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☐ Original Article ☐

# A Mutation in QRDR in the ParC Subunit of Topoisomerase IV was Responsible for Fluoroquinolone Resistance in Clinical Isolates of *Streptococcus pneumoniae*

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*Forty-one strains of* Streptococcus pneumoniae *were isolated at Seoul National University Children's Hospital from 1991 to 1997. Isolates were divided into six groups based on MICs of three quinolones, ciprofloxacin, ofloxacin and norfloxacin. Sequencing showed that the isolates which were intermediately resistant to three quinolones or resistant to at least one kind of quinolone had one missense mutation, Lys137→Asn (AAG→AAT) substitution in the ParC subunit of topoisomerase IV without additional mutation in QRDR of the GyrA subunit of DNA gyrase. In conclusion, the ParC subunit of DNA topoisomerase IV is the primary target site for fluoroquinolone in* S. pneumoniae *and Lys137→Asn substitution renders the quinolone resistance in* S. pneumoniae.

**Key Words:** *S. pneumoniae,* fluoroquinolone resistance, ParC, GyrA, DNA gyrase, topoisomerase IV, QRDR

*Streptococcus pneumoniae* is one of major gram-positive human pathogens responsible for respiratory tract infections such as pneumonia, sinusitis, otitis, bronchitis, bacteremia, and meningitis in children and adults (Zeller *et al.* 1997). The effective treatment of these infections has been antibiotic therapy. Until the 1970s, most clinical isolates of *S. pneumoniae* were susceptible to penicillin, but the emergence of resistant strains to penicillin and other antibiotics, as well as their spread around the world, has become a major concern for antimicrobial therapy (Chong *et al.* 1995; Lee *et al.* 1995; Song *et al.* 1997) and has increased the need for study of the resistance mechanism (Pan and Fisher, 1996).

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Fluoroquinolones are synthetic broad-spectrum antibiotics which are very active against both gram-negative and -positive bacteria (Finch, 1995; Zeller *et al.* 1997). Initially, pneumococci were very susceptible to fluoroquinolones in vitro regardless of their penicillin-susceptibility. However, the increased use of fluoroquinolones has resulted in the rapid emergence of resistant strains (Janoir *et al.* 1996; Pan *et al.* 1996).

A number of reports have shown that mutations in DNA gyrase and topoisomerase IV are the mechanism of fluoroquinolone resistance in bacteria. In gram-negative bacteria, such as *Aeromonas salmonicida* (Opprgaard and Sorum, 1994), *Pseudomonas aeruginosa* (Kureish *et al.* 1994), *Salmonella typhimurium* (Reyna *et al.* 1995), and *Escherichia coli* (Kim *et al.* 1996a; Pan and Fisher, 1996; Park *et al.* 1996; Lee and Lee, 1998), DNA gyrase and topoisomerase IV have been regarded as the primary and secondary targets of fluoroquinolones, respectively. In pneumococci too, fluoroquinolone resistance mechanisms are mutational alterations of DNA gyrase and topoisomerase IV, and decrease in drug

accumulation inside cells (Janoir *et al.* 1996; Munoz and Campa, 1996; Pan *et al.* 1996; Zeller *et al.* 1997). Contrary to gram-negative bacteria, topoisomerase IV has been reported as the primary target and DNA gyrase as the secondary target in gram-positive bacteria such as *Staphylococcus aureus* (Ferrero *et al.* 1994) and *S. pneumoniae* (Tankovic *et al.* 1996). Since most studies were done with laboratory-derived resistant strains, some investigators have questioned whether the same kinds of mutation occurred in resistant clinical isolates. In this study, we investigated the presence of mutation in QRDRs of *parC* of topoisomerase IV and *gyrA* of DNA gyrase in the clinical isolates of fluoroquinolone-resistant pneumococci.

## MATERIALS AND METHODS

### Bacterial strains and antimicrobial susceptibility test

Forty-one strains of *S. pneumoniae* isolated between 1991 and 1997 from a variety of clinical materials at Seoul National University Children's Hospital were used in this study. MICs of fluoroquinolones were determined by the broth microdilution method using Mueller-Hinton broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 5% lysed horse blood according to the method of the National Committee for Clinical Laboratory Standards (1998). The NCCLS breakpoints for ciprofloxacin, ofloxacin, and norfloxacin were: 1, 2, and 4 μg/mL were susceptible and 4, 8, and 16 μg/mL were resistant, respectively.

### Genomic DNA isolation

Genomic DNA was isolated from *S. pneumoniae* as described elsewhere (Kaufmann *et al.* 1994; Kim *et al.* 1996b) with slight modification. Cells were pelleted by centrifugation at 3,000 × *g* for 30 min and suspended in 100 μL TEG [100 mM Tris (pH 7.4), 10 mM EDTA, and 25% glucose] containing 50 μg/mL of lysozyme. After 10 μL of mutanolysin (1000 U/mL) was added, the reaction mixture was incubated at 37°C for 1 h, and then 500 μL of GES reagent (5 M guanidine thiocyanate, 0.1 M EDTA, 0.5% sarkosyl) was added. After cells were lysed, 250 μL of 7.5 M ammonium acetate was added to the cells and the cells were left on ice for at least 10 min. Then, 500 μL of phenol-chloroform-isoamyl alcohol was added to the cells and mixed gently by inversion. After the reaction mixture was centrifuged, the aqueous phase was transferred to a new test tube and reextracted with phenol-chloroform-isoamyl alcohol until the interface was clear. Then the reaction mixture was extracted with 500 μL of chloroform and the aqueous phase was transferred to a new microcentrifuge tube. A 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold absolute ethanol were added to the reaction mixture, which was placed at −20°C overnight to precipitate DNA. DNA was collected by centrifugation, resuspended in sterile millipore filtered water and stored at −20°C until use.

### PCR of the QRDRs of *gyrA* and *parC*

PCRs were carried out in a final volume of 100 μL containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 1.75 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM dNTP, 100 pmol each of oligonucleotide primers (Pan and Fisher, 1996), 1 U of Vent DNA polymerase, and 1 μg of genomic DNA. A primer VGA3 (5′-CCGTCGCATTCTTTACG-3′) in the sense orientation and a primer VGA4 (5′-TGCTGGCAAGACCGTTGC-3′) in the antisense orientation were used to amplify a 382 bp fragment in QRDR of *gyrA*. A primer M0363 (5′-TGGGTTGAAGCCGGTTCA-3′) in the sense orientation and a primer M4271 (5′-TGCTGGCAAGACCGTTGG-3′) in the antisense orientation were used to amplify a 366 bp fragment in the QRDR of *parC*. Amplification reaction consisted of preincubation at 94°C for 1 min to enhance denaturation of the genomic DNA, 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C (for *gyrA*) or 53°C (for *parC*) for 1 min, and extension at 72°C for 1 min. The reaction mixtures were incubated at 72°C for 7 min for additional extension. PCR products were analyzed with electrophoresis in a 2% agarose gel and stained with ethidium bromide.

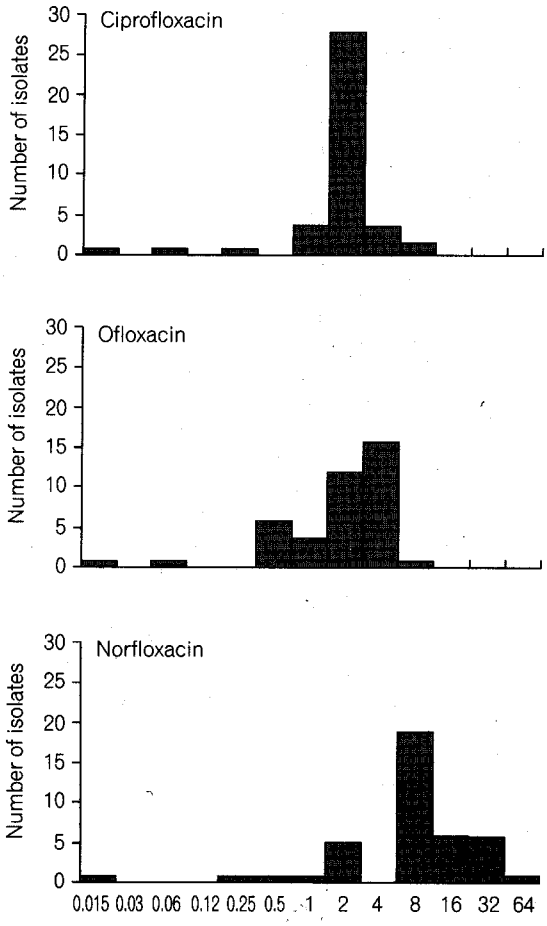


*Fig. 1. Distribution of ciprofloxacin, ofloxacin, and norfloxacin MICs for 41 clinical isolates of* S. pneumoniae. *MICs were determined by the broth microdilution test according to the method of the National Committe for Clinical Laboratory Standards (1998).*

### DNA sequence analysis

PCR products were purified from a gel slice with GENECLEAN II KIT (Bio101, Vista, CA, U.S.A.) and 180 ng of the product was used for sequencing. Nucleotide sequencing was performed by dideoxynucleotide chain termination method (Sanger *et al.* 1977) using ABI PRISM DNA Sequencing Kit and 373 DNA Sequencing System (Perkin-Elmer, Foster City, CA. U.S.A).

## RESULTS

### Fluoroquinolone susceptibility

MICs for 41 isolates of *S. pneumoniae* ranged from 0.015 to 8 μg/mL for ciprofloxacin, from 0.015 to 64 μg/mL for norfloxacin, and from 0.015 to 8 μg/mL for ofloxacin (Fig. 1). Twelve out of 41 isolates (32%) were resistant to at least one kind of fluoroquinolones. Six isolates (15%) were resistant to

**Table 1. Fluoroquinolone susceptibilities of *S. pneumoniae* isolates**

| Category | Number (%) of isolates | | |
|---|---|---|---|
| | Ciprofloxacin | Ofloxacin | Norfloxacin |
| Susceptible | 7 (17) | 24 (59) | 9 (22) |
| Intermediate | 28 (68) | 16 (39) | 19 (46) |
| Resistant | 6 (15) | 1 (2) | 13 (32) |

**Table 2. Mutation identified in QRDR of *parC* in clinical isolates of *Streptococcus pneumoniae***

| Fluoroquinolone susceptibility group | MIC range (μg/mL) | | | No. of isolates | *gyrA* change | *parC* change | |
|---|---|---|---|---|---|---|---|
| | Ciprofloxacin | Ofloxacin | Norfloxacin | | Nucleotide | Nucleotide | Amino acid |
| I | 0.015-2 | 0.015-4 | 0.015-8 | 20 | –* | – | – |
| II | 2 | 4 | 8 | 8 | – | AAG→AAT | Lys137→Asn |
| III | 2 | 0.5-2 | 16-32 | 4 | – | AAG→AAT | Lys137→Asn |
| IV | 2 | 4 | 16 | 3 | – | AAG→AAT | Lys137→Asn |
| V | 4-8 | 4 | 16-64 | 5 | – | AAG→AAT | Lys137→Asn |
| VI | 8 | 8 | 16 | 1 | – | AAG→AAT | Lys137→Asn |

*: No change.

ciprofloxacin and 13 isolates (32%) were resistant to norfloxacin, while one isolate was resistant to ofloxacin (Table 1). Only one isolate was resistant to all three fluoroquinolones. The isolates were divided into six groups based on the MICs of fluoroquinolone (Table 2).

### Mutations in QRDRs of *gyrA* and *parC* genes in fluoroquinolone-resistant isolates

Sequencing the QRDRs of GyrA and ParC of 41 isolates showed only one mutation in *parC* in all of the intermediate and resistant isolates without any additional mutation in *gyrA*. All of the isolates showed the same missense mutation, Lys137 (AAG) →Asn (AAT) substitution in the ParC subunit of topoisomerase IV (Table 2).

## DISCUSSION

A problem associated with the use of fluoroquinolones is the selection of spontaneous resistant mutants. Several studies with pneumococci have shown that low-level resistance can result from mutations in toposiomerase IV (Janoir *et al.* 1996; Munoz and Campa, 1996; Pan *et al.* 1996; Perichon *et al.* 1997). Recently, the fluoroquinolone efflux mechanism was described as a cause of low-level resistance in laboratory-derived and clinically-isolated pneumococci (Brenwald *et al.* 1998). Increased levels of resistance occur following the acquisition of additional mutations in *gyrA*, which encodes the A subunit of DNA gyrase (Janoir *et al.* 1996; Munoz and Campa, 1996; Pan *et al.* 1996).

In *S. pneumoniae,* several mutations have been reported to be responsible for quinolone resistance in vitro. These were Ser83→Tyr or →Phe, and Glu87→Lys in GyrA (Finch, 1995) and Ser80→Tyr, Ser80→Phe, Asp83→Asn, Asp80→His, Ala84→Thr, Arg95→Cys, and Lys137→Asn in ParC (Munoz and Campa, 1996; Pan *et al.* 1996), and Lys137→Asn in ParE (Perichon *et al.* 1997). Recent in vitro experiments by Pan and Fisher (1998) showed that fluoroquinolone caused stepwise mutations of Ser83→Phe or →Tyr in GyrA and Ser79→Tyr, or→Phe and Asp83→Asn in ParC.

Taba and Kusano found Ser81→Phe, Glu85→Lys, and Trp93→Arg substitutions in GyrA and Ser79→Phe or →Tyr and Lys137→Asn substitutions in ParC in four clinically-isolated ciprofloxacin- and sparfloxacin-resistant strains (Taba and Kusano, 1998). However, in this study, we found only Lys137→Asn substitutuion in ParC in clinical isolates. The difference in the mutations might be due to absence of high level resistant strains in this study.

In gram-positive bacteria, topoisomerase IV is known as the primary target for fluoroquinolone and any mutation in topoisomerase IV without accompanying mutation in GyrA increases MIC in a small degree (Janoir *et al.* 1996). In other words, *S. pneumoniae* could become highly resistant to fluoroquinolone only when mutations occur in both topoisomerase IV and DNA gyrase. As mentioned above in laboratory-derived strains, Lys137→Asn was responsible for the intermediate resistance to ciprofloxacin (MIC = 2 μg/mL) while an additional mutation at Ser83 in QRDR of GyrA was necessary for high resistance (MIC = 32 and 128 μg/mL) (Munoz and Campa, 1996). In this study with clinical strains, Lys137→Asn in ParC alone showed norfloxacin resistance with MICs ranging from 2 to 64 μg/ml without any additional mutation in *gyrA,* suggesting the presence of other resistance mechanisms on some of the strains. This site (Lys137) in ParC of pneumococci corresponds to 141 in GyrA of *E. coli* and *S. pneumoniae* which were very close to the DNA binding site of GyrA (Tyr122). The positive charge of Lys137 in ParC might be involved in quinolone binding, and that's why a mutation to Asn with a neutral charge could cause decreased quinolone binding resulting in fluoroquinolone resistance. In conclusion, clinical isolates of low-level fluoroquinolone-resistant pneumococci have a mutation at a site of QRDR of *parC*.

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