In vitro activity of sitafloxacin against clinical strains of Streptococcus pneumoniae with defined amino acid substitutions in QRDRs of gyrase A and topoisomerase IV

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Objectives: Fluoroquinolone-resistant Streptococcus pneumoniae are increasing worldwide rapidly. In vitro activities of sitafloxacin were evaluated against clinical isolates of S. pneumoniae resistant to levofloxacin (MIC of levofloxacin ≥4 mg/L), which were characterized genetically.

Methods: The quinolone resistance determining regions (QRDRs) of gyrA, gyrB, parC and parE of these strains were analysed by PCR-based sequencing. MICs of sitafloxacin and other quinolones were determined by a microdilution broth method.

Results: All 18 strains had at least one amino acid substitution in the QRDRs of GyrA and ParC, which included Ser-81 → Tyr/Phe and Glu-85 → Lys in GyrA and Ser-79 → Phe/Ile/Tyr, Asp-83 → Tyr, Asn-91 → Asp, Ser-107 → Phe, Lys-137 → Asn and Ala-142 → Ser in ParC. Most isolates had Asp-435 → Asn/Ile-460 → Val/Ala-596 → Thr substitutions in ParE, while no amino acid substitution in GyrB was noted in all isolates. Ten isolates for which levofloxacin MICs were 16 or 32 mg/L had multiple mutations in both GyrA and ParC. The MIC80 value of sitafloxacin for levofloxacin-resistant isolates was 0.25 mg/L. The range of MICs of sitafloxacin for isolates resistant to levofloxacin (MIC 4–32 mg/L) was 0.016–0.5 mg/L.

Conclusions: These findings warrant further studies to evaluate the usefulness of sitafloxacin in the treatment of levofloxacin-resistant S. pneumoniae infection.

Keywords: levofloxacin-resistant S. pneumoniae, drug resistance, sitafloxacin, target alteration, efflux pump

Introduction

Streptococcus pneumoniae is the major cause of respiratory tract infections, bacteraemia and bacterial meningitis. For a long time, penicillin was the most effective drug against such infections. However, the incidence of multidrug-resistant S. pneumoniae is currently increasing throughout the world.1 The rapid spread of pneumococcal clones resistant to β-lactams and macrolides has promoted the use of selected fluoroquinolones for the treatment of pneumococcal infections. Therefore, fluoroquinolones with antipneumococcal activity, such as levofloxacin, moxifloxacin, gatifloxacin and gemifloxacin, may play an important role in the management of pneumococcal disease.2

Accordingly, the increase in S. pneumoniae resistance to fluoroquinolones that has been reported recently is of great concern. So far, two mechanisms responsible for the reduced susceptibility to fluoroquinolones have been identified in clinical isolates: target alteration and/or reduced drug accumulation due to drug efflux.3 The targets of fluoroquinolones are DNA gyrase and topoisomerase IV, which are encoded by gyrA, gyrB, parC and parE. Fluoroquinolone-resistant strains show amino acid substitutions in quinolone resistance determining regions

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(QRDRs) of DNA gyrase and topoisomerase IV. Multiple mutations within QRDRs of both gyrA and parC result in high-level resistance to levofloxacin. In this study, the *in vitro* activities of sitafloxacin, a newer quinoline, were evaluated against clinical isolates of *S. pneumoniae* resistant to levofloxacin (MICs of levofloxacin ≥4 mg/L), which were characterized genetically.

Materials and methods

**Bacterial strains**

Eighteen clinical isolates of *S. pneumoniae* with resistance to fluoroquinolones (MICs of levofloxacin ≥4 mg/L) were used in this study. The strains were collected from isolates from various specimens submitted to the clinical laboratory of Ryukyu University Hospital from January 1994 through December 2004. The isolates were confirmed to be *S. pneumoniae* by colony morphology, optochin susceptibility and bile solubility, and the presence of the autolysin gene *hla* was confirmed by PCR (Wakunaga Pharmaceuticals, Co., Hiroshima, Japan). The bacteria were grown on 5% sheep blood agar (Kyokuto Co., Tokyo, Japan) at 37°C in an atmosphere enriched with 5% CO₂. A levofloxacin-susceptible clinical strain, *S. pneumoniae* WP90, was used for sequencing analysis to compare its amino acid sequence with those of the other strains. The quality control strain *S. pneumoniae* ATCC 49619 was also used for MIC determination.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility was determined by the 2-fold broth microdilution method according to the guidelines of the CLSI (formerly the NCCLS). Cation-adjusted Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA) was supplemented with 3% lysed horse blood. Microdilution trays (final volume, 100 μL/well) were inoculated with an automatic MIC-2000 inoculator (Dynametech Laboratories, Inc., Alexandria, VA, USA). Final inocula contained ~5×10⁴ cfu/well. The MIC of each drug was defined as the lowest concentration resulting in the complete inhibition of visible growth after 18 h of incubation. MICs were also determined in the presence and absence of 10 mg/L reserpine (Sigma Aldrich Japan, K. K., Tokyo) to evaluate the presence of an efflux mechanism. A change in concentration resulting in the complete inhibition of visible growth after 18 h of incubation. MICs were also determined in the presence and absence of 10 mg/L reserpine (Sigma Aldrich Japan, K. K., Tokyo) to evaluate the presence of an efflux mechanism. A change in concentration result-

**DNA sequencing and analysis**

Mutations in the QRDRs of the gyrA, gyrB, parC and parE genes of fluoroquinolone-resistant strains were investigated by the PCR method. The primer sequences used in this study were described previously. Bacterial genomic DNA was prepared from several colonies of *S. pneumoniae* grown on a blood agar plate by boiling with Chelex-100 (Bio-Rad, Hercules, CA, USA). Subsequently, 5 μL of the extract was added to 50 μL of a PCR solution [1× PCR buffer, 200 μM of each dNTP, 2.5 U of Taq polymerase (Takara Biomedical, Kyoto, Japan) and 0.5 μM of sense and reverse primers]. PCR conditions were as follows: 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The PCR products were electrophoresed on an agarose gel to confirm the presence of the product, and were then purified with a PCR purification kit (Qiagen Sciences Inc., Germantown, MD, USA) to prepare a sequencing template. The sequencing reaction was conducted with a Rhodamine Terminator Cycle Sequencing FS Ready Reaction kit (PE Biosystems, Foster city, CA, USA). The reaction mixtures were placed in a thermal cycler and denatured at 94°C for 2 min. They were then subjected to 25 PCR cycles (94°C for 10 s, 50°C for 5 s and 60°C for 4 min). The nucleotide sequences were determined with an ABI PRISM3100 DNA sequencer.

Results

Sequencing of the region encoding the QRDRs of gyrA, gyrB, parC and parE was carried out to investigate the involvement of gene mutations in fluoroquinolone-resistant clinical isolates. The results of sequencing analysis were reproducible. Table 1 summarizes the substitutions of deduced amino acid sequences within QRDRs of GyrA, ParC and ParE of the 18 levofloxacin-resistant strains. All 18 strains had at least one amino acid substitution in the QRDRs of GyrA and ParC, which included Ser-81→Tyr/Phe and Glu-85→Lys in GyrA and Ser-79→Phe/Ile/Tyr, Asp-83→Tyr, Asn-91→Asp, Ser-107→Phe, Lys-137→Asn and Ala-142→Ser in ParC. Among them, 10 strains had amino acid substitutions in both QRDRs of GyrA and ParC. Most isolates had Asp-435→Asn/Ile→460→Val/Ala-596→Thr substitutions in ParE, while no amino acid substitution in GyrB was noted in all isolates. Multiple amino acid substitutions in both GyrA and ParC were detected in 10 strains with levofloxacin MICs of 16 or 32 mg/L.

The MIC₅₀ of sitafloxacin for levofloxacin-resistant isolates was 0.25 mg/L. The range of MICs of sitafloxacin for isolates highly resistant to levofloxacin (MIC 16–32 mg/L) was 0.125–0.5 mg/L. The addition of reserpine did not change the susceptibility of the strains to quinolones (data not shown). MICs of levofloxacin, sitafloxacin, sparfloxacin and gatifloxacin against the control strain ATCC 49619 were 0.5, 0.032, 0.25 and 0.125 mg/L, respectively.

Discussion

Predominantly, the fluoroquinolone resistance of *S. pneumoniae* clinical isolates is attributed to amino acid substitutions at positions Ser-81 in GyrA and Ser-79 in ParC to either Phe or Tyr. The present study showed that the same amino acid substitutions were detected in Japanese levofloxacin-resistant strains, confirming the significant role of these substitutions within QRDRs of GyrA and ParC. The Glu-85→Lys substitution in GyrA was also detected in high-level levofloxacin-resistant strains, suggesting this mutation is associated with the quinolone resistance as well as Ser-81→Phe/Tyr. The Ser-79→Ile substitution in ParC (strains V7 and HC37) was first detected in this study, and this substitution seemed to be associated with the quinolone resistance. The Lys-137→Asn substitution in ParC is detected frequently in levofloxacin-susceptible strains; therefore, this mutation may be unrelated to the resistance. Other mutations in ParC (Asp-91→Asp, Ser-107→Phe and Ala-142→Ser) were detected in strains with high resistance to levofloxacin (MICs 16–32 mg/L). The significance of these substitutions is still unclear. Multiple substitutions in ParC may increase the resistance, even when each single substitution would cause no effect. Further studies are required to clarify the significance of these
mutations. The Ile-460—Val substitution in ParE is prevalent in Japanese isolates less susceptible to fluoroquinolones. Amino acid substitutions in ParE detected in the present study (Asp-435—Asn, Ile-460—Val and Ala-596—Thr) may be related to low-level quinolone resistance.

Sitafloxacin, a newer quinolone, is potent against Gram-positive cocci as well as Gram-negative bacilli, and excellent activity against *S. pneumoniae* has been reported. In the present study, this drug had significantly lower MICs for *S. pneumoniae* with resistance to levofloxacin, compared with the other quinolones tested, and these findings support previous reports showing a similar potency of this drug against levofloxacin-resistant *S. pneumoniae* with defined multiple mutations within both gyrase A and topoisomerase IV. Gemifloxacin is also potent against such strains with double mutations both in gyrase A and topoisomerase IV. MICs of sitafloxacin were higher for strains with a single Ser-81 mutation in GyrA (strains TZ2–11 and 3568) than those with a single Ser-79 mutation in ParC (strains 2E19, 4426, DS-1, 4511 and V91), suggesting that the primary target of sitafloxacin may be GyrA rather than ParC. The MIC of sitafloxacin for strain VY5 with a Ser-81—Tyr substitution in GyrA was lower than those for strains with Ser-81—Phe substitutions. Ser-81—Tyr may not affect the affinity of GyrA for sitafloxacin. In contrast, Glu-85—Lys substitutions in GyrA resulted in higher MICs of sitafloxacin.

In summary, levofloxacin-resistant *S. pneumoniae* isolated in Japan had multiple amino acid substitutions in QRDRs of GyrA, ParC and ParE, as described previously in other countries. Sitafloxacin was potent against levofloxacin-resistant *S. pneumoniae* with multiple mutations in QRDRs of gyrase A and topoisomerase IV. Further studies are warranted to evaluate the usefulness of sitafloxacin against the infections caused by levofloxacin-resistant *S. pneumoniae*.

### Transparency declarations

Conflict of interest. None to declare.

### References


