This association was observed even when the analysis was done independently in the *P. mirabilis*, *M. morganii* and *P. stuartii* subsets (data not shown). However, the presence of *qnrD* does not appear to be related to ESBL production: *P. mirabilis* Q1084 expressed the CTX-M-2 enzyme whilst *P. vulgaris* Q5169 did not produce any ESBL.

3.5. Concluding remarks

To the best of our knowledge, this is the first report of *qnrD* in the Americas. In a previous search of PMQR genes in clinical enterobacteria with unusual phenotypes of quinolone susceptibility [10], we did not find *qnrD*. However, the bacterial collection used in that study was highly biased towards resistance or decreased susceptibility to quinolones and included only two *P. mirabilis* and one *M. morganii*. Such a collection of selected isolates is not comparable with that included in the current study (consecutive isolates specifically collected to perform a prevalence study) and therefore the results from both studies cannot be compared as well, at least in epidemiological terms. The *qnrD* gene has never been found in other prevalence studies on PMQR genes from Argentina that addressed enterobacterial species not belonging to the tribe *Proteeae*, such as *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp. [16], *Escherichia coli*, *Shigella* spp. and *Salmonella* spp. [17]. This fact strengthens the notion that the species belonging to the tribe *Proteeae* might constitute a natural reservoir of this gene [4–9].

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Competing interests

None declared.

Ethical approval

Not required.

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