Molecular analysis of low-level fluoroquinolone resistance in clinical isolates of *Moraxella catarrhalis*

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We investigated antimicrobial susceptibility and the molecular mechanism underlying low-level resistance to fluoroquinolones in 70 non-duplicate clinical isolates of *Moraxella catarrhalis*. The isolates were collected in a general hospital in Tokyo, Japan, between January and October 2013 from 38 men and 32 women; most of the isolates (48 out of 70, 68.5%) were obtained from post-nasal drips of children. The antimicrobial susceptibility of *M. catarrhalis* isolates was determined with an Etest, and low-level fluoroquinolone-resistant isolates were subtyped by PFGE. Mutations in the *gyrA* and *parC* genes were determined by PCR and sequencing. PCR products of the *gyrA* and *parC* genes from the low-level fluoroquinolone-resistant isolates were transformed into a fluoroquinolone-susceptible strain. Among the 70 isolates, five (7.1%) exhibited elevated fluoroquinolone MICs (levofloxacin, 1.0 mg l⁻¹; ciprofloxacin, 0.5 mg l⁻¹) and different PFGE patterns. The patients from whom these five isolates were isolated had not undergone treatment with fluoroquinolones for the past 6 months. Each of the five low-level fluoroquinolone-resistant isolates had a *gyrA* gene mutation resulting in a Thr-to-Ile substitution at aa 80 (T80I) in the GyrA protein, while no changes were detected in the *parC* gene. A transformant carrying the *gyrA* gene containing the T80I substitution, which corresponded to Ser83 in *Escherichia coli*, displayed an elevated fluoroquinolone MIC and contained the T80I alteration in GyrA. Thus, our findings reveal that the low-level resistance to fluoroquinolones in *M. catarrhalis* is due to an amino acid substitution of Thr80 to Ile in GyrA. This is the first evidence of low-level fluoroquinolone resistance in *M. catarrhalis*.

**INTRODUCTION**

*Moraxella catarrhalis* is a Gram-negative aerobic diplococcus that frequently colonizes the post-nasal cavity in children. It is one of the pathogens responsible for upper and lower respiratory tract infections, conjunctivitis and otitis media (Catlin, 1990). Fluoroquinolones are very effective for treating these infections, and fluoroquinolone regimens have been increasingly used in various countries including Japan (Jacoby, 2005).

Fluoroquinolones act primarily by inhibiting DNA gyrase and DNA topoisomerase IV (Drlica & Zhao, 1997). These two enzymes work together in the replication, transcription, recombination and repair of DNA, which are essential for bacterial growth. Both are large, complex enzymes composed of two pairs of subunits. The subunits of DNA gyrase are GyrA encoded by the *gyrA* gene and GyrB encoded by the *gyrB* gene (Jacoby, 2005). The corresponding subunits of topoisomerase IV are ParC and ParE, which are encoded by the *parC* and *parE* genes, respectively (Jacoby, 2005). In Gram-negative cocci, such as *Neisseria gonorrhoeae* and *Neisseria meningitidis*, mechanisms of fluoroquinolone resistance involve amino acid substitutions in the quinolone-resistance-determining region (QRDR) of GyrA and ParC (Belland *et al.*, 1994; Deguchi *et al.*, 1995; Wang *et al.*, 2006; Shultz *et al.*, 2005) and reduction of fluoroquinolone accumulation in the cells (Tanaka *et al.*, 1994).

Although *M. catarrhalis* strains are generally susceptible to various antimicrobials including fluoroquinolones (Hoban *et al.*, 2001; Watanabe *et al.*, 2012), fluoroquinolone-resistant *M. catarrhalis* strains (with levofloxacin MICs of >2 mg l⁻¹) have been reported from the USA and Taiwan.
(DiPersio et al., 1998; Hsu et al., 2012). However, to date, the precise mechanism of fluoroquinolone resistance in M. catarrhalis has not been reported, since fluoroquinolone-resistant isolates of this species are rarely isolated. Therefore, we investigated the molecular mechanisms of low-level resistance to fluoroquinolone in M. catarrhalis. Our report is the first describing the mechanisms resulting in low-level resistance to fluoroquinolone in M. catarrhalis.

**METHODS**

**Bacterial strains.** A total of 70 non-duplicate clinical isolates of M. catarrhalis were used in this study. They were isolated from post-nasal drip, sputum, and otorrhoea specimens between January and October 2013 at the Tokyo Metropolitan Health and Medical Corporation Toshima Hospital, Tokyo. Isolates were identified using Gram staining and an ID Test-HN-20 Rapid ‘Nissui’ (Nissui). All isolates and the reference strain ATCC 49143 were maintained in 10% skimmed milk and stored at −80°C.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing for levofloxacin and ciprofloxacin was examined using an Etest (AB Biodisk) on Mueller–Hinton agar plates containing 5% sheep blood, according to the manufacturer’s recommendations. *Staphylococcus aureus* ATCC 29213 was used as a control. The breakpoints used for categorization of susceptibility as defined by the Clinical and Laboratory Standards Institute (CLSI), were ≤2.0 mg l⁻¹ for levofloxacin and ≤1.0 mg l⁻¹ for ciprofloxacin (CLSI, 2010). Furthermore, levofloxacin susceptibility of five low-level fluoroquinolone-resistant M. catarrhalis isolates (levofloxacin MIC, 1 mg l⁻¹) and their transformants was also determined using the disk-diffusion method (5 µg levofloxacin; BD).

**PFGE.** The isolates were analysed by PFGE, using a previously described procedure (Martí et al., 2013). Briefly, each DNA preparation was digested with SpeI (New England Biolabs) overnight at 37°C in 300 µl restriction endonuclease buffer containing 2.5 U enzyme. The Gene Path system (Bio-Rad Laboratories) was used to separate the DNA fragments.

**PCR and DNA sequencing of gyrA and parC genes.** PCR amplification and direct sequencing were performed to identify mutations in the QRDRs (aa 6–201 of GyrA and 5–191 of ParC, *Escherichia coli* numbering; GenBank accession no. U00096) of the gyrA and parC genes in low-level fluoroquinolone-resistant *M. catarrhalis* isolates (S22, S53, S62, S68 and S70). Primers to amplify the two genes were designed using the *M. catarrhalis* BBH DNA sequence (GenBank accession no. CP002005) (Table 1). DNA extraction was performed by boiling. PCR amplification of the gyrA and parC genes was performed using PrimeSTAR HS DNA polymerase (TaKaRa) on a Gene Amp PCR System 9600-R thermal cycler (Perkin-Elmer) and using the following cycling conditions: 94°C denaturation for 2 min, followed by 32 cycles of denaturation at 94°C for 10 s, annealing at 56°C for 5 s and extension at 72°C for 45 s, with a final extension at 72°C for 2 min. PCR products were purified using Wizard SV Gel and the PCR Clean-Up systems (Promega) and then sequenced using a 3730 DNA Analyzer (Applied Biosystems). A similarity search for the deduced amino acid sequences was conducted using BLAST. Alignments of peptide sequences with the reference sequences of *M. catarrhalis* BBH (GenBank accession no. CP002005), *N. gonorrhoeae* FA1090 (AE004969), *N. meningitidis* Z2491 (AL157999) and *E. coli* K-12 (U00096) were performed using the BioEdit software to identify substitution positions.

**Transformation experiments.** Transformation experiments were performed as described previously by Saito et al. (2012) with some modification. Briefly, as a recipient strain, the fluoroquinolone-susceptible *M. catarrhalis* ATCC49143 was suspended in 1 ml brain heart infusion (BHI) broth to the equivalent of a McFarland standard of 1.0 and incubated for 1 h at 35°C. A PCR product (approx. 1.5 µg) containing the gyrA gene with the T80I mutation was generated by using primers for amplification of gyrA gene from the low-level fluoroquinolone-resistant isolate S22, purified and added to the incubation broth. The purified PCR product of the parC gene in S22 was used as the negative control in this experiment. After incubation for 10 h, the broth was centrifuged at 6000 g at 25°C for 5 min, and the pellet was plated on BH agar plate containing levofloxacin (0.5, 1 and 2 mg l⁻¹). After incubation overnight, susceptibility to levofloxacin and mutations in the gyrA and parC genes were determined for colonies that grew on the plate, as described above.

**RESULTS AND DISCUSSION**

In total, 70 non-duplicate *M. catarrhalis* isolates were collected from 38 males and 32 females. Rates of isolation of *M. catarrhalis* from each specimen were as follows: 48 isolates (68.5%) from post-nasal drip, 20 (28.6%) from sputum, and 1 (1.4%) each from otorrhoea and eye discharge. Fifty (71.4%) *M. catarrhalis* isolates were recovered from young children (10 years old or younger) and 20 (28.6%) from children older than 10 years and from adults.

Five of the *M. catarrhalis* isolates (7.1%; S22, S53, S62, S68 and S70) exhibited elevated fluoroquinolone MICs (levofloxacin, 1 mg l⁻¹; ciprofloxacin, 0.5 mg l⁻¹; Fig. 1). All of the five isolates were obtained from post-nasal drips of patients who were 6 years of age or younger, and who had not been treated with fluoroquinolones in the past 6

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Position*</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>F-Mc_gyrA</td>
<td>ATTCGGTCAGTCCAAATTG</td>
<td>8</td>
<td>588</td>
</tr>
<tr>
<td></td>
<td>R-Mc_gyrA</td>
<td>TTGGATCTTCGGGTATG</td>
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<td></td>
</tr>
<tr>
<td>parC</td>
<td>F-Mc_parC</td>
<td>GATTGATACCTGTTCCGTG</td>
<td>12</td>
<td>564</td>
</tr>
<tr>
<td></td>
<td>R-Mc_parC</td>
<td>CAGACCTTGGACAACCTCGTG</td>
<td>575</td>
<td></td>
</tr>
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</table>

*Primer positions were determined by the start codon of gyrA and parC genes in *M. catarrhalis* BBH complete genome (GenBank accession no. CP002005).
months. Notably, these isolates differed in PFGE patterns from one another (data not shown). The diameter of the inhibition zone for levofloxacin was 22–23 mm in the five isolates, in comparison with 34 mm in the fluoroquinolone-susceptible strain, ATCC49143 (Table 2). Isolates with MICs exceeding the MIC breakpoint for fluoroquinolones were not isolated in this study.

The nucleotide sequences and predicted amino acid sequences in the QRDRs of the \textit{gyrA} and \textit{parC} genes for the five low-level fluoroquinolone-resistant isolates were compared with those of the wild-type \textit{M. catarrhalis} strain ATCC49143 (Table 2). All five isolates contained a C239T mutation in the \textit{gyrA} gene sequence that was predicted to result in a Thr-to-Ile substitution at aa 80 (T80I) of GyrA. A comparison with the GyrA sequences in \textit{N. gonorrhoeae}, \textit{N. meningitidis} and \textit{E. coli} is shown in Fig. 2. The T80I change in GyrA in the five isolates corresponded to Ser91 in \textit{N. gonorrhoeae}, Thr91 in \textit{N. meningitidis} and Ser83 in \textit{E. coli}. We did not identify mutations in the sequences of the \textit{parC} gene in the five isolates.

The \textit{gyrA} gene predicted to result in a GyrA protein with the T80I substitution was amplified by PCR from the low-level fluoroquinolone-resistant isolate S22. Transformation of the fluoroquinolone-susceptible ATCC49143 strain with this PCR product resulted in more than 100 colonies on a BHI agar plate containing levofloxacin at a concentration

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**Fig. 1.** Result of antimicrobial susceptibility testing for the 70 clinical isolates of \textit{M. catarrhalis} investigated in this study.

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**Table 2.** \textit{gyrA} gene mutations in five low-level fluoroquinolone-resistant isolates (S22, S53, S62, S68 and S70) and in one \textit{gyrA}-mutated transformant

The table shows the position and effects of the \textit{gyrA} gene mutation on the amino acid sequence of GyrA, and on MICs and the size of the inhibition zone for the fluoroquinolone levofloxacin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Codon*</th>
<th>Levofloxacin</th>
<th>Diameter of inhibition zone (mm)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77</td>
<td>80</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>His</td>
<td>Gly</td>
<td>Asp</td>
</tr>
<tr>
<td></td>
<td>CAT</td>
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<td>GAT</td>
</tr>
<tr>
<td>ATCC49143</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>S22</td>
<td>- - -</td>
<td>- - Ile (-T-)</td>
<td>- -</td>
</tr>
<tr>
<td>S53</td>
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<td>- -</td>
</tr>
<tr>
<td>S62</td>
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<tr>
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<td>S70</td>
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<td>- -</td>
</tr>
<tr>
<td>49143TF</td>
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<td>- - Ile (-T-)</td>
<td>- -</td>
</tr>
<tr>
<td></td>
<td>0.047</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

*The position of the mutation in the \textit{gyrA} gene sequence in the five isolates is C239 (C239T).
†The disk contained 5 μg levofloxacin.
of 0.5 mg l\(^{-1}\), while no colonies grew on plates with levofloxacin concentrations of \(\geq 1\) mg l\(^{-1}\). The transformant 49143TF with the T80I substitution in GyrA exhibited a levofloxacin MIC of 1 mg l\(^{-1}\) and a smaller zone of inhibition (22 mm) than ATCC49143 (Table 2). The parC gene sequence in the gyrA transformants was identical to that in the parental strain.

Fluoroquinolones with broad-spectrum bactericidal activities have been widely used to treat various infections. However, the increased prevalence of fluoroquinolone-resistant bacteria has recently become a serious health concern worldwide (Belland et al., 1994; Deguchi et al., 1995; Wang et al., 2006; Shultz et al., 2005). In this study, although all isolates were susceptible to fluoroquinolone by CLSI criteria, 7.1\% (5/70) exhibited elevated MICs to fluoroquinolones (Fig. 1). To date, \textit{M. catarrhalis} isolates that are resistant or less susceptible to fluoroquinolones have not been reported in Japan. However, our findings suggest that, in the near future, \textit{M. catarrhalis} isolates may confer fluoroquinolone resistance to other clones. Since this study reports frequency of fluoroquinolone resistance only in a specific region, we plan to continue our studies using isolates isolated from different hospitals.

To date, the breakpoint criterion for disk diffusion in fluoroquinolones for \textit{M. catarrhalis} has not been defined in CLSI. In this study, low-level fluoroquinolone-resistant \textit{M. catarrhalis} displayed a levofloxacin inhibition zone that was 22–23 mm in diameter. Therefore, we suggest that a diameter of less than 22 mm is indicative of fluoroquinolone resistance in \textit{M. catarrhalis}. Although we did not investigate fluoroquinolone-resistant isolates from other parts of the world, we propose that the diameter of the inhibition zone around levofloxacin may be used as a criterion for screening for fluoroquinolone-resistant isolates.

Previous studies have reported the mechanisms of fluoroquinolone resistance in several Gram-negative bacteria, such as \textit{N. gonorrhoeae}, \textit{N. meningitidis} and \textit{E. coli} (Belland et al., 1994; Deguchi et al., 1995; Wang et al., 2006; Shultz et al., 2005; Liu et al., 2012). In addition, one previous report indicates that GyrA is the primary site of action of fluoroquinolones in Gram-negative bacteria and that ParC is a secondary target; moreover, the report suggests that multiple QRDR changes are accompanied by an increase in fluoroquinolone resistance (Deguchi et al., 1996). In the present study, we have shown that five low-level fluoroquinolone-resistant isolates contained a T80I substitution in GyrA. This mutation is known to confer resistance to fluoroquinolones in \textit{N. gonorrhoeae} (Ser91), \textit{N. meningitidis} (Thr91) and \textit{E. coli} (Ser83). Moreover, \textit{M. catarrhalis} transformants with the T80I alteration in GyrA showed elevated MICs of levofloxacin. Therefore, our findings indicate that the alteration in GyrA in \textit{M. catarrhalis} isolates is important for increasing MICs of fluoroquinolones, and the substitution at aa 80 in GyrA may be a first step towards fluoroquinolone resistance in \textit{M. catarrhalis}.

On the other hand, overexpression of efflux system components is also involved in fluoroquinolone resistance in several bacteria such as \textit{E. coli}, \textit{Proteus mirabilis} and \textit{Pseudomonas} spp. (Jellen-Ritter & Kern, 2001; Saiito et al., 2006; Aeschlimann et al., 2003; Kumita et al., 2009). Although the role of the efflux system was not investigated in this study, we believe that T80I in GyrA causes low-level fluoroquinolone resistance in \textit{M. catarrhalis} because the levofloxacin MIC for the 49143TF strain was identical to those for the five clinical isolates.

In conclusion, our findings reveal that low-level resistance to fluoroquinolones in \textit{M. catarrhalis} is due to amino acid substitutions in GyrA (i.e. T80I). To our knowledge, this is the first report investigating the relationship between low-level fluoroquinolone resistance and an alteration in GyrA in clinical isolates of \textit{M. catarrhalis}. Since the increased use of fluoroquinolones has increased resistance to these antimicrobials in several organisms, future studies are required to investigate the rate with which fluoroquinolone resistance develops in \textit{M. catarrhalis} isolates.

### Acknowledgements

The authors declare no conflicts of interest associated with this study.

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**Fig. 2.** Amino acid sequence alignment of partial GyrA in the five clinical isolates (S22, S53, S62, S68 and S70), \textit{M. catarrhalis} BBH (GenBank accession no. CP002005), \textit{N. gonorrhoeae} FA1090 (AE004969), \textit{N. meningitidis} Z2491 (AL157959) and \textit{E. coli} K–12 (U00096). Aa 80 corresponds to Ser91 in \textit{N. gonorrhoeae} FA1090, Thr91 in \textit{N. meningitidis} Z2491 and Ser83 in \textit{E. coli} K–12. Identical amino acids are shown by dots.
REFERENCES


