Fluoroquinolone Resistance in *Streptococcus pneumoniae* from a University Hospital, Thailand

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The most frequent markers of fluoroquinolone resistance in S. pneumoniae are chromosomal mutations in the quinolone-resistance-determining regions of DNA gyrase and topoisomerase IV encoding for the gyrA, gyrB and parC, parE genes. In 2008, 6.5% of the Streptococcus pneumoniae isolates in a Bangkok university hospital were resistant to ofloxacin. Using PCR and DNA sequencing, we identified mutations in both the gyrA and parC genes of four ofloxacin- and ciprofloxacin-resistant S. pneumoniae isolates (minimum inhibitory concentrations > 32 µg/ml). Mutations were found in the gyrA gene at positions Ser81Phe, Glu85Gly, Glu85Lys and in the parC gene at position Ser79Tyr. Three isolates had mutations in both genes. Two of the isolates were serotype 6B and two were serotypes not contained in currently licensed pneumococcal vaccines. This is the first report of the mechanisms of fluoroquinolone resistance in S. pneumoniae in Thailand.

Keywords: *Fluoroquinolone, gyrA gene, parC gene, Streptococcus pneumoniae, Ofloxacin, Ciprofloxacin*

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Streptococcus pneumoniae is the most common bacterial pathogen causing pneumonia, bacteremia and meningitis, and results in high morbidity and mortality worldwide. The increasing prevalence of resistance to multiple antimicrobial agents (*e.g.*, penicillin, macrolides, tetracycline and trimethoprim-sulfamethoxazole), has resulted in frequent use of newer fluoroquinolones for the empirical treatment of *S. pneumoniae* infections[1]. Fluoroquinolones are bactericidal drugs that are highly effective in the treatment of pneumococcal infection. Yet, they also appear to select rapidly for resistant pneumococcal strains, which may lead to treatment failure. Fluoroquinolone resistance is mediated primarily by point mutations in genes encoding the subunits of the drug's target enzymes, DNA gyrase (*gyrA* and *gyrB* genes) and topoisomerase IV (*parC* and *parE* genes), together termed quinolone-resistance-determining regions (QRDR). The mutations most frequently implicated in resistance include *gyrA* Ser81 to Phe or Tyr and at *parC* Ser79 to Phe or Tyr[2-6].

Although fluoroquinolone resistance is currently relatively uncommon in most settings, rates will likely increase with the widespread use of broader spectrum fluoroquinolones. A 2003 publication described the prevalence of fluoroquinolone-resistant *S. pneumoniae* as 14.3% in Hong Kong, < 1% in USA and Canada, and absent in some European countries and Australia[7]. We describe the prevalence of fluoroquinolone resistance and results of a preliminary study of resistance mechanisms (*i.e.*, mutations in the *gyrA* and *parC* genes) among *S. pneumoniae* isolates at a university hospital in Thailand.

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Material and Method

Clinical isolates

S. pneumoniae isolates were collected during January-December 2008 from various clinical specimens (normally sterile sites and non-sterile sites) at Siriraj Hospital, a tertiary care hospital in Bangkok, Thailand. Specimens were from patients of all ages. Isolates were identified by optochin and bile solubility testing according to standard microbiological techniques and stored at -70°C in 5% trypticase soy broth plus 20% by volume glycerol until testing[8]. Multiple isolates from the same illness episode of one patient were counted

only once.

Drug susceptibility tests

In vitro drug susceptibility was performed by the disk diffusion method on Mueller Hinton agar (Becton Dickinson Ltd., USA) supplemented with 5% sheep blood as described by the Clinical Laboratory Standards Institute (CLSI)[9]. *S. pneumoniae* ATCC 49619 was used as a quality control. The interpretation criteria for disk diffusion results and ofloxacin MIC results were based on the 2008 CLSI guidelines[9]. For ciprofloxacin, MIC $\geq$ 4 µg/ml was considered resistant[10] and *S. aureus* ATCC 25923 was used as a control.

Study of GyrA and parC genes

To study the mechanisms of resistance in QRDR (the *gyrA* and *parC* genes), polymerase chain reaction (PCR) was performed by extracting the total genomic DNA from each isolate using a Puregene DNA Purification kit (Gentra Systems Inc, USA). Primers for the *gyrA* gene were[5]: forward5'-GTGGAATATTGGTTGCCATC-3' and reverse 5'-GATGAAGGCAAGTTTTATCG-3'; for the *parC* gene: forward 5'-TGGGTTGAAGCCGGTTCA-3' and reverse 5'-TGCTGGCAAGACCGTTGG-3'. The amplification protocol was 95°C denaturation for 5 min, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C and with final extension at 72°C for 7 min. *S. pneumoniae* ATCC 49619 was used as a quality control. The PCR product was purified by NucleoSpin Extract II PCR clean-up kit (Macherey-Nagel Ltd., USA). Sequencing of the *gyrA* and *parC* genes was carried out using an automated DNA sequencer (ABI Prism™ 3730*xl* DNA sequencers, USA). DNA sequences were analyzed by software available over the internet at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/BLAST) and multiple sequence alignment was analyzed by BioEdit software, ver. 5.0.9 (Isis Pharmaceuticals, CA, USA). *S. pneumoniae* ATCC 49619 was used as a fluoroquinolone-sensitive control.

S. pneumoniae serotyping

Capsular serotypes of fluoroquinolone-resistant isolates were determined by the quellung reaction, using the Pneumotest kit plus specific type and factor antisera according to the manufacturer's instructions (Staten Serum Institute, Denmark). Typing was done with the addition of a loopful (a few microliters) of methylene blue 0.3% weight/volume into the bacterial suspension on a glass slide to improve visualization using a microscope (OYMPUS BX 50 Model U-MD08, Oympus Corporation, Japan) with an oil immersion lens (magnification, 10x100). Isolates that were not one of the serotypes included in the 23-valent pneumococcal polysaccharide vaccine (Merck Ltd, USA) or the 13-valent pneumococcal conjugate vaccine (Wyeth-Ayerst Ltd., USA)], were considered nontypable.

Results

A total of 170 *S. pneumoniae* isolates were available for evaluation. Fifty-five isolates were from sterile sites, 46 (83.7%) of which were from blood. For the 115 isolates from nonsterile sites, 75 (65.2%) were isolated from sputum. Overall, 66% were isolated from male. Results of disk diffusion revealed that 159 (93.5%) of isolates were susceptible to ofloxacin. Five (3%) and 6 (3.5%) isolates were intermediate and resistant to ofloxacin, respectively. All ofloxacin-resistant isolates were obtained from sputum. This compares to 99% ofloxacin susceptibility among isolates collected during 1997-2001, as described in a previous report[11]. Four ofloxacin-resistant *S. pneumoniae* isolates were randomly selected for molecular analysis. The four patients had a mean age of 59.8 $\pm$ 16.8 years (range 41-82 years), three were male, and all had respiratory tract infections. All four isolates were from sputum cultures and were also resistant to penicillin, erythromycin, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole by disk diffusion. All isolates were susceptible to linezolid and vancomycin. Two of the isolates were serotype 6B, while the other two were non of the 13-vaccine serotypes.

DNA sequence analysis of the 502 bp of the *gyrA* gene revealed a coding region of 167 amino acids. Sequencing of the control *S. pneumoniae* ATCC 49619 revealed 100% nucleotide and amino acid sequence identity with the published sequence of the *gyrA* gene in *S. pneumoniae* R6, which is a fluoroquinolone-sensitive reference strain (accession no. AF053121, Fig. 1A). Single or double mutations were found in all four clinical isolates. DNA sequence analysis of the 367 bp of the *parC* gene revealed a coding region of 122 amino acids. *S. pneumoniae* ATCC 49619 shared 99% nucleotide and amino acid sequence identity with the published sequence of the *parC* gene in *S. pneumoniae* R6 (accession no. AF053121, Fig. 1B). A single mutation was found in three of four clinical isolates (Numbers 1, 3, and 4).

The mutations identified in the four fluoroquinolone-resistant isolates are summarized in Table 1. For the *gyrA* gene, mutations were found at

Ser81Phe (TCC→TTC), Glu85Gly (GAA→GGA) and Glu85Lys (GAA→AAA). One clinical isolate (No. 3) had *gyrA* mutations at Ser81Phe and Glu85Lys. For the *parC* gene, only one mutation was found: Ser79Tyr (TCT→TAT).

Discussion

This study is the first to describe mutations in the *gyrA* and *parC* genes of fluoroquinolone-resistant *S. pneumoniae* isolates in Thailand. Previous studies on genetic changes conferring fluoroquinolone resistance in *S. pneumoniae* have identified mutations in *gyrA* at Ser81Phe or Glu85Lys and in *parC* at Ser79Phe or Asp83Tyr[2-6,12-14]. The four isolates in this study were resistant to second generation of fluoroquinolones (*i.e.*, ciprofloxacin and ofloxacin) and contained mutations previously described in isolates resistant to third and fourth generation fluoroquinolones (*e.g.*, levofloxacin and moxifloxacin): Ser81Phe, Glu85Gly in *gyrA* and Ser79Tyr in *parC* gene[15-17]. Unfortunately, we did not test whether our isolates were also resistant to the 3rd and 4th generation fluoroquinolones. The isolates in this report had high-level resistance to ofloxacin and ciprofloxacin (MIC > 32 µg/ml, determined by E-test method using the manufacturer's guideline), compared to the highest MIC of 8 µg/ml for both drugs in our previous report[11]

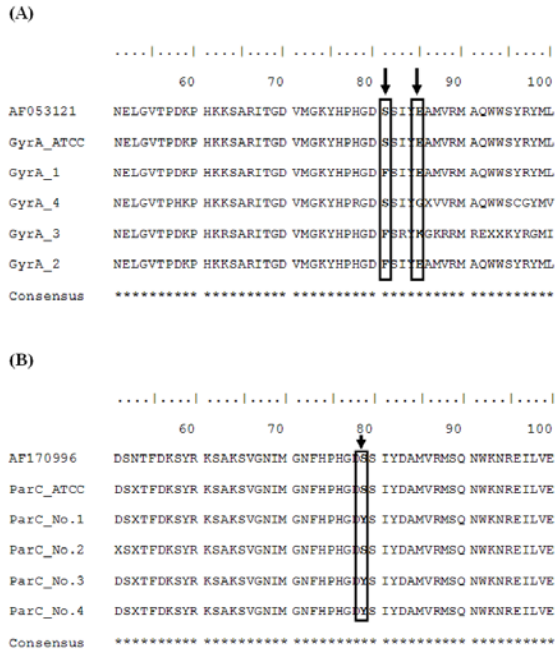

Fig. 1 Multiple amino acid sequence alignment of the translated GyrA protein (1A) and the ParC protein (1B) from *S. pneumoniae* R6 (GenBank accession no. AF053121), ATCC 49619 and clinical isolates No. 1-4; boxes indicate amino acid substitutions. Asterisks indicate identity among sequences.

Multiple mutations were identified in three of the four isolates studied. Two isolates had two mutations (one nucleotide substitution each in the *gyrA* and *parC* genes), and one isolate had three mutations (two nucleotide substitutions in the *gyrA* gene and one in the *parC* gene). Despite the lack of a *parC* mutation in one isolate in this study, it seems likely that there are other mutations outside the sequence recognized by the PCR and DNA sequencing methods. Other possible mechanisms of fluoroquinolone resistance have been reported, including mutations in the *gyrB* and *parE* genes and an active efflux mechanism that pumps drug out of the organism, resulting in lower intracellular concentration and a smaller increase in MIC[18,19]. These possible resistance mechanisms merit further study.

Two of the fluoroquinolone-resistant *S. pneumoniae* isolates in this study were serotype 6B and two were non-vaccine types, which differ from

Table 1. DNA sequence analysis of pneumococcal *gyrA* and *parC* genes

Clinical isolate No.	Serotypes	Amino acid substitution		Nucleotide substitution	
		gyrA	*parC*	*gyrA*	*parC*
1	6B	Ser81Phe	Ser79Tyr	TCC→TTC	TCT→TAT
2	Non-typable	Glu85Gly	none	GAA→GGA	none
3	6B	Ser81Phe Glu85Lys	Ser79Tyr	TCC→TTC GAA→AAA	TCT→ TAT
4	Non-typable	Ser81Phe	Ser79Tyr	TCC→TTC	TCT→TAT

previous reports in Thailand. One study described a single ofloxacin-resistant isolate, which was serogroup 19[11], while a second study described one serotype 6A isolate that was resistant to ciprofloxacin and levofloxacin[20]. The surveillance is needed to monitor for trends in fluoroquinolone resistance, which have implications for clinical management and vaccine policy.

This report provides the first description of mechanisms of fluoroquinolone-resistance in *S. pneumoniae* in Thailand. The data will help improve understanding of *S. pneumoniae* drug resistance, which is an important step toward the development of new drugs.

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การดื้อยากลุ่ม fluoroquinolone ในเชื้อ Streptococcus pneumoniae สายพันธุ์ซึ่งแยกได้จากผู้ป่วยในโรงพยาบาลของมหาวิทยาลัยในประเทศไทย

สมพร ศรีเฟื่องฟุ้ง, ชาญวิทย์ ตรีพุทธรัตน์, กุลกัญญา โชคไพบูลย์กิจ, โสภิตา คำรังษี

การศึกษาเพื่อหาความไวของเชื้อ Streptococcus pneumoniae จำนวน 170 สายพันธุ์ ซึ่งแยกได้จากผู้ป่วยในโรงพยาบาลศิริราชระหว่างเดือนมกราคมถึงธันวาคม ปี พ.ศ. 2551 ต่อยา ofloxacin พบว่าเชื้อมีความไวร้อยละ 93.5 สำหรับผลศึกษาการกลายพันธุ์ของจีน gyrA และ parC ที่ทำให้เกิดการดื้อยากลุ่ม fluoroquinolone เช่น ofloxacin และ ciprofloxacin ในเชื้อจำนวน 4 สายพันธุ์ ซึ่งแยกได้จากผู้ป่วย ซึ่งมีค่าความเข้มข้นน้อยที่สุดของยาต้านจุลชีพ ที่สามารถยับยั้งการเจริญของแบคทีเรียได้ (minimal inhibitory concentration หรือเรียกย่อว่า MIC) มากกว่า 32 ไมโครกรัม/มล. นั้นจะพบว่าจีน gyrA มีการเปลี่ยนแปลงกรดอะมิโนที่ตำแหน่ง Ser81Phe และ Glu85Gly ส่วนในยีน parC มีการเปลี่ยนแปลงกรดอะมิโนที่ตำแหน่ง Ser79Tyr และมี 3 สายพันธุ์ที่มีการกลายพันธุ์ทั้งสองจีนนอกจากนี้ยังพบว่า เชื้อจำนวน 4 สายพันธุ์ ซึ่งนำมาศึกษานั้นเป็นเชื้อซีโรทัยป์ 6B จำนวน 2 สายพันธุ์ และ nonvaccine type จำนวน 2 สายพันธุ์ ผลของการศึกษานี้เป็นรายงานแรกเกี่ยวกับกลไกการดื้อยากลุ่ม fluoroquinolone ของเชื้อ S. pneumoniae ในประเทศไทย