Plasmid-Mediated Quinolone Resistance in Australia

JOSE-MANUEL RODRIGUEZ-MARTINEZ,1,2 LAURENT POIREL,1 ALVARO PASCUAL,2 and PATRICE NORDMANN1

ABSTRACT

The aim of this study was to search for plasmid-encoded quinolone resistance determinants QnrA and QnrS in fluoroquinolone-resistant and extended-spectrum β-lactamase (ESBL)-producing enterobacterial isolates recovered in Sydney, Australia, in 2002. Twenty-three fluoroquinolone-resistant, of which 16 were also ESBL-positive, enterobacterial and nonrelated isolates were studied. PCR with primers specific for qnrA and qnrS genes and primers specific for a series of ESBL genes were used. A qnrA gene was identified in two ESBL-positive isolates, whereas no qnrS-positive strain was found. The QnrA1 determinant was identified in an Enterobacter cloacae isolate and in a carbapenem-resistant Klebsiella pneumoniae isolate, both of which expressed the same ESBL SHV-12. Whereas no plasmid was identified in the E. cloacae isolate, K. pneumoniae K149 possessed two conjugative plasmids, one that harbored the qnrA and blsSHV-12 genes whereas the other expressed the carbapenemase gene blsIMP-4. The qnrA gene, was located in both cases downstream of the orf513 recombinase gene and upstream of the qnrA1 gene, a structure identical to that found in sul1-type integron In36 and qnrA-positive strains from Shanghai, China. However, the gene cassettes of the sul1-type integrons were different. This study identified the first plasmid-mediated quinolone resistance determinant in Enterobacteriaceae in Australia.

INTRODUCTION

Quinolone resistance usually results in Enterobacteriaceae from mutations in genes coding for chromosomally encoded type II topoisomerases or for efflux pumps and porins.2,3 However, recent reports indicate that quinolone resistance may be also plasmid mediated.3,9,13 The first plasmid-mediated quinolone resistance protein Qnr (termed recently QnrA) was identified from urine in a Klebsiella pneumoniae isolate from Birmingham, Alabama by L. Martinez-Martinez and G. Jacoby.7 The plasmid-mediated quinolone resistance gene qnrA encodes a 218-amino-acid protein of the pentapeptide family that protects gyrase from quinolone inhibition.14 Recently, another plasmid-mediated quinolone resistance determinant, QnrS, has been reported from Japan.3 Molecular studies showed that the qnrA gene was located with other resistance determinants in sul1-type integrons harboring a duplication of the 3′-conserved sequence of integrons and orf513 gene.1,2,13 This latter gene encoding a putative recombinase involved in site-specific acquisition of resistance genes.

Plasmid-mediated determinant QnrA has been identified from remotely related areas, such as the United States, China, Thailand, Korea, Turkey, Germany, and France.9,16 The origin of the QnrA determinant has been identified recently as being the water-borne environmental species Shewanella algae.11 The aim of this study was to determine the prevalence of the qnrA and qnrS genes in clinical isolates recovered in Sydney, Australia, to evaluate further the dissemination of these novel plasmid-mediated quinolone resistance determinants.

MATERIALS AND METHODS

Bacterial strains

Twenty-three ciprofloxacin-resistant nonduplicate enterobacterial strains isolated in 2002 in the metropolitan area of Sydney, Australia, and recovered during a 2-month period from urine samples were studied, including 11 Klebsiella pneumoniae, 8 Escherichia coli, 1 Enterobacter cloacae, 1 Enterobacter agglomerans, 1 Proteus mirabilis and 1 Citrobacter freundii strain. They were randomly taken from the strain collection of the Antimicrobial Reference Laboratory, Department of Microbiology, The Prince of Wales Hospital, Randwick, Australia. In addition, previously studied E. coli EC158 and K. pneumoniae K149 strains isolated

1Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, Université Paris XI, K.-Bicêtre, France.
2University Hospital Virgen Macarena, University of Sevilla, Sevilla, Spain.
in Melbourne in September, 2002, that both produce metallo-β-lactamase IMP-4 were included in this study.10 Sixteen isolates (69.5%) produced extended-spectrum β-lactamases (ESBLs) and were detected as reported.1 E. coli NCTC50192 harboring four plasmids of 154, 66, 38, and 7 kb was used as size marker for plasmids. E. coli J53 A2b was used as the host in the conjugation experiments and E. coli DH10B in the transformation experiments.5 E. coli LO was used as a qnrA1-positive and E. coli (pBCH2.6) as qnrS-positive control strains.1

Susceptibility testing

MICs for nalidixic acid and fluoroquinolones were determined by an agar dilution technique and interpreted, when available, according to the guidelines of the CLST.8 MICs of β-lactams, chloramphenicol, tetracycline, tobramycin, and gentamicin were determined by an agar dilution technique with Mueller-Hinton agar (Sanofi-Diagnostic Pasteur), as described previously.5 PCR experiments were carried out using specific primers for the qnrA and qnrS genes. Primers QnrA-A and QnrA-B3 for the qnrA gene and QnrS-A2 (5′-AGT GAT CTC ACC TTC ACC GC-3′) and QnrS-B2 (5′-CAG GCT GCA ATT TTT ATA CC-3′) for the qnrS gene amplifying internal fragments of 661 and 550 bp, respectively, were used. Once the corresponding gene was identified using a series of primers for genes coding for ESBLs,4 primers specific for β-lactamase gene blsSHV (SHV-A, 5′-ATG CTT TAT WTT CGC CTT GAT GTG-3′, and SHV-B, 5′-TTA GCG TTG CCA GTG CTC G-3′) were used in combination with qnrA-specific primers to evaluate a putative colinearity between those genes. The PCR products were sequenced with an Applied Biosystems sequencer (ABI 377).

Plasmid and Southern blot analyses

Plasmid analysis of the clinical isolates, transconjugants, and transformants was performed by using the Kieser technique,5 followed by an agarose gel electrophoresis analysis. Southern blot analysis was performed using whole-cell DNA restricted with BamHI and NdeI restriction enzymes, agarose gel electrophoresis, and membrane transfer, followed by hybridization using qnrA and blsSHV-12-specific probes.6

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>E. cloacae Qn17 qnrA+</th>
<th>K. pneumoniae K149 (p149) qnrA+</th>
<th>E. coli J53 transconjugant (p149)qnrA+</th>
<th>E. coli J53 A2b</th>
<th>E. coli DH10B (p149SN12)qnrA+</th>
<th>E. coli DH10B</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAL</td>
<td>&gt;256</td>
<td>32</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>CIP</td>
<td>16</td>
<td>1.25</td>
<td>0.12</td>
<td>0.25</td>
<td>128</td>
<td>0.002</td>
</tr>
<tr>
<td>OFX</td>
<td>&gt;32</td>
<td>1</td>
<td>0.01</td>
<td>0.5</td>
<td>0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>NFX</td>
<td>32</td>
<td>2</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
<td>0.004</td>
</tr>
<tr>
<td>MFX</td>
<td>&gt;32</td>
<td>1</td>
<td>0.03</td>
<td>0.5</td>
<td>0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>SPX</td>
<td>&gt;32</td>
<td>1</td>
<td>0.01</td>
<td>0.5</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>AMX</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>512</td>
<td>4</td>
<td>&gt;512</td>
<td>4</td>
</tr>
<tr>
<td>AMC</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>8</td>
<td>128</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>PIP</td>
<td>64</td>
<td>256</td>
<td>2</td>
<td>&gt;512</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>TZP</td>
<td>8</td>
<td>128</td>
<td>1</td>
<td>128</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CAZ</td>
<td>256</td>
<td>512</td>
<td>8</td>
<td>&gt;512</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>CTX</td>
<td>32</td>
<td>256</td>
<td>4</td>
<td>128</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>FEP</td>
<td>0.5</td>
<td>64</td>
<td>2</td>
<td>32</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>IMP</td>
<td>0.25</td>
<td>8</td>
<td>0.06</td>
<td>0.12</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>TM</td>
<td>&gt;128</td>
<td>128</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GM</td>
<td>&gt;128</td>
<td>128</td>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>&gt;128</td>
<td>128</td>
<td>128</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TET</td>
<td>&gt;128</td>
<td>128</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

aNAL, nalidixic acid; CIP, ciprofloxacin; OFX, ofloxacin; NFX, norfloxacin; MFX, moxifloxacin; SPX, sparfloxacin, AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; CAZ, cefazidime; CTX, ceftaxime; FEP, ceftipime; IMP, imipenem; TM, tobramycin; GM, gentamicin; C, chloramphenicol; TET, tetracycline. Breakpoints used for quinolone and fluoroquinolone susceptibility testing were the following: for nalidixic acid, susceptible if MIC ≤16 mg/L and resistant if MIC ≥32 mg/L; for ciprofloxacin, moxifloxacin, and sparfloxacin, susceptible if MIC ≤1 mg/L and resistant if MIC ≥8 mg/L; for ofloxacin, susceptible if MIC ≤2 mg/L and resistant if MIC ≥8 mg/L; for norfloxacin, susceptible if MIC ≤4 mg/L and resistant if MIC ≥16 mg/L.

bE. coli J53 (p149) transconjugant was obtained from K. pneumoniae K149.

cE. coli DH10B (p149SN12) recombinant strain contained a SacI insert expressing the QnrA determinant.

dND, Not determined.
Conjugation and transformation experiments

Transfer of the qnrA1 gene into reference strain E. coli J53 ΔK18 by conjugation was attempted by liquid and solid mating-out assays. Transconjugants were selected on trypticase soy (TS) agar plates containing sodium azide (100 mg/L) and amoxicillin (100 mg/L) or chloramphenicol (30 mg/L). In addition, plasmid extracts of qnrA1-positive strains were used for transformation assays in E. coli DH10B, as described, giving rise to transformants selected on plates containing amoxicillin (100 mg/L) or chloramphenicol (30 mg/L).6

Cloning experiments and sequence analysis

Cloning experiments were performed with the restriction enzymes SacI or BamHI from whole-cell DNAs extracted as previously described using the vector pBK-CMV, followed by expression of recombinant plasmids in E. coli DH10B and selection on TS agar containing kanamycin (30 mg/L) and nalidixic acid (4 mg/L), gentamicin (8 mg/L), or chloramphenicol (30 mg/L). Antibiograms obtained by disk diffusion were performed with E. coli DH10B harboring recombinant plasmids, and sizes of the plasmid inserts were determined by restriction analysis. Both strands of each recombinant plasmid were sequenced. The nucleotide sequences were analyzed with software available over the internet at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Retrospective analysis of the antibiotic resistance profile of the SHV-12-positive E. coli transconjugant obtained from K. pneumoniae isolate K149 (IMP-4 and SHV-12 positive) recovered in Australia10 indicated that the transferred plasmid p149 conferred reduced susceptibility to several quinolones whereas the IMP-4-positive plasmid did not. Thus, PCR experiments were performed to screen for the putative qnrA gene that gave a positive result for the K. pneumoniae K149 isolate but not for the IMP-4-positive E. coli EC158 strain recovered during the same period of time. Consequently, a survey was conducted to evaluate prevalence of QnrA and QnrS determinants among a collection of ciprofloxacin-resistant enterobacterial isolates recovered in Melbourne over a 2-month period. This screening gave negative results for the qnrS gene (the first survey of that type), whereas one of the 23 clinical strains analyzed was positive for the qnrA gene (8.7%). This isolate, E. cloacae strain Qn17, was also positive for the blaoxy-12 gene. The nucleotide sequences of the two qnrA-like genes were identical to that of the known qnrA, latter termed qnrA1.9 K. pneumoniae K149 was resistant to ampicillin, cefazidime, ticarcillin, ticarcillin plus clavulanic acid, imipenem, fosfomycin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, tobramycin, gentamicin, kanamycin, rifampin, and sulfonamides of intermediate susceptibility to nalidixic acid and resistant to fluoroquinolones (Table 1). E. cloacae Qn17 was resistant to amoxicillin, cefotaxime, cefazidime, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, aminoglycosides, rifampin, sulfonamides, nalidixic acid, and fluoroquinolones.

Conjugation, plasmid analysis, and Southern hybridization experiments (data not shown) did not identify any plasmid in E. cloacae Qn17, whereas a 160-kb conjugative plasmid was identified in K. pneumoniae K149 that possessed both the qnrA and blaoxy-12 genes. However, PCR mapping did not reveal any co-linearity between those two genes. In addition, PCR combinations with primers specific for a class 1 integron did

FIG. 1. Comparison of sul1-type integrons that contain a qnrA gene. The identical structure identified in K. pneumoniae K149 and in E. cloacae Qn17 isolates is indicated in comparison with those of In36 and In37 from Shanghai.14 pMG252 is from the QnrA-positive E. coli strain from Alabama.12
not identify any gene cassette upstream of orf513. Another 150-kb plasmid was identified in *K. pneumoniae* K149, which harbored the carbapenemase gene *blaIMP-4* located inside a class 1 integron structure, as reported previously.16

Quinolone resistance was transferred with a conjugation frequency (the number of transconjugants divided by the number of the donor cells) of $5 \times 10^{-5}$. Transconjugant *E. coli* J53 (p149) containing the *qnrA* gene displayed resistance to ampicillin, ceftazidime, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, tobramycin, and gentamicin. It showed reduced susceptibility to nalidixic acid and fluoroquinolones (Table 1), but susceptibility to sulfonamides.

Cloning experiments using DNA from *K. pneumoniae* K149 gave rise to an *E. coli* (p149SN12) recombinant strain that showed reduced susceptibility to quinolones (Table 1). Analysis of the sequence of the 3,495-bp insert indicated that *qnrA1* was located in a complex Inφ family class 1 integron similar to In36,15 with the presence of an *ampR* gene downstream of *qnrA* (Fig. 1). The identical fragment was detected in *E. cloacae* Qn17. However, further PCR mapping did not identify the overall structure of Inφ or In37 in both isolates. In particular, PCR failed to detect IS6100 downstream of *qnrA*, whereas it has been detected in the *qnrA*-positive integrons In36 and In37.15

This study emphasizes that the QnrA determinants are also found in enterobacterial isolates in the Australian continent. QnrA-like determinants were identified in ESBL-producing enterobacterial isolates, as reported previously.9 It is noteworthy that it has been detected in a carbapenem-resistant *K. pneumoniae* isolate, emphasizing that some strains may harbor a large pool of resistance genes leading to panresistance. Another interesting feature observed in that study is the possible chromosomal location of the *qnrA1* gene in *E. cloacae* Qn17, suggesting that the *qnrA*-structure could have integrated into the chromosome of that isolate, possibly by a transposition process, as already suggested.15

**ACKNOWLEDGMENTS**

This work was funded by a grant from the Ministère de l’Éducation Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, France, and by a grant from the European Community (6th PCRD, LSHM-CT-2003-503335). Strains were kindly provided by the Antibiotic Reference Laboratory, Department ofMicrobiology, The Prince of Wales Hospital, Randwick NSW. L.P. is a researcher from the INSERM (Paris, France) and J.M.R.M. was a recipient of a travel grant from the Spanish Society for Clinical Microbiology and Infectious Diseases in 2004.

**REFERENCES**


Address reprint requests to:
Dr. Patrice Nordmann
Service de Bactériologie-Virologie
Hôpital de Bicêtre
78, rue du Général Leclerc
94275 Le Kremlin-Bicêtre, France

E-mail: nordmann.patrice@bct.ap-hop-paris.fr
This article has been cited by:


