Emergence of fluoroquinolone-resistant Streptococcus pyogenes in Japan by a point mutation leading to a new amino acid substitution

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Objectives: Streptococcus pyogenes causes various diseases in humans. While the prevalence of fluoroquinolone-resistant S. pyogenes isolates has been increasing since 2000 in the USA and Europe, it has remained very low in Japan. We isolated a fluoroquinolone-resistant S. pyogenes strain and analysed its genetics.

Methods: TU-296, a strain of S. pyogenes resistant to levofloxacin (MIC 16 mg/L), was isolated from the throat of a patient in their thirties with pharyngitis in autumn 2007. We carried out susceptibility tests for various antimicrobial agents and PCR analysis of the genes gyrA, gyrB, parC and parE in the quinolone resistance-determining region, followed by sequencing of the PCR products to find mutation(s) and the resulting amino acid substitution(s). We then sequenced the PCR product of the emm gene and determined the emm genotype.

Results: S. pyogenes TU-296 was found to have the following mutations and amino acid substitutions: adenine to cytosine in gyrA and cytosine 367 to thymine in parC, resulting in Glu-85→Ala in GyrA and Ser-79→Phe in ParC. The genotype of the isolate was emm11.

Conclusions: Amino acid substitutions in fluoroquinolone-resistant S. pyogenes have already been reported from Europe and the USA, including Ser-81→Phe or Tyr and Met-99→Leu in GyrA, as well as Ser-79→Phe, Tyr or Ala and others in ParC. Numerous point mutations were found in parC and parE of S. pyogenes TU-296. In addition, a new amino acid substitution was detected (Glu-85→Ala in GyrA). To our knowledge, there have been no previous reports of this substitution in a clinical isolate of S. pyogenes.

Keywords: group A Streptococcus, substitution of amino acid, GyrA, ParC, S. dysgalactiae subsp. equisimilis

Introduction

Streptococcus pyogenes causes pharyngitis, scarlet fever and severe infections such as streptococcal toxic shock syndrome and necrotizing fasciitis. This organism has many pathogenic features, such as adherence to epithelial cells, the ability to invade tissues and the ability to produce extracellular virulence factors, including streptococcal pyogenic exotoxins (SpeA, SpeB and SpeC) and streptolysin O. S. pyogenes possesses M-, R- and T-proteins on its outer membrane. M-protein is related to thermostability, anti-opsonin activity, adherence to epithelial cells and other virulence features, with >100 types having been found.

Fluoroquinolone resistance in S. pyogenes is caused by amino acid substitutions in four polypeptides called GyrA, GyrB, ParC and ParE, which are respectively coded for by at least one point mutation in the genes gyrA, gyrB, parC and parE located in the quinolone resistance-determining region (QRDR). The genes gyrA and gyrB code for DNA gyrase (topoisomerase II) subunits A and B, while parC and parE code for topoisomerase IV subunits C and E.

In 2000, the first fluoroquinolone-resistant S. pyogenes was reported by Yan et al. in the USA.1 Following this, reports of such isolates from other parts of the USA and Europe have gradually been increasing.3–4 In contrast, reports of fluoroquinolone-resistant S. pyogenes from Japan have been rare.5
Fluoroquinolone resistance of *Streptococcus pyogenes*

We recently found a levofloxacin-resistant (MIC 16 mg/L) clinical isolate of *S. pyogenes* at Tohoku University Hospital in Japan. In the present study, we examined the molecular features of its resistance mechanism.

Materials and methods

**Bacterial isolate**

When a patient in their thirties was diagnosed as having pharyngitis in autumn 2007, a throat swab was taken for bacterial testing. *S. pyogenes* was isolated from the specimen and was found to be resistant to levofloxacin with an MIC of 16 mg/L. The patient was treated with amoxicillin, and subsequently the infection by *S. pyogenes* was cleared. The patient had been hospitalized repeatedly and had received various antimicrobial agents, including fluoroquinolones.

The isolate, *S. pyogenes* TU-296, was stored at −80°C and was cultured on 5% sheep blood agar at 35°C for 20 h under 5% CO₂.

**Antimicrobial susceptibility testing**

The MICs of antimicrobial agents were determined by the microdilution method according to the recommendations of the CLSI (2009). *Streptococcus pneumoniae* ATCC 49619 was used for quality control. The quinolones tested were norfloxacin (KYORIN Pharmaceutical Co. Ltd, Tokyo, Japan), ofloxacin, levofloxacin and nalidixic acid (Daichi-Sankyo Co. Ltd, Tokyo, Japan), enoxacin and sparfloxacin (Dainippon Sumitomo Pharma, Tokyo, Japan), ciprofloxacin, lomefloxacin and cinoxacin (Sigma-Aldrich Japan K.K., Tokyo, Japan), prulifloxacin, moxifloxacin (Meiji Seika Kaisha Ltd, Tokyo, Japan), and gatifloxacin (Bayer Health Care, Berlin, Germany) and garenoxacin (Taisei Toyama Pharmaceutical Co. Ltd, Tokyo, Japan). The MICs of these antimicrobial agents were determined by using a microdilution method. The MIC of gatifloxacin was measured at BML Co. Ltd (Saitama, Japan), while the MICs of erythromycin, clarithromycin, azithromycin, josamycin, clindamycin, quinupristin/dalfopristin, telithromycin, penicillin, cefotaxime, cefepime and meropenem were measured using Eiken dry plates (Eiken Chemical Co. Ltd, Tokyo, Japan).

**DNA extraction from an isolate**

Aliquots of sterile distilled water (100 µL) were dispensed into 0.2 mL microtubes. A few colonies of *S. pyogenes* TU-296 were suspended in the water and heated for 10 min at 95°C in a thermal cycler, cooled on crushed ice and centrifuged at 15,000 rpm for 1 min. Then the supernatant was used as a DNA template.

**PCR amplification of gyrA, gyrB, parC and parE in the QRDR and the emm gene**

Amplification of the gyrA, gyrB, parC and parE genes in the QRDR was performed by PCR using primer sets described previously. Amplification was performed for 30 cycles, each cycle consisting of pre-heating at 94°C for 10 min, denaturation at 94°C for 1 min, annealing at 61°C for 1 min (for gyrA, parC and parE) or at 66°C for 1 min (for gyrB) and extension at 72°C for 1 min, followed by an additional extension at 72°C for 10 min. The PCR products were confirmed to be a single band by mini-submarine electrophoresis.

**DNA sequencing**

The PCR products were purified with a QIAquick® PCR purification kit (Qiagen GmbH, Germany), according to the manufacturer’s instructions. DNA sequencing of the purified products (gyrA, gyrB, parC, parE and emm) was carried out with a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Company Ltd, CA, USA) in an ABI 3730xl Genetic Analyzer (Applied Biosystems), according to the manufacturer’s instructions.

**Analysis of data**

The data obtained by DNA sequencing were compared with the QRDR of the genomes of various bacteria by the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). emm typing was performed with the online CDC emm database (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm). Finally, each sequence of gyrA and parC was compared with those of GenBank accession nos. AF220945 and AF220946 for gyrA and parC, respectively, of fluoroquinolone-susceptible *S. pyogenes* ATCC 700294.

Sequences of parC and parE in *S. pyogenes* TU-296 were analysed by the ClustalW program and TreeView X.

**Results**

The MICs of nalidixic acid and cinoxacin were 256 and 512 mg/L, respectively; the MICs of norfloxacin, ofloxacin and enoxacin were 32, 16 and 32 mg/L, respectively; the MICs of levofloxacin, lomefloxacin and sparfloxacin were 16–64 mg/L; and the MICs of prulifloxacin, moxifloxacin, gatifloxacin and garenoxacin were 0.25–4 mg/L. Garenoxacin had the lowest MIC of 0.25 mg/L among the quinolones. Macrolide, lincosamide, streptogramin, ketolide and β-lactam antibiotics were active against this isolate. The quinupristin/dalfopristin combination showed intermediate activity.

A base substitution (cytosine for adenine at position 476) was found in the partial sequence of gyrA in *S. pyogenes* TU-296 (accession no. AB514146), resulting in substitution of alanine (GCA) for glutamic acid (GAA) at position 85 (Glu-85→Ala) in GyrA. Another base substitution (thymine for cytosine at position 367) was found in the partial sequence of parC (DDBJ accession no. AB518044), resulting in substitution of phenylalanine (TTC) for glutamic acid (GAA) at position 85 (Glu-85→Ala) in ParC. The sequences of parC and parE from *S. pyogenes* TU-296 showed high homology with *Streptococcus dysgalactiae* subsp. *equisimilis* according to the bits score, E-value, phylogenetic tree with the ClustalW program and TreeView X (see Figure 1).

The genotype of the M-protein of *S. pyogenes* TU-296 was determined as emm11 by comparison with the CDC emm database.

Table 1 summarizes previous reports of amino acid substitutions attributed to point mutations in the QRDR of gyrA and parC and the susceptibility profile of *S. pyogenes* TU-296 to various quinolones in the present study. Reports on surveillance performed in Spain, Belgium and the USA are indicated in the last three rows of the table. Our new strain occupies an intermediate position on the scale of resistance to fluoroquinolones, and we suspect that the cause of resistance is the substitution Glu-85→Ala in GyrA.

**72°C for 10 min. The PCR products were confirmed to be a single band by mini-submarine electrophoresis.**
Figure 1. Phylogenetic tree analysis of parC in the QRDR of S. pyogenes TU-296 and other streptococci. The parC sequence of fluoroquinolone-resistant S. pyogenes TU-296 with substitution of Ser-79 → Phe (DDBJ accession no. AB518044) was compared with that of parC in other streptococci by ClustalW and BLAST. TU-296 was isolated at Tohoku University Hospital, Japan. The figure shows part of the phylogenetic tree for parC. This figure was adapted from the phylogenetic tree created by TreeView X. Evolutionary distances were computed by the Kimura 2 parameter method. GBS, S. agalactiae; GAS, S. pyogenes; GGS, S. dysgalactiae subsp. equisimilis; Sp, Streptococcus porcinus; Su, Streptococcus uberis; pc, partial code; cg, complete genome.
Table 1. Comparison of emm type, amino acid substitution and quinolone susceptibility of the resistant strain from the present study with previous reports

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year of isolation</th>
<th>Country</th>
<th>Strain</th>
<th>emm type</th>
<th>Substitution due to point mutation in QRDR</th>
<th>MIC (µg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>2007</td>
<td>Japan</td>
<td>TU-296</td>
<td>11</td>
<td>GyrA: Glu85Ala; ParC: Ser79Phe</td>
<td>CIP: 16; LVX: 18; NOR: 2; SPX: 16; GAT: 2; MFX: 2; CLX: 16; OFX: &gt;32; GRX: 0.25</td>
<td></td>
</tr>
<tr>
<td>Yan et al.</td>
<td>2000</td>
<td>USA</td>
<td>NIH-R-01-GAS</td>
<td>UD</td>
<td>Ser81Ala, Met99Leu; Ser79Phe</td>
<td>&gt;32; &gt;32; &gt;256; &gt;32</td>
<td>1.00; &gt;32</td>
</tr>
<tr>
<td>Biedenbach et al.</td>
<td>2000</td>
<td>Sweden</td>
<td>89-7025A</td>
<td>UD</td>
<td>none</td>
<td>4; 2</td>
<td>1; 2</td>
</tr>
<tr>
<td>Richter et al.</td>
<td>2002</td>
<td>USA</td>
<td>UD</td>
<td>12</td>
<td>Ser81Tyr; Ser79Phe, Ala121Val</td>
<td>32; 16; 4</td>
<td>2; 3</td>
</tr>
<tr>
<td>Reinert et al.</td>
<td>2000</td>
<td>Germany</td>
<td>M8141</td>
<td>89</td>
<td>Ser81Tyr; Ser79Phe, GLU91Asp, Ser140Pro</td>
<td>2; 2; 32; 1; 0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>Rivera et al.</td>
<td>2003</td>
<td>Spain</td>
<td>342-D</td>
<td>3.1</td>
<td>Ser80Pro; none</td>
<td>4; 3; 64; 1</td>
<td>0.38; 0.125</td>
</tr>
<tr>
<td>Alonso et al.</td>
<td>2004</td>
<td>Spain</td>
<td>ES9A-1473</td>
<td>28</td>
<td>Ser81Tyr; Ser79Phe, Asp91Asn</td>
<td>32; 32; 8; 4</td>
<td>10</td>
</tr>
<tr>
<td>Wojna et al.</td>
<td>2007</td>
<td>Japan</td>
<td>none</td>
<td>58</td>
<td>Ser81Tyr; Ser79Phe</td>
<td>32; 32; 8; 4</td>
<td>5</td>
</tr>
<tr>
<td>Alberti et al.</td>
<td>1998–1999</td>
<td>Spain</td>
<td>30 strains</td>
<td>3, 6, 73, 28, 75, 22, 78, 13, 12, st118</td>
<td>none</td>
<td>Ser79Ala, Ser79Phe, Asp91Asn</td>
<td>≥4</td>
</tr>
<tr>
<td>Malhotra-Kumar et al.</td>
<td>1999–2002</td>
<td>Belgium</td>
<td>55 strains</td>
<td>6, 75, 28, 12, 1, 4, 9, 22, 76</td>
<td>none</td>
<td>Ser79Ala, Ser79Tyr, Asp91Asn, Ala121Val, Gly128Asp, Ser140Pro</td>
<td>2–8; 1–4; 8–64; 0.5–1; 1–4</td>
</tr>
<tr>
<td>Orschin et al.</td>
<td>2002–2003</td>
<td>USA</td>
<td>42 strains</td>
<td>6</td>
<td>none</td>
<td>Ser79Ala, Ser79Phe, Asp83Asn, Asp91Asn</td>
<td>1.5–6; 2–4; 0.38–0.75; 0.25–0.5</td>
</tr>
</tbody>
</table>

CIP, ciprofloxacin; LVX, levofloxacin; NOR, norfloxacin; SPX, sparfloxacin; GAT, gatifloxacin; MFX, moxifloxacin; CLX, clinafloxacin; OFX, ofloxacin; GRX, grepafloxacin; GRN, garenoxacin; ND, not determined; UD, undescribed.

*aSusceptibility testing was performed by Etest.*
Discussion
The TU-296 strain of S. pyogenes was resistant to many quino-
lones, with the exception of moxifloxacin, gatifloxacin and gar-
enoxy. The following substitutions have already been
reported in clinical isolates: Ser-81→Ala, Tyr or Phe and
Met-99→Leu in GyrA; and Ser-79→Ala, Tyr or Phe,
Asp-83→Asn, Asp-91→Asn, Ala-121→Val, Gly-128→Val and
Ser-140→Pro in ParC.1–10 However, the substitution
Glu-85→Ala in GyrA of our fluoroquinolone-resistant S. pyo-
genesis TU-296 (DDBJ accession no. AB514146) has not
been reported previously. Substitution of Glu-85
in GyrA has been extremely low compared with that in the USA and
Europe. According to a previous report5 from Japan, the amino
acyl substitutions detected were Ser-81→Phe in GyrA and
Ser-79→Phe in ParC. This finding was similar to reports from the USA and Europe.

The sequences of parC (DDBJ accession no. AB518044) and
parE in S. pyogenes TU-296 revealed many point mutations, and were homologous with S. dysgalactiae subsp. equisimilis,
showing a high bits score and a low E-value by cluster analysis.
Similar cases have been reported previously. In Japan, the fre-
cuency of fluoroquinolone-resistant S. pyogenes clinical isolates has been extremely low compared with that in the USA and Europe. According to a previous report5 from Japan, the amino
acyl substitutions detected were Ser-81→Phe in GyrA and
Ser-79→Phe in ParC. This finding was similar to reports from the USA and Europe.

In conclusion, we report the first S. pyogenes clinical isolate with substitution Glu-85→Ala in GyrA. Amino acid substitu-
tions in GyrA and ParC of S. pyogenes strains showing an
MIC of levofloxacin of 2 or 4 mg/L have been reported pre-
viously. We need to carefully monitor the prevalence of fluo-
oroquinolone resistance among clinical isolates of S. pyogenes in the future.

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Transparency declarations
We do not have any financial interests or associations that might pose a
conflict of interest.

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