qnrD, a Novel Gene Conferring Transferable Quinolone Resistance in Salmonella enterica Serovar Kentucky and Bovismorbificans Strains of Human Origin

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L. M. Cavaco, H. Hasman, S. Xia, and F. M. Aarestrup

Research Group for Antimicrobial Resistance and Molecular Epidemiology, National Food Institute, Technical University of Denmark, Copenhagen V, Denmark; Department of Veterinary Pathobiology, Faculty of Life Science, University of Copenhagen, Denmark; and Branch for Enteric Disease Control and Prevention, Henan, Center for Disease Control and Prevention, Nongye East Road, East New District, Zhengzhou, Henan 450016, People’s Republic of China

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In a previous study, four Salmonella isolates from humans in the Henan province of China showed reduced susceptibility to ciprofloxacin (MIC, 0.125 to 0.25 µg/ml) but were susceptible to nalidixic acid (MIC, 4 to 8 µg/ml). All isolates were negative for known qnr genes (A, B, and S), aac(6’)/Ib-cr, and mutations in gyrA and parC. Plasmid DNA was extracted from all four isolates and transformed into Escherichia coli TG1 and DH10B cells by electroporation, and transformants were selected on 0.06 µg/ml ciprofloxacin containing brain heart infusion agar plates. Resistance to ciprofloxacin could be transferred by electroporation, and a similar 4,270-bp plasmid was found in all transformants. By sequence analysis, the plasmid was found to carry an open reading frame that had similarities to other qnr genes and that encoded a 214-amino-acid pentapeptide repeat protein. This gene, designated qnrD, showed 48% similarity to qnrA, 61% similarity to qnrB1, and 41% similarity to qnrS1. Further subcloning of the qnrD coding region into the constitutively expressed tetA gene of vector pBR322 showed that the gene conferred an increase in the MIC of ciprofloxacin by a factor of 32 (from an MIC of 0.002 to an MIC of 0.06 µg/ml). For comparison, qnrA1 and qnrS1 were also subcloned into pBR322 and transformed into DH10B cells, conferring MICs of 0.125 and 0.5 µg/ml, respectively. A phylogenetic analysis of all known qnr sequences was performed and showed that qnrD was more closely related to the qnrB variants but formed an independent cluster. To our knowledge, this is the first description of this qnrD gene.

Quinolone resistance in the Enterobacteriaceae is mostly mediated by point mutations in the quinolone resistance-determining regions (QRDR) of the gyrase and topoisomerase IV genes, leading to a target modification. Other resistance mechanisms include efflux pump mechanisms, and more recently, target protection mechanisms encoded by the qnr genes and enzymatic modifications encoded by aac(6’)/Ib-cr have also been found to contribute to resistance to drugs belonging to this antimicrobial class. First, qnrA1 from a clinical strain of Klebsiella pneumoniae isolated in Alabama was described by Martinez-Martinez et al. in 1998 (17). This strain carried plasmid pMG252, which contained the gene encoding quinolone resistance, later named qnrA1 (17).

In 2005, Hata et al. described another qnr gene from a Shigella flexneri 2b isolate isolated during an outbreak of food poisoning in Japan. This strain contained a plasmid, designated pAH0376, containing a gene with high similarity to qnr, which was designated qnrS. The QnrS protein was also a 214-amino-acid protein with 59% similarity to QnrA that conferred low-level resistance to fluoroquinolones (11).

In 2006, Jacoby and colleagues described a third gene encoding quinolone resistance, qnrB. This gene was first found in a Klebsiella pneumoniae isolate from India and encoded a 214-amino-acid protein of the pentapeptide repeat family, which had 41% amino acid identity with qnrA and 39% amino acid identity with qnrS (14).

To date, a total of 6 qnrA, 4 qnrS, and 20 qnrB variants have been described in the literature and are listed in the database maintained at the website http://www.lahey.org/qnrStudies (13). Furthermore, other similar chromosomal genes such as Vibrio vulnificus qnr, Vibrio parahaemolyticus qnr, Vibrio cholerae qnr, Photobacterium profundum qnr, Enterococcus faecalis qnr, and Enterococcus faecalis qnr were described previously (1, 3, 9, 13, 19–22).

Later, other transferable resistance genes were found to cause reduced susceptibility to quinolones. The aminoglycoside acetyltransferase variant aac(6’)/Ib-cr is able to modify ciprofloxacin and norfloxacin. Moreover, in 2007, in Japan, but also in Belgium, another gene, qepA, was discovered to encode a putative specific efflux pump, which is able to reduce susceptibility to hydrophilic quinolones (18, 26). Furthermore, a second variant, named qep42, from France was recently described (4).

Recently, Wang and colleagues described another qnr gene, qnrC, which was found in Proteus mirabilis; however, its sequence is not yet publicly available, but it was found that qnrC encodes a 221-amino-acid protein with different amino acid identities from qnrD, which indicates that the gene is different from qnrD (25).

Here, we report the finding of a plasmid containing a novel...
quino lol resistance gene, qnrD, which has been found to cause reduced susceptibility to fluoroquinolones in isolates of Salmonella enterica serovar Bovismorbificans and Kentucky strains isolated from humans in the Henan province of China. The complete plasmid was sequenced, and the novel qnrD gene was cloned along with both the qnrA1 and qnrS1 genes, which were cloned for comparisons of the susceptibility patterns in vitro. The novel qnrD gene shares similarities with the previously described qnr genes and encodes a putative pentapeptide repeat protein that is able to confer reduced susceptibility to fluoroquinolones. A phylogenetic analysis shows that it clusters separately from the known qnr genes and variants.

**MATERIALS AND METHODS**

**Strains.** Salmonella enterica strains HN-GSS2006-142, HN-GSS2007-0.033, HN-GSS2007-0.056, and HN-GSS2007-0.057 were isolated in 2006 to 2007 from human infections in China in the Henan province. These strains belonged to serovars Kentucky (HN-GSS2007-0.033) and Bovismorbificans (remaining strains) and were included in this study based on their resistance pattern (low-level quinolone resistance gene, qnr).

**Susceptibility testing.** MICs were determined in broth microdilution assay for ciprofloxacin, nalidixic acid, norfloxacin, and ofloxacin according to Clinical and Laboratory Standards Institute (CLSI) standards (8) by using 96-well plates (Sensititre; Trek Diagnostics), and self-made panels were used for the wider range of dilutions of ciprofloxacin, nalidixic acid, norfloxacin, and ofloxacin.

**DNA preparation and transformation.** Plasmid DNA was extracted from all strains using the Qiagen (Hilden, Germany) Midi kit. Initial transformation experiments were performed by electroporation (Gene Pulser; Bio-Rad) of the plasmid DNA into competent TG1 cells (Stratagene, Cambridge, United Kingdom). Transformants were selected on brain heart infusion agar plates containing 0.06 μg/ml ciprofloxacin. Plasmid DNA from transformants was extracted and restricted with several restriction enzymes (EcoRV, HindIII, EcoRI, and SmaI) (New England Biolabs, Hitchin, United Kingdom) to observe restriction patterns and choose an enzyme for the restriction of fragments before cloning (data not shown).

**Cloning.** For restriction and cloning experiments, the plasmid DNA of Salmonella enterica serovar Bovismorbificans strain GSS-HN-2007-0.057 was used. Plasmid DNA of the transformant TI1-HN-GSS-2007-0.057 was restricted using EcoRV (New England Biolabs, Hitchin, United Kingdom). The restriction product containing the plasmid fragments was purified using a QIAquick purification kit (Qiagen, Valencia, CA) and ligated into pACYC177 vector DNA (New England Biolabs, Hitchin, United Kingdom) digested with SmaI (New England Biolabs, United Kingdom) and dephosphorylated with shrimp alkaline phosphatase (USB Corporation). The ligation products of pACYC177 with the inserts were electroporated into competent E. coli TG1 cells (Stratagene, Cambridge, United Kingdom), selected on ampicillin (50 μg/ml)- and on ciprofloxacin (0.06 μg/ml)-containing plates, and replicated on kanamycin (50 μg/ml)-containing brain heart infusion agar plates (Becton Dickinson, Sparks, MD) to identify inserts.

**Total plasmid sequencing.** The cloned fragments were amplified using plasmid DNA as a template for a long PCR (Phusion high-fidelity PCR kit; Finnzymes, Espoo, Finland) with primers designed with the kanamycin resistance gene to amplify the insert and the flanking regions of the SmaI restriction site (Table 1) with HF buffer (Phusion high-fidelity PCR kit; Finnzymes, Espoo, Finland) and ligated into pACYC177 vector DNA (New England Biolabs, Hitchin, United Kingdom). The restriction product containing the plasmid fragments was amplified using a QIAquick purification kit (Qiagen, Valencia, CA) and ligated into pACYC177 vector DNA (New England Biolabs, Hitchin, United Kingdom) digested with SmaI (New England Biolabs, United Kingdom) and dephosphorylated with shrimp alkaline phosphatase (USB Corporation). The ligation products of pACYC177 with the inserts were electroporated into competent E. coli TG1 cells (Stratagene, Cambridge, United Kingdom), selected on ampicillin (50 μg/ml)- and on ciprofloxacin (0.06 μg/ml)-containing plates, and replicated on kanamycin (50 μg/ml)-containing brain heart infusion agar plates (Becton Dickinson, Sparks, MD) to identify inserts.

**TABLE 1. Primers used for amplification/sequencing and cloning of plasmid p2007057**

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<thead>
<tr>
<th>Primer</th>
<th>Sequencea</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>PACYC177 SmaI insert fw</td>
<td>5'-CGTACTCCCTGATGATGCATG-3'</td>
<td>Seq inserted fragment</td>
</tr>
<tr>
<td>PACYC177 SmaI insert rev</td>
<td>5'-GGCCTCAAAATTTGAGATTTAC-3'</td>
<td>Seq inserted fragment</td>
</tr>
<tr>
<td>P1</td>
<td>5'-GCTTTTTACATTGGATTTTTCT-3'</td>
<td>Seq plasmid</td>
</tr>
<tr>
<td>P2</td>
<td>5'-CGTCTTCTGCCTACAAAAAT-3'</td>
<td>Seq plasmid</td>
</tr>
<tr>
<td>P3</td>
<td>5'-CGTATGAACTCCTGTTATACAGG-3'</td>
<td>Seq plasmid</td>
</tr>
<tr>
<td>P4</td>
<td>5'-TGCTAACCTGCTTATATACGG-3'</td>
<td>Seq plasmid</td>
</tr>
<tr>
<td>P6</td>
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<td>P7</td>
<td>5'-AGGCGGGAAGTCTCAAAATG-3'</td>
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<td>P8</td>
<td>5'-ATATCGACAGTGTTGTGATC-3'</td>
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<td>5'-CCTGAAAGGCCGTACG-3'</td>
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<td>5'-AAGTCTCACACTCTGCTTAG-3'</td>
<td>Seq plasmid</td>
</tr>
<tr>
<td>P11</td>
<td>5'-CTTTTGAGACTCCTGGCTC-3'</td>
<td>Seq plasmid</td>
</tr>
<tr>
<td>P12</td>
<td>5'-CGTGTGCAACTTGCTGATAT-3'</td>
<td>Cloning qnrD</td>
</tr>
<tr>
<td>qnrD start EcoRV</td>
<td>5'-GGGGATACCTTTAAAGGTTGTATTTACATGGATC-3'</td>
<td>Amplification of qnrD gene</td>
</tr>
<tr>
<td>qnrD end Sall</td>
<td>5'-CCCCGTCGACTTGTAGTACACCATTG-3'</td>
<td>Cloning qnrD</td>
</tr>
<tr>
<td>qnrD rev</td>
<td>5'-AAACAGCTGAGCCGCTG-3'</td>
<td>Amplification of qnrD gene</td>
</tr>
<tr>
<td>qnrA start EcoRV</td>
<td>5'-CCCATATGCTTTGAATTTGGGTG-3'</td>
<td>Cloning qnrA1</td>
</tr>
<tr>
<td>qnrA end Sall</td>
<td>5'-CGGCGATCGTCCCTTACAAAGGGTAC-3'</td>
<td>Cloning qnrA1</td>
</tr>
<tr>
<td>qnrS start PvuII</td>
<td>5'-CCCGCTGACATTAGTCAGGATATACACAACA-3'</td>
<td>Cloning qnrS1</td>
</tr>
<tr>
<td>qnrS end Sall</td>
<td>5'-CCCCGTCGACATTAGTCAGGATATACACAACA-3'</td>
<td>Cloning qnrS1</td>
</tr>
</tbody>
</table>

* The recognition sites for the restriction enzymes are underlined in the nucleotide sequences of the primers used for cloning.
plasmid DNA obtained from the transformants to observe the similarities between the plasmids that were isolated from the original isolates and p2007057. The fragments obtained in the amplification rounds were sent for sequencing at Macrogen Laboratories (Seoul, South Korea).

Sequence analysis. Sequence analysis was performed using the Vector NTI program tools Contig Express and AlignX (VectorNTI Suite Informax, Inc.). BLAST searches were performed using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) using both the blastn and the tblastx algorithms.

Phylogenetic analysis. All representative sequences of the known qnr genes described above were obtained from GenBank in FASTA format. Phylogenetic analysis was conducted using the neighbor-joining method using MEGA 4 software, version 4 (23).

Cloning of the qnrD, qnrA1, and qnrS1 genes. Subcloning of the gene and flanking regions was performed to confirm if it conferred the observed quinolone resistance phenotype. Primers were designed to amplify the entire ORF and some of the flanking region (62 nucleotides upstream and 46 nucleotides downstream) carrying an EcoRV restriction site in the forward primer and a SalI site in the reverse primer (Table 1). PCR was performed in a 50-μl PCR mixture including 5 μl 10× reaction buffer (Ampliqon), 0.5 μl forward and reverse primer, 0.5 μl deoxynucleoside triphosphates, 1.5 mM MgCl2, 0.2 μl Taq polymerase (Ampliqon), and 2 μl DNA template. PCR was run with the same program using a Trio thermocycler (Biometra): 94°C for 5 min; 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 30 cycles; and 72°C for 10 min. The amplified fragment digested with EcoRV and SalI (New England BioLabs, Hitchin, United Kingdom) was then ligated into the tetA gene of vector pBR322 (New England Biolabs, Hitchin, United Kingdom) downstream of the tetA promoter digested with EcoRV and SalI (New England Biolabs, Hitchin, United Kingdom). The ligation product was electroporated into competent electrocompetent TG1 cells with the extracted plasmid DNA obtained from isolate GSS-HN-2007-033, which was considered and divided into pentapeptide repeats. The conserved amino acids according to the consensus sequence (A/C/S/T/V)(D/N)(L/F)(S/T/R)(G/R) (22) are in boldface type, and the most characteristic pentapeptide units are underlined.

FIG. 1. QnrD amino acid sequence. (a) Alignment of amino acid sequences encoded by the qnrA1, qnrB1, qnrS1, and qnrD genes obtained using AlignX with Vector NTI software. (b) Hypothetical structure of the QnrD protein. The amino acid sequence was represented and divided into pentapeptide repeats. The conserved amino acids according to the consensus sequence (A/C/S/T/V)(D/N)(L/F)(S/T/R)(G/R) (22) are in boldface type, and the most characteristic pentapeptide units are underlined.

RESULTS

Susceptibility testing. The Salmonella enterica serovar Kentucky (n = 1) and Bovismoribificans (n = 3) isolates included in this study showed reduced susceptibility to ciprofloxacin (MIC, 0.125 or 0.25 μg/ml) but were susceptible to nalidixic acid (MIC = 8 μg/ml). All four isolates were susceptible to the remaining drugs tested.

All PCR screenings performed previously for qnrA, qnrB, qnrS, aac(6′)Ib-cr, and qepA were negative for all four isolates.

No amino acid substitutions were found in the QRDR of the gyrA or parC topoisomerase gene. One amino acid substitution outside of the QRDR in the parC gene (T57S) was detected in all but one isolate (GSS-HN-2007-033). This mutation was previously described but not likely related to quinolone resistance since it was found in susceptible strains previously (2).

Plasmid extraction and transfer of resistance. All four strains contained one or several small plasmids as observed by plasmid extraction. The transfer of resistance by the transformation of electrocompetent TG1 cells with the extracted plasmid DNA was successful for all the strains. The transformants obtained were able to grow on plates containing 0.06 μg/ml ciprofloxacin. Electrophoresis of plasmid DNA extracts from the transformants showed the presence of a small plasmid of about 4.3 kb in all transformants obtained and an additional small-sized plasmid that was cotransferred to a transformant obtained from isolate GSS-HN-2007-033, which was consid-
ered not to be related to the resistance phenotype observed since it was present in only one of the transformants, and no difference in their phenotypes was noted for the other transformants containing only the 4.3-kb plasmid. Further cloning and sequencing were proceeded on the 4.3-kb plasmid, which was suspected to contain the quinolone resistance determinant.

Restriction, cloning, and sequencing of plasmid. Restriction of plasmid DNA with EcoRV resulted in two distinct fragments of about 3.2 kb and 1.1 kb. All isolates carried a plasmid with a similar restriction pattern using the EcoRV enzyme, and due to subsequent PCR mapping, we believe that the plasmids carried by the other isolate were similar to the plasmid that was cloned and sequenced.

Cloning of the digested plasmid DNA from strain HN-GSS-2007-057 into the SmaI site located in the aph(3’)-Ia kanamycin resistance gene of vector plasmid pACYC177 allowed us to select two ampicillin-resistant and kanamycin-susceptible clones carrying a fragment of about 3.2 kb.

Amplification and sequencing of the cloned fragment in both directions showed that the same fragment (although in inverted positions) was present in both selected clones. Further sequencing resulted in the full sequencing of the 3.2-kb fragment, which was suspected to contain the quinolone resistance determinant, but also of the remaining 1.1 kb-portion of the plasmid, which was totally sequenced and assembled and was deposited in the GenBank library under accession number FJ228229. The sequence of the cloned 3.2-kb fragment contained an ORF encoding a 214-amino-acid protein that showed similarity to previously identified qnr genes. Its nucleotide sequence showed 45% similarity to qnrA1, 65% similarity to qnrB1, and 38% similarity to qnrS1. The nucleotide and translated amino acid sequences were compared to the those of qnr genes known and aligned by using Clustal W for comparisons (Fig. 1).

Phylogenetic analysis. The gene found was, to our knowledge, unknown but was related to the known qnr genes and encoded a putative protein of the pentapeptide repeat family, and therefore, we have conducted a molecular evolutionary genetic analysis using MEGA 4 software according to methods described previously by Tamura et al. (23), which is represented as a phylogenetic tree (Fig. 2).
The qnr gene variants clustered in the different major gene groups. However, the novel gene clustered separately from the major groups but showed a closer similarity to the qnrB group.

Total plasmid sequencing. In the plasmid sequence analysis, we observed that it contained five ORFs, as shown in Fig. 3. Apart from the qnrD gene, no other known structures and no further resistance genes have been found in the plasmid sequence (Fig. 3). Surprisingly, we were also unable to locate any similarity to replication proteins or repeat sequences previously deposited in the GenBank database.

Susceptibility testing of transformants with natural and cloned plasmid clones. The MIC of ciprofloxacin in E. coli DH10B cells increased in the transformant carrying the plasmid from strain GSS-HN-2007-057 and also in the strain carrying plasmid pBR322 with the cloned qnrD gene from 0.002 μg/ml to 0.06 μg/ml (by a factor of 32). The increase in the MIC of ciprofloxacin was slightly higher for E. coli DH10B cells carrying pBR322 with qnrA1 (MIC = 0.125 μg/ml) and E. coli DH10B cells carrying pBR322 with qnrS1 (MIC = 0.5 μg/ml). The MIC of norfloxacin increased from 0.015 μg/ml to 0.25, 0.5, and 0.06 μg/ml for qnrA, qnrS, and qnrD, respectively, and the MIC of ofloxacin increased from 0.015 μg/ml to 0.25, 0.5, and 0.125 μg/ml, respectively. The changes in the MIC of nalidixic acid were smaller than those observed for the fluoroquinolones (only 1 to 2 dilution steps) in all strains carrying qnr genes (Table 2).

<table>
<thead>
<tr>
<th>Antimicrobial drug</th>
<th>E. coli</th>
<th>E. coli</th>
<th>E. coli</th>
<th>E. coli</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DH10B</td>
<td>DH10B(pBR322)</td>
<td>DH10B(pBR322)</td>
<td>DH10B(pBR322)</td>
<td>DH10B(pBR322)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
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<td>0.004</td>
<td>0.125</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.015</td>
<td>0.015</td>
<td>0.25</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Ofloxacin</td>
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<td>0.032</td>
<td>0.25</td>
<td>0.5</td>
<td>0.125</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2</td>
<td>2</td>
<td>8 (4)</td>
<td>8 (4)</td>
<td>4 (2)</td>
</tr>
</tbody>
</table>

* All MIC determinations were performed in broth microdilution assays according to CLSI standards (8).

DISCUSSION

We have identified and cloned a novel gene conferring resistance to ciprofloxacin in four Salmonella enterica serovar Bovismorbificans and Kentucky isolates obtained from human infections in the Henan province in China (25). qnrD showed similarities to qnrA, qnrB, and qnrS genes and encoded a 214-amino-acid pentapeptide repeat protein. The phylogenetic analysis showed that it clustered independently from the known qnr gene variants but shared the highest similarity with the qnrB variants.

It was demonstrated that the plasmid and also the cloned gene were able to confer an increase in the MIC of ciprofloxacin by a factor of about 32 without a major increase in the MIC of nalidixic acid, which is within the expected phenotype of a qnr-related gene. However, the comparison with the cloned qnrA and qnrS genes showed that qnrD resulted in slightly lower increases in the MIC.

Previously known qnr genes have been found in Salmonella isolates of different origins; however, these genes seem to be carried by different genetic elements (5, 12, 15). Previously sequenced plasmids carrying qnr genes showed different backbones and possibilities of origin (7, 10, 15, 16). Furthermore, the qnr genes have been related to integron structures; however, the plasmid found to carry this novel gene showed a small size and carried no integron structures or further resistance determinants.

Until recently, qnr genes have been observed at low prevalences in most reported screenings; however, further genes and variants might still be discovered, increasing the pool of determinants conferring resistance to quinolones. Although their clinical implications are still unknown, the spread of such resistance determinants is concerning.

ACKNOWLEDGMENTS

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