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Original Article

Emergence of quinolone-resistant strains in *Streptococcus pneumoniae* isolated from paediatric patients since the approval of oral fluoroquinolones in Japan

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ABSTRACT

Tosufloxacin (TFLX) is a fluoroquinolone antimicrobial agent. TFLX granules for children were initially released in Japan in 2010 to treat otitis media and pneumonia caused by drug-resistant bacteria, e.g. penicillin-resistant *Streptococcus pneumoniae* and beta-lactamase-negative, ampicillin-resistant *Haemophilus influenzae*. The evolution of bacterial resistance since TFLX approval is not known. To clarify the influence of quinolones administered to children since their approval, we examined the resistance mechanism of TFLX-resistant *S. pneumoniae* isolated from paediatric patients as well as patient clinical characteristics. TFLX-resistant strains (MIC $\geq$ 2 mg/L) were detected among clinical isolates of *S. pneumoniae* derived from children ($\leq$15 years old) between 2010 and 2014. These strains were characterised based on quinolone resistance-determining regions (QRDRs), i.e. *gyrA*, *gyrB*, *parC*, and *parE*. In addition, the antimicrobial susceptibility, serotype, and multilocus sequence type of strains were determined, pulsed-field gel electrophoresis was performed, and patient clinical characteristics based on medical records were assessed for cases with underling TFLX-resistant strains. Among 1168 *S. pneumoniae* isolates, two TFLX-resistant strains were detected from respiratory specimens obtained from paediatric patients with frequent exposure to TFLX. Both strains had mutations in the QRDRs of *gyrA* and *parC*. One case exhibited gradual changes in the QRDR during the clinical course. This is the first study of quinolone-resistant *S. pneumoniae* isolated from children, including clinical data, in Japan. These data may help prevent increases in infections of quinolone-resistant *S. pneumoniae* in children; specifically, the results emphasise the importance of administering fluoroquinolones only in appropriate cases.

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1. Introduction

Streptococcus pneumoniae is the major causal pathogen of otitis media, pneumonia, and invasive pneumococcal diseases, such as sepsis and meningitis, in children. Penicillin-nonsusceptible *S. pneumoniae* have increased rapidly since around 1990 and are a major problem in many countries [1–3]. Recently, paediatric respiratory infections due to macrolide-resistant *Mycoplasma pneumoniae* and beta-lactamase non-producing ampicillin-resistant *Haemophilus influenzae* have become critical issues in Japan [4,5]. Accordingly, oral fluoroquinolone tosufloxacin (TFLX) was initially released for children in January 2010. Since its introduction, many paediatricians and otolaryngologists prescribe TFLX owing to its clinical effectiveness, good compliance based on a twice-daily dosage, and relatively pleasant taste. Frequent use of TFLX may lead to an increased risk of drug-resistant bacteria. Therefore, the development of quinolone-resistant *S. pneumoniae* in children is a concern in Japan. A decrease in TFLX susceptibility has been observed for *S. pneumoniae* derived from children with

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community-acquired pneumonia [6]. However, the precise frequency of quinolone-resistant *S. pneumoniae* in children in Japan is unknown.

In this study, we analysed the gene sequences of quinolone resistance-determining regions (QRDRs) in TFLX-resistant *S. pneumoniae* isolated from paediatric patients to determine the mechanism of TFLX resistance. Secondly, we analysed *S. pneumoniae* isolates obtained from a single patient over time to elucidate the dynamic process by which TFLX resistance is acquired. The frequent use of oral quinolones by children led to changes in the QRDR and an increase in the minimum inhibitory concentration (MIC) of quinolone in *S. pneumoniae.*

2. Materials and methods

2.1. Samples

In total, 1168 clinical isolates of *S. pneumoniae* derived from children (≤15 years old) were isolated at the Chiba Children's Hospital between 2010 and 2014. The specimen categories for *S. pneumoniae* strains are shown in Table 1. Among these isolates, two TFLX-resistant strains (MIC ≥ 2 mg/L) were analysed; one strain, referred to as strain 1, was isolated in 2010 and the other strain, referred to as strain 2, was isolated in 2014. Six pneumococcal strains isolated from a single patient who had TFLX-resistant strain 2 were also analysed (Fig. 1). Patient characteristics (i.e. age, sex, underlying disease, previous exposure to TFLX, and history of pneumococcal conjugate vaccination) were retrospectively analysed based on medical records for patients with a confirmed TFLX-resistant strain. Patient names were de-identified. This study was approved by the Chiba Children's Hospital Ethics Committee (approval number 2016-11-31).

These isolates were stored at −80 °C in 10% skim milk until use. Each isolate was grown on Trypticase Soy Agar with 5% sheep blood (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) for 24 h at 37 °C in 5% CO_2. Each isolate was identified as *S. pneumoniae* using an optochin susceptibility test and a bile solubility test. Polymerase chain reaction (PCR) assays targeting the *lytA* gene, which encodes the major pneumococcal autolysin (LytA), were also used to identify *S. pneumoniae.* All strains were susceptible to optochin, bile-soluble, and positive for the *lytA* gene, and were therefore identified as *S. pneumoniae.*

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) standard method and the MIC was determined. The MIC breakpoints for TFLX were determined in accordance with the recommendations for respiratory infections established by the Japanese Society of Chemotherapy [7].

Table 1
Specimen categories of *Streptococcus pneumoniae* strains.

Source	Strain numbers (%)
Sputum	706 (60.4)
Nasopharynx	217 (18.5)
Nasal discharge	146 (12.5)
Ear discharge/middle ear effusion	33 (2.8)
Pharynx/tonsil/gingiva	20 (1.7)
Blood	19 (1.6)
Eye discharge	12 (1.0)
Cerebrospinal fluid	2 (0.1)
Others	13 (1.1)
Total	1168 (100.0)

2.3. Capsular serotyping and molecular typing

Serotypes were determined using the slide agglutination reaction with the *S. pneumoniae* antisera ‘Seiken’ set (Denka Seiken, Tokyo, Japan) and the Quellung reaction using pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark).

Molecular typing was performed by multi-locus sequence typing (MLST) as described by Enright et al. [8]. Seven housekeeping genes, i.e. *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*, were amplified by PCR for MLST. The sequencing reaction was carried out using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The Applied Biosystems 3130xl Genetic Analyzer was used for sequencing. Allelic numbers and sequence types (STs) were determined using the MLST website (http://spneumoniae.mlst.net/).

2.4. DNA extraction

Each isolate was subcultured on Trypticase Soy Agar with 5% sheep blood (Nippon Becton Dickinson) for 24 h at 37 °C in 5% CO_2. Bacterial genomic DNA was extracted from several colonies of each isolate by the boiling method. Extracted DNA was stored at −20 °C prior to PCR amplification.

2.5. Quinolone resistance-determining region (QRDR) sequencing

The QRDRs of *gyrA*, *gyrB, parC*, and *parE* were amplified by PCR. The primers for each loci were synthesised as described by Pan et al. and Balsalobere et al. [9,10]. The primers are described in Table 2 and were generated using KOD FX Neo® (TOYOBO, Osaka, Japan). The sequencing reaction was performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed using the Applied Biosystems 3130xl Genetic Analyzer. The sequences were compared with those of wild-type *S. pneumoniae* R6 using data obtained from GenBank (accession number NC003098).

2.6. Detection of other drug-resistance genes

Mutations in genes encoding penicillin-binding proteins (i.e. *pbp1a*, *2x,* and *2b*), the targets of beta-lactams, and the presence of *mef*(A) and *erm*(B), which are associated with resistance to macrolides in *S. pneumoniae*, were detected by PCR using the Penicillin-resistant *S. pneumoniae* Detection Reagent Kit (Wakunaga Pharmaceutical, Osaka, Japan).

2.7. Pulse-field gel electrophoresis (PFGE)

The clonal relatedness among isolates was determined by PFGE using the restriction enzyme *Sma*I [11]. The DNA fragments were separated on a 1% agarose gel. Electrophoresis was performed in 0.5 × Tris/borate/EDTA buffer at 6 V/cm and 14 °C for 20 h with pulse time of 5.3–34.9 s and linear ramping using the CHEF-DRIII system (Japan Bio-Rad Laboratories, Inc., Tokyo, Japan). A DNA size standard lambda ladder was used as a reference marker.

3. Results

3.1. Bacteriological analysis and patient clinical characteristics for TFLX-resistant S. pneumoniae

Two TFLX-resistant strains (isolated in 2010 and 2014) were detected from 1168 clinical isolates of *S. pneumoniae.* Accordingly, the frequency of quinolone-resistant *S. pneumoniae* was 0.2%.

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