An outbreak of *Enterobacter cloacae* infections with variable susceptibility to fluoroquinolones occurred in the University Medical Center Utrecht in the Netherlands in 2002. Our investigation showed that a *qnrA* gene was present in 78 (94%) of 83 outbreak isolates and that a *qnrA*-encoding plasmid transferred to other strains of the same species and other species. The earliest isolate carrying this same plasmid was isolated in 1999. *qnrA* was located in a complex integron consisting of the *intI1*, *aadB*, *qacEΔ1*, *sul1*, *orf513*, *qnrA1*, *ampR*, *qacEΔ1*, and *sul1* genes that were not described previously. On the same plasmid, 2 other class 1 integrons were present. One was a new integron associated with the *bla*~CTX-M-9~ extended-spectrum β-lactamase.

Multidrug-resistance among *Enterobacteriaceae*, including resistance to quinolones, is increasing. Although quinolone resistance is predominantly caused by chromosomal mutations, it may also result from a plasmid-encoded *qnr*-gene (1). The QnrA determinant, a 218–amino acid protein, protects DNA gyrase and topoisomerase IV from the inhibitory activity of quinolones (2). However, expression of *qnrA* alone is frequently insufficient to reach Clinical and Laboratory Standards Institute breakpoints for ciprofloxacin resistance. Since first identified in 1994 in the United States, *qnrA*-like genes have been sporadically identified in Enterobacteriaceae worldwide (3–9).

At the end of 2002, an outbreak of aminoglycoside-resistant *Enterobacter cloacae* infections with variable susceptibility to ciprofloxacin was detected in the University Medical Center Utrecht (UMCU), the Netherlands, involving >80 patients (10). The first aim of this study was to test the hypothesis that the variable susceptibility to ciprofloxacin of the outbreak strain was associated with plasmid-mediated *qnrA* and if so, to characterize the gene’s molecular background and determine its ability to transfer in vitro as well as in vivo. Maximum circumstantial evidence for horizontal transfer in vivo with the outbreak strain as donor would be obtained if the following observations were made: 1) different species or strains collected from the same patient harbored the same *qnrA*-encoding plasmid; 2) this same *qnrA*-encoding plasmid was not found in patients without an epidemiologic link to the outbreak. The second aim of this study was to determine to what extent the *qnrA* gene is an emerging resistance problem in our hospital.

**Materials and Methods**

**Bacterial Isolates**

A total of 1,167 isolates were tested for a *qnrA* gene. Group I consisted of 178 *E. cloacae* pulsed-field gel electrophoresis (PFGE) typed isolates obtained from January 2001 to August 2003 from 159 patients (10). Of these, 83 tobramycin-resistant isolates obtained from 83 patients belonged to 1 clonal lineage (cluster I, outbreak strain). Five of these patients also carried a tobramycin-susceptible variant of the clonal lineage (IA). The remaining 95 *E. cloacae* isolates contained 5 small clusters of 2 isolates each (III–VII), 1 cluster with 6 isolates (VIII), 1 cluster with 3 isolates (II), and 70 unique strains.

Groups II and III consisted of aminoglycoside-resistant, gram-negative bacteria identified in the hospital database that were other than the outbreak strain; these bacteria were isolated from patients with an outbreak strain (group II) as well as from patients not involved in the outbreak but admitted in the same period (January 2001–August 2003) (group III). Aminoglycoside resistance was the selection criterion because the outbreak strain was aminoglycoside-resistant.

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resistant, and these isolates are stored routinely in our laboratory. Group IV consisted of 867 *Enterobacteriaceae* isolates comprising 8 different species collected from 3 different origins: 269 clinical isolates from UMCU (1994–2000), 514 isolates from 23 European hospitals (1997–1998), and 84 fecal screening isolates from 53 patients at admission at UMCU (2000) (11).

Identification and Susceptibility Testing

Identification and susceptibility testing of isolates obtained through 2000 were performed by using the VITEK1 System with AMS R09.1 software (bioMérieux, Marcy-L’etoile, France); isolates obtained after 2000 were tested by using the Phoenix 100 Automated Microbiology System version V3.22 software (Becton Dickinson Biosciences, Sparks, MD, USA). For susceptibility testing, Clinical and Laboratory Standards Institute guidelines were used (12). In the conjugation experiments, MICs were determined by using Etest (AB Biodisk, Solna, Sweden).

Genotyping and Characterization of β-Lactamases

*E. cloacae* isolates were typed by PFGE. *Citrobacter freundii*, *Escherichia coli*, and *Klebsiella pneumoniae* were typed by PFGE and random amplified polymorphic DNA (13). To determine the kind of β-lactamases the outbreak strain expressed, isoelectric focusing (IEF) was performed with Phastgels (pH gradient 3–9) with the PhastSystem (Pharmacia AB, Uppsala, Sweden) (14). β-lactamases of isoelectric pH (pl) 5.6 (TEM-1), pl 7.6 (SHV-2A), and pl 8.2 (blacTX-M-9) and a broad range pl calibration set (Amersham Biosciences, Little Chalfont, UK) were used. β-lactamases were detected with nitrocefin (Oxoid, Basingstoke, UK).

Detecting and Characterizing Resistance Genes

Target DNA for polymerase chain reaction (PCR) assays was extracted by heating bacterial suspensions for 10 min at 95°C. *qnrA*, *blacTX-M-9*, and *aadB* were detected by PCR with primers and annealing temperatures described in the Table. The outbreak strain carried an integron containing an *aadB* gene encoding aminoglycoside resistance (17). Primers were developed to detect *aadB* gene and the downstream 3′-conserved segment (CS) of the integron in the same PCR (*aadB*-3′CS). PCR assays were performed for 30 or 35 cycles. The AmpC PCR tests were performed as described earlier, except that a single PCR format was used (18).

The *blacTX-M* gene from *E. cloacae* 02-477 was sequenced by using CTX-M-9 group sequence primers (Table). The flanking regions of the *qnrA* gene and the *blacTX-M-9* gene were determined by using a PCR and DNA sequencing strategy based on the sequences from In7, In36, In37, In60, and an integron from *E. coli* O159 (5,9,19–22). To confirm that the gene cassettes were part of a complex integron with *qnrA* or *blacTX-M-9*, we used the Expand Long Template PCR system (Roche, Woerden, the Netherlands) that employed primers to amplify sequences between the *qnrA* or *blacTX-M-9* and the possible gene cassettes. All amplified products were (partly) sequenced for confirmation. Sequencing was performed with Qiagen Quick (Qiagen, Westburg b.v., Leusden, the Netherlands) purified PCR products by using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit and a 3100 capillary DNA sequencer (Applied Biosystems, Nieuwerkerk a/d Yssel, the Netherlands).

Conjugation Experiments

For conjugation experiments, an *E. coli* K12 and a tobramycin-susceptible clinical *E. cloacae* (03-702) isolate of PFGE cluster I* were used as recipients. An *E. cloacae* (02-477) belonging to PFGE cluster I was used as donor. Conjugation was performed as described (23). MacConkey agar plates containing tobramycin (8 µg/mL) were used for counter selection, and transconjugants were selected on colony form. Conjugation was confirmed by a *qnrA*-specific PCR. Secondly, transconjugant *E. coli* C02-477A was used as a donor for *qnrA*-negative *E. cloacae* 03-702 belonging to cluster I*. Transconjugants were selected by using 15 µg/mL ampicillin-clavulanic acid and 5 µg/mL tobramycin. Transconjugants were characterized as described above.

Detecting Resistance Genes on Plasmid by Southern Hybridization

Plasmids were isolated with the Qiagen Plasmid Maxi Kit (Qiagen). Plasmid DNA was separated on 1% PFGE agarose (Bio-Rad Laboratories, Richmond, CA, USA) in 0.5× Tris-borate-EDTA, 0.05 mmol/L thiourea buffer at 14°C in CHEF DR-II apparatus (Bio-Rad). Run time was 22 h with a voltage of 6 V/cm and a linearly ramped pulse time of 30 to 70 s. The DNA was blotted and hybridized. The probes were PCR amplification products obtained with primers used to detect *aadB*-3′CS, *blacTX-M-9*, and *qnrA* genes (Table). Products were labeled with the AlkPhosDirect Reaction Kit (Amersham Biosciences) and detected with CPD-Star (Amersham Biosciences).

Results

*qnrA1* in Outbreak Strain

For 78 (94%) of the 83 *E. cloacae* isolates in cluster I (outbreak strain), the *qnrA*-specific PCR was positive. To confirm results from the PCR, 2 fragments were sequenced. The obtained sequences were identical to the published sequence of *qnrA1* (GenBank accession no. AY070235).
Susceptibility testing showed that 87% of the 83 outbreak isolates were resistant or intermediate resistant to ciprofloxacin (43% resistant, 43% intermediate resistant), 100% were resistant to tobramycin, 63% to gentamicin, 2% to amikacin, 100% to ceftriaxone, 12% to trimethoprim-sulfamethoxazole, and 0% to carbapenems. A total of 81 (98%) of the 83 isolates harbored an \( \text{aadB} \) containing integron.

IEF showed the presence of a \( \beta \)-lactamase with a pI of \( \approx 8.2 \), which suggested the presence of either an AmpC \( \beta \)-lactamase or a CTX-M type extended-spectrum \( \beta \)-lactamase. No AmpC-specific amplification products were obtained. Eighty-two (99%) of the 83 isolates harbored a \( \text{bla}_{\text{CTX-M}} \) gene. DNA sequencing showed the presence of \( \text{bla}_{\text{CTX-M-9}} \).

The plasmid (pQC) of conjugant \( \text{E. coli} \) C02-477A was isolated, and its size was estimated at 180 kb by agarose gel electrophoresis. Southern blotting that used specific probes confirmed that pQC contained the \( qnrA1 \) gene, the \( \text{bla}_{\text{CTX-M-9}} \) gene, and the integron with an \( \text{aadB} \) gene cassette (data not shown). Sequences flanking the \( qnrA1 \) and \( \text{bla}_{\text{CTX-M-9}} \) genes were comparable with 3 previously described class 1 integrons (Figure 1). The first integron (In-UMCU-1 accession no. AY987395), containing the \( qnrA1 \) gene, had the same additional structures as In36, \( \text{orf513} \), \( qnrA1 \), \( \text{ampR} \), plus a second copy of the 3′-conserved segment. The In36 integron contained the gene cassettes \( \text{drf16} \) and \( \text{aadA2} \), while In-UMCU-1 contained only the \( \text{aadB} \) gene cassette. In addition, the DNA sequences between the second \( \text{sul1} \) gene and \( \text{orf5} \) (bp 9606–9624 of In36) differed from the sequence of In-UMCU-1 (5). The second integron (In-UMCU-2, accession no. DQ108615), which contained \( \text{bla}_{\text{CTX-M-9}} \), was comparable to In60, but In60 contained the \( \text{drf16} \) and \( \text{aadA2} \) gene cassettes, while In-UMCU-2 contained the \( \text{aadB} \) gene cassette (21). The third integron (In-UMCU-3, accession no. DQ019420), which contained the gene cassettes \( \text{sat} \), \( \text{psp} \), and \( \text{aadA2} \), was described previously in an enterotoxigenic \( \text{E. coli} \) O159 isolated in Japan (22). PCR amplification of the \( \text{aadA2} \) gene of the donor, recipient, and transconjugants indicated that this third integron was also located on pQC.

### Evidence for Transfer of \( qnrA \) in vitro

In vitro conjugation experiments showed that pQC could be transferred both from and to the outbreak strain (Figure 2). pQC was successfully transferred from

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**Table. Oligonucleotides used for polymerase chain reaction amplification and sequencing**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>5′–3′ sequences</th>
<th>GenBank accession no.</th>
<th>Nucleotide positions</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>( QnrA )</td>
<td>( qnrR )</td>
<td>AGG AAG CGC CGC TGA GAT TG</td>
<td>AY070235</td>
<td>762–743</td>
<td>56</td>
<td>281</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>( qnrF )</td>
<td>CTA TGC CGA TCT GCG CTA TG</td>
<td>AY070235</td>
<td>482–501</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>( aadB )</td>
<td>( aadB )</td>
<td>TGG AGG AGT TGG ACT AT</td>
<td>AY173047</td>
<td>251–267</td>
<td>55</td>
<td>432</td>
<td>This study</td>
</tr>
<tr>
<td>( 3′CS )</td>
<td></td>
<td>AAG CAG ACT TGA CCT GA</td>
<td>M73619</td>
<td>1342–1326</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-uni-F} )</td>
<td>CTA TGC CGA TCT GCG CTA TG</td>
<td>U95364</td>
<td>214–232</td>
<td>50</td>
<td>538</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-uni-R} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-2F} )</td>
<td>ATG CTA ACT CAG AGC ATT CG</td>
<td>X92507</td>
<td>6–25</td>
<td>58</td>
<td>884</td>
<td>(16)</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-2R} )</td>
<td>TTA TG CAT GAA AAA CGG TG</td>
<td>X92507</td>
<td>889–870</td>
<td></td>
<td></td>
<td>(16)</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-10-1F} )</td>
<td>ATG GTT AAA AAA TCA CGT CG</td>
<td>X92506</td>
<td>63–82</td>
<td>60</td>
<td>872</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-10-4R} )</td>
<td>AAA CGG TG TGT GTG ACG AT</td>
<td>X92506</td>
<td>934–918</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-9F} )</td>
<td>AGA CGA GTG CGG TGC AGC AA</td>
<td>AJ416345</td>
<td>217–236</td>
<td>67</td>
<td>773</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{bla}_{\text{CTX-M-9}} )</td>
<td>( \text{ctx-m-9R} )</td>
<td>GAT TCT CGC CGC TGA AGC CA</td>
<td>AJ416345</td>
<td>989–970</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
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<td>( \text{ctx-m-9-1F} )</td>
<td>TGG TGA CAA AGA GAG TGC AAG GC</td>
<td>AJ416345</td>
<td>133–154</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>( \text{Sequence ( } \text{bla}_{\text{CTX-M-9}} ) group</td>
<td>( \text{ctx-m-9-MF} )</td>
<td>GGA GGC GTG ACG GCT TTT</td>
<td>AJ416345</td>
<td>576–593</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>( \text{Sequence ( } \text{bla}_{\text{CTX-M-9}} ) group</td>
<td>( \text{ctx-m-9-MR} )</td>
<td>AAA AGG CGT AAC CAC GCC TCC</td>
<td>AJ416345</td>
<td>593–576</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>( \text{Sequence ( } \text{bla}_{\text{CTX-M-9}} ) group</td>
<td>( \text{ctx-m-9-4R} )</td>
<td>TCA CAG CCC TTC GGC GAT</td>
<td>AJ416345</td>
<td>1007–990</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>
E. cloacae 02-477 to recipient E. coli K12. The resulting transconjugant E. coli was subsequently used as donor to transfer pQC to a type I A E. cloacae (03-702), which resulted in a successful transfer of pQC. pQC conferred increased ciprofloxacin MICs (from 6- to 10-fold) and resistance to tobramycin, tetracycline, and ceftriaxone to the transconjugants (Figure 2). Acquisition and loss of pQC were associated with 2 changes in the PFGE pattern.

Evidence for Transfer of \textit{qnrA1} in Vivo

Different species or strains collected from the same patient harbored the same pQC. From 22 of the 53 patients with an outbreak strain, 35 other tobramycin-resistant, gram-negative clinical isolates were available. Eleven different strains obtained from 11 patients were positive for \textit{qnrA1}, \textit{bla}_{CTX-M-9}, and \textit{aadB}-3′-CS. These comprised 4 different species: \textit{C. freundii} (n = 1), \textit{Enterobacter aerogenes} (n = 1), \textit{E. coli} (n = 7), and \textit{K. pneumoniae} (n = 2). Plasmid isolation from 6 \textit{E. coli} and 1 \textit{K. pneumoniae} yielded a plasmid of the same size as the pQC in the outbreak strain. Because of its large size and possibly a very low copy number, only small amounts of plasmid DNA could be isolated. These amounts were insufficient to perform further comparative analyses by restriction fragment analysis or Southern blotting.

Some \textit{E. cloacae} strains with a strong epidemiologic link to the outbreak strain were also pQC positive. All isolates belonging to clusters III, VII, and VIII contained pQC as well as 5 \textit{E. cloacae} isolates with a unique genotype. Plasmid isolation of 3 strains again showed a plasmid of the same size as the outbreak pQC. Three of the 5 unique isolates were obtained from patients who also harbored the outbreak strain.

The \textit{qnrA} gene, the \textit{aadB}-containing integron, and the \textit{bla}_{CTX-M-9} could not be detected in PFGE cluster IA, which is closely related to the outbreak strain (Figure 3). The loss of these genes was associated with increased susceptibility to ciprofloxacin, tobramycin, ceftriaxone, and tetracycline. In addition, an identical change in the PFGE pattern was observed, as in the in vitro experiments. These results suggest that the host may lose pQC in vivo.

\textbf{qnrA1 Recent Emergence as Clinical Problem}

pQC was not found in isolates obtained from patients without an epidemiologic link to the outbreak. No \textit{qnrA1} gene was detected in any of 83 aminoglycoside-resistant gram-negative organisms (44 \textit{E. coli}, 19 \textit{K. pneumoniae}, 4 \textit{Proteus mirabilis}, 2 \textit{Klebsiella oxytoca}, 2 \textit{E. cloacae}, 1 \textit{Enterobacter} sp., 7 \textit{C. freundii}, 4 \textit{Serratia marcescens}) obtained from 74 patients admitted to wards not involved in the outbreak during the outbreak period. Neither was \textit{qnrA1} detected in any of the 269 UMCU isolates or the 84 community isolates.

Only 1 \textit{qnrA1}-positive isolate was found in the 514 European isolates. This \textit{qnrA1}-positive isolate was an \textit{E. cloacae} organism isolated in 1999 at a surgical ward at UMCU that belonged to cluster III. The other 2 cluster III isolates were isolated at the same surgical ward during the outbreak period.

\textbf{Discussion}

We report a nosocomial outbreak with a R-plasmid–encoded \textit{qnrA1} gene. This plasmid (pQC) was first detected in an \textit{E. cloacae} isolated in 1999 and subsequently in another \textit{E. cloacae} strain that caused a large outbreak in our hospital, starting in 2001. Strong evidence is provided that this outbreak strain was the source from which pQC disseminated to other strains of the same species and other species by horizontal gene transfer. The \textit{qnrA1} gene was not detected in any of the hospital isolates (1994–2003) tested without an epidemiologic link to the
outbreak strain, indicating that qnrA1 is a new emerging resistance trait in our hospital.

pQC contained 3 different class 1 integrons. One integron was identical to an integron detected in an E. coli O159 from Japan (22). The 2 other integrons were complex integrons, In-UMCU-1 and In-UMCU-2, which were not described previously. Complex integrons are composed of a 5′-CS, gene cassettes, 3′-CS, qacΔE, sulII, additional genes, qacΔE, and sulII. These additional genes differ from gene cassettes by lacking a 59-bp element and having their own promoter (24). The qnrA1 gene in In-UMCU-1 was also present as an additional gene, as was the case for the 3 previous characterized qnrA1 genes in In36, In37, and the complex integron of pQR1 (5,9). The sequences of these genes were identical for In-UMCU-1, In36, and In37, and slightly different for the integron on pQR1. The gene cassette content of the 4 integrons, however, was different, although all 4 possessed a gene encoding aminoglycoside resistance. All qnrA1-positive isolates reported in the literature also show resistance to cephalosporins (1,4–9,25–27). Therefore, qnrA1 seems to be closely associated with resistance to cephalosporins and aminoglycosides. How these comparable but different complex integrons arose is unclear. Either the same additional genes became associated with different integrons or the gene cassettes in an original complex integron were exchanged.

Our study confirmed previous findings that the presence of qnrA1 does not necessarily lead to MICs above Clinical and Laboratory Standards Institute breakpoints for resistance to ciprofloxacin (1,3,7,25). Therefore, the presence of qnrA1 had no therapeutic consequences for the patients from whom these isolates were obtained. However, the increased MIC may provide the host bacterium a selective advantage in an environment of low concentrations of quinolones, increasing the bacterial numbers and therefore the absolute chance of a chromosomal mutation encoding resistance (7,25). The presence of a qnrA1-carrying plasmid might even enhance the mutation rate encoding quinolone resistance (1). Furthermore, acquisition of qnrA1 by a host bacterium that already contains quinolone resistance mechanisms may raise MICs above the LCSI breakpoints (25,28,29). As shown in this study, the same plasmid may cause fluctuation in susceptibility in MICs in different recipients because of variation in porin expression or mutations in the gyrase or efflux pump-encoding genes (2).

In conclusion, in a hospital setting the qnrA1 gene is advantageous for the host bacterium. Because of this gene’s location on promiscuous R-plasmids, it is likely to emerge worldwide.

Acknowledgments

We thank George A. Jacoby and Fred C. Tenover for providing control strains for detecting extended AmpCs and extended-spectrum β-lactamases.

Mr Paauw is a doctoral candidate at the Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, UMCU, the Netherlands. His research is focused on detecting and characterizing genetic features that can enhance virulence, resistance, and epidemic behavior of Enterobacteriaceae.

References


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