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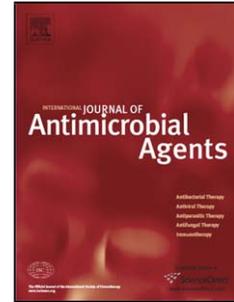
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First description of *Klebsiella pneumoniae* clinical isolates carrying both *qnrA* and *qnrB* genes in Portugal [☆]

Sónia Ferreira ^{a,b,c}, Mark Toleman ^b, Elmano Ramalheira ^c, Gabriela J. Da Silva ^d, Timothy Walsh ^b, Sónia Mendo ^{a,*}

^a *University of Aveiro, Department of Biology, CESAM, 3810-193 Aveiro, Portugal*

^b *School of Medicine, Department of Medical Microbiology, Cardiff University, Heath Park, Cardiff CF14 4XN, UK*

^c *Hospital Infante D. Pedro EPE, 3810 Aveiro, Portugal*

^d *Center of Pharmaceutical Sciences, University of Coimbra, Health Sciences Campus, Faculty of Pharmacy, Department of Microbiology, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal*

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* Corresponding author. Present address: CESAM & Departamento de Biologia, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal. Tel.: +351 234 370 970; fax: +351 234 372 587.

E-mail address: smendo@ua.pt (S. Mendo).

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ABSTRACT

In the present study, 21 multidrug-resistant *Klebsiella pneumoniae* isolates were recovered from patients hospitalised in the Intensive Care Unit of Hospital Infante D. Pedro in Aveiro, Portugal. Fifteen isolates carried *qnr* genes. Four strains harboured the quinolone resistance genes *qnrA* and *qnrB*, both located on a large plasmid in two strains (KP4 and KP10) and on different plasmids in two strains (KP5 and KP6). These findings indicate an extremely high prevalence of *qnr* genes associated with various mobile elements such as *ISCR1* and class 1 integrons.

1. Introduction

Plasmid-mediated quinolone resistance encoded by *qnrA*, *qnrB* or *qnrS* genes has been reported worldwide [1,2]. Qnr proteins are members of the pentapeptide repeat protein family that protect topoisomerases and thereby reduce their susceptibility to fluoroquinolones [1,3]. *qnrA* was the first *qnr* gene described in a *Klebsiella pneumoniae* in 1998 by Martínez-Martínez et al. [4]. Subsequently, it has been shown to be widely spread worldwide as part of a complex *sul1*-type integron containing *ISCR1* [1,5]. *qnrB* and *qnrS* have also been reported in many countries from Europe, Asia and the USA [1]. Dissemination of plasmids carrying these genes can potentiate the rapid development of higher-level quinolone resistance in bacterial strains currently classified as susceptible [6]. Moreover, transferrable *qnr* genes are usually carried by conjugative plasmids (50–180 kb) that often encode extended-spectrum β -lactamases (ESBLs) or AmpC-type β -lactamases [7]. In fact, epidemiological studies of the distribution of *qnr* determinants show that *qnr*-positive strains frequently express ESBLs [8]. Here we report the presence of *qnr* genes in quinolone-resistant *K. pneumoniae* and investigate their association with different β -lactamases.

2. Material and methods

2.1. *Klebsiella pneumoniae* clinical isolates

Twenty-one *K. pneumoniae* were isolated from various biological products, including urine, wound swabs, respiratory secretions, blood and sterile body

fluids, collected from patients in the Intensive Care Unit of Hospital Infante D. Pedro (Aveiro, Portugal). Species identification was performed using the VITEK 2 system (bioMérieux, Marcy l'Etoile, France). The population analysed was non-repetitive and only a single isolate per patient was included in the study.

2.2. Drug susceptibility testing

Susceptibility testing was performed according to the guidelines of the Clinical and Laboratory Standard Institute [9]. ESBL production was confirmed by Etest with cefotaxime/cefotaxime + clavulanic acid, ceftazidime/ceftazidime + clavulanic acid and cefepime/cefepime + clavulanic acid strips, according to manufacturer's instructions (AB BIODISK, Solna, Sweden).

2.3. Hybridisation assays

Resolution of S1-digested genomic DNA of *K. pneumoniae* isolates was performed with a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK). Chromosomal and plasmid DNA were separated by electrophoresis in a 1% agarose gel in 0.5 × TBE buffer (pH 8.0) at 200 V and 14 °C for 20 h, with the pulse time ramped from 5 s to 45 s. The resulting pulsed-field gel electrophoresis (PFGE) gel was used to assess the *qnr* gene location, which was determined by DNA–DNA hybridisation using a radiolabelled probe consisting of polymerase chain reaction (PCR)-generated *qnrA* and *qnrB* genes.

2.4. Polymerase chain reaction and DNA sequencing

PCR detection of class 1 integrons was performed as previously described [10]. The presence of *qnr* genes was detected by PCR using the primers described by Wu et al. [11]. All isolates were screened by PCR for the presence of SHV, CTX-M, TEM and OXA β -lactamases as well as AmpC groups (DHA, MOX, FOX, CMY and ACC) [12]. Nucleotide sequences of PCR products were determined and compared with other sequences deposited in the EMBL GenBank.

2.5. Conjugation assays

Conjugation assays were performed with *Escherichia coli* J53 Azi^r (azide-resistant) as recipient and KP4, KP5, KP6 and KP10 as donors. Strains were routinely grown in Luria–Bertani broth. Culture plates contained tryptic soy agar or Mueller–Hinton (MH) agar (Becton Dickinson and Co., Sparks, MD). Selective media plates (MH agar) contained 100 μ g/mL sodium azide and one of the following antibiotics: ciprofloxacin (0.125 μ g/mL); sulfamethoxazole (100 μ g/mL); or chloramphenicol (40 μ g/mL).

3. Results and discussion

All 21 isolates were resistant to nalidixic acid [minimum inhibitory concentration (MIC) \geq 32 μ g/mL] and ciprofloxacin (MIC \geq 4 μ g/mL). Nineteen isolates were intermediate resistant (8 μ g/mL) and two were resistant (\geq 16 μ g/mL) to norfloxacin. Six strains were identified as being ESBL-producers.

Fifteen of the twenty-one isolates tested positive for the presence of *qnr* genes (Table 1). Twelve carried *qnrA1*, which in eight isolates was found downstream of *In37* and *ISCR1* that codes for a transposase and provides the promoter sequences for high-level expression of the plasmid-mediated *qnrA* gene in Enterobacteriaceae. All isolates co-harboring *qnrA*, *aac(6′)-Ib-cr* and *bla_{OXA-30}* also harboured *ISCR1* and a class 1 integron. Three isolates carried *qnrB* alone. The *qnrS* gene was not detected. Previous reports describe the expression of *qnrA* and *qnrS* [3], and the co-existence of *qnrB4* and *qnrS* in the same *K. pneumoniae* isolate in two different plasmids has also been reported [2]. However, here we report four isolates positive for both *qnrA* and *qnrB* simultaneously (KP4, KP5, KP6 and KP10). Digestion with S1 revealed the presence of plasmids ranging in size from 50 kb to 450 kb in the strains possessing both genes (data not shown). S1-restricted plasmids were hybridised with labelled *qnrA*- and *qnrB*-specific probes revealing that both genes are plasmid-encoded and that *qnrA1* and *qnrB4* are located on the same plasmid in two of the strains (KP4 and KP10) and on different plasmids in two strain (KP5 and KP6) (data not shown). The *qnrA1* gene was found on a 450 kb plasmid in strains KP4, KP5, KP6 and KP10. The *qnrB4* gene was located on the same 450 kb plasmid in two of the strains (KP4 and KP10) and on a 75 kb plasmid in strains KP5 and KP6. This is the first report of the presence of both *qnrA1* and *qnrB4* on the same plasmid. Conjugation was attempted using *E. coli* J53 Azi^r as recipient, however none of the *qnr* genes could be transferred under the different experimental laboratory conditions tested. β -Lactamase screening in all isolates revealed that *bla_{TEM}* was present in 38% of the isolates and *bla_{SHV}* in 85.7%. *bla_{OXA}* was found in 38% of the

isolates and was found to be part of the In37 array in all of them. Interestingly, although a genetic linkage between *bla*_{CTX-M} and *aac(6')-Ib-cr* has been reported previously [13–15], *bla*_{CTX-M} was not detected. However, all cephalosporin-resistant isolates harboured *qnr* genes, thus suggesting a linkage between the presence of *qnr* and resistance to cephalosporins as previously reported [1,3]. Strains KP4 and KP5, although negative for *bla*_{CTX-M}, harboured *qnrA*, *qnrB* and *bla*_{DHA-1} encoding a plasmidic AmpC-type β -lactamase that confers resistance to cephamycins and extended-spectrum cephalosporins, explaining the resistance to cephalosporins exhibited by these isolates. Moreover, *int1* was detected in 80% of the isolates, and 38% carried more than one integron. A total of 18 gene cassettes occurring in three different cassette arrays were identified, with the variants of *aadA* and *dfrA* genes being most frequent. The complex integron In37 [*aac(6')-Ib-cr*, *bla*_{OXA-30}, *catB3*, *arr-3*]:ISCR1:*qnrA1* was found in eight isolates; this structure has been previously described in an *E. coli* isolate from China (accession no. AY259086). Our results showed a high association between *aac(6')-Ib-cr*, *bla*_{OXA-30}, class 1 integron, ISCR1 and *qnrA*, as previously reported [15], suggesting the dissemination of this structure worldwide. Also, most of the *qnr*-positive isolates carried a *bla* gene encoding an ESBL or an *ampC* gene coding DHA-1, reinforcing once again a possible relationship between *qnr* genes and broad spectrum β -lactamases [1,16]. These findings indicate an extremely high prevalence of *qnr* genes associated with various mobile elements such as ISCR1 and class 1 integrons. Moreover, the role of co-existence of both *qnrA* and *qnrB* for the strain still needs to be clarified,

however it can act as a reservoir that will further disseminate these genes to sensitive bacteria.

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Competing interests

None declared.

Ethical approval

Not required

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Table 1Characteristics of *qnrA*-, *qnrB*- and *qnrA/qnrB*-positive *Klebsiella pneumoniae* strains

Isolate	MIC ($\mu\text{g/mL}$)			Genetic element detected							
	NA	CIP	NOR	<i>int11</i>	ISCR	<i>bla</i> _{OXA}	<i>bla</i> _{CTX-M}	<i>bla</i> _{TEM}	<i>bla</i> _{SHV}	<i>bla</i> _{DHA}	<i>qnr</i>
KP2	≥ 32	≥ 4	8	–	–	–	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-1}	–	<i>qnrA1</i>
KP4	≥ 32	≥ 4	8	+	–	–	–	–	–	<i>bla</i> _{DHA-1}	<i>qnrA1</i> , <i>qnrB4</i>
KP5	≥ 32	≥ 4	≥ 16	+	+	<i>bla</i> _{OXA-30}	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-1}	<i>bla</i> _{DHA-1}	<i>qnrA1</i> , <i>qnrB4</i>
KP6	≥ 32	≥ 4	8	+	+	<i>bla</i> _{OXA-30}	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-1}	–	<i>qnrA1</i> , <i>qnrB4</i>
KP7	≥ 32	≥ 4	≥ 16	+	+	<i>bla</i> _{OXA-30}	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-5}	–	<i>qnrA1</i>
KP8	≥ 32	≥ 4	8	+	+	–	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-1}	<i>bla</i> _{DHA-1}	<i>qnrA1</i>
KP9	≥ 32	≥ 4	8	–	–	–	–	<i>bla</i> _{TEM-1}	–	–	<i>qnrB4</i>
KP11	≥ 32	≥ 4	8	+	+	–	–	–	<i>bla</i> _{SHV-1}	–	<i>qnrA1</i>
KP10	≥ 32	≥ 4	8	+	+	<i>bla</i> _{OXA-30}	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-1}	–	<i>qnrA1</i> , <i>qnrB4</i>
KP14	≥ 32	≥ 4	8	+	+	<i>bla</i> _{OXA-30}	–	–	<i>bla</i> _{SHV-1}	<i>bla</i> _{DHA-1}	<i>qnrA1</i>
KP16	≥ 32	≥ 4	8	+	+	<i>bla</i> _{OXA-30}	–	–	<i>bla</i> _{SHV-1}	<i>bla</i> _{DHA-1}	<i>qnrA1</i>
KP17	≥ 32	≥ 4	8	+	–	–	–	–	<i>bla</i> _{SHV-5}	–	<i>qnrB4</i>
KP19	≥ 32	≥ 4	8	+	+	<i>bla</i> _{OXA-30}	–	–	<i>bla</i> _{SHV-1}	<i>bla</i> _{DHA-1}	<i>qnrA1</i>
KP20	≥ 32	≥ 4	8	+	+	<i>bla</i> _{OXA-30}	–	<i>bla</i> _{TEM-1}	<i>bla</i> _{SHV-1}	–	<i>qnrA1</i>

KP21	≥32	≥4	8	+	-	-	-	-	<i>bla</i> _{SHV-1}	<i>bla</i> _{DHA-1}	<i>qnrB4</i>
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MIC, minimum inhibitory concentration; NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; -, negative for the presence of the screened gene; +, positive for the presence of the screened gene.