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ORIGINAL ARTICLE

# Fluoroquinolone-nonsusceptible *Streptococcus pneumoniae* isolates from a medical center in the pneumococcal conjugate vaccine era

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**KEYWORDS**
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*Streptococcus pneumoniae*;
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**Abstract** *Background/Purpose: Streptococcus pneumoniae* is one of the most common pathogens to cause mucosal and invasive infection in humans. Resistance to fluoroquinolones (FQ) is associated with clinical failure when treating pneumococcal diseases and increase of mortality.
*Methods:* We collected clinical isolates of *S. pneumoniae* from January 2011 to July 2015 at Chang Gung Memorial Hospital, Taoyuan, Taiwan. Susceptibility to FQ was examined by disk diffusion method. Levofloxacin or moxifloxacin-nonsusceptible *S. pneumoniae* isolates were analyzed by serotyping, multilocus sequence typing, and sequencing of the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE*.
*Results:* During the study period, 42 FQ-nonsusceptible pneumococcal isolates were identified. The rate increased from 1.6% of total pneumococcal isolates (2 of 127) in 2011 to 4.6% (13 of 283) in 2014, then decreased to 1.5% (3 of 202) in the first half of 2015. These isolates belonged to 13 serotypes, and serotype 14 (12 of 42, 33.3%) was the most prevalent. Most of the isolates belonged to international clones or their variants. After QRDR analysis, there were 19 isolates in five clusters that shared both the same sequence type and QRDR mutation.
*Conclusions:* FQ resistance initially emerged in either vaccine or nonvaccine serotypes. The majority of isolates were international clones or related variants, suggesting that resistance

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was disseminated through clonal spread. The wide use of pneumococcal conjugate vaccine since 2013 appears to have reduced the spread of FQ-nonsusceptible pneumococci.


## Introduction

Fluoroquinolones (FQs), especially so-called *respiratory FQs* (levofloxacin, gatifloxacin, and moxifloxacin), have good *in vitro* activity against *Streptococcus pneumoniae*, one of the most important pathogens for bacterial pneumonia, meningitis, acute otitis media, and septicemia in humans. Resistance to FQ has been reported since the mid-1990s in North America and Europe in clonally distinct strains.[1,2] *S. pneumoniae* with FQ resistance has also been reported in Asia and Africa.[3,4] Further clinical reports suggest that resistance to FQ is associated with clinical failure when treating pneumococcal infections using these agents.[5,6]

The antimicrobial effects of FQ originate from the inhibition of bacterial DNA gyrase and topoisomerase IV. The two subunits of DNA gyrase, GyrA and GyrB, are encoded by *gyrA* and *gyrB* genes, whereas the two subunit of topoisomerase, ParC and ParE, are encoded by *parC* and *parE*. Mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* are closely related to FQ resistance in *S. pneumoniae*. Efflux pumps are also documented to be related to FQ resistance. However, the influence of efflux pumps to moxifloxacin and levofloxacin resistance is relatively less, compared with the QRDR mutations.[7]

The pneumococcal seven-valent conjugate vaccine (PCV7) has been introduced to Taiwan to prevent pneumococcal infection since 2005. Introduction of pneumococcal 13-valent conjugate vaccine (PCV13) for use in 2011 further reduced the infections caused by non-PCV7 serotypes.[8] In this article we report the serotypes, sequence types, and antimicrobial susceptibility of FQ-nonsusceptible *S. pneumoniae* isolates collected from a medical center in Taiwan in the pneumococcal conjugate vaccine era. The resistance mechanisms of FQ-nonsusceptibility were studied by sequencing the QRDR of *gyrA, gyrB, parC*, and *parE* genes.

## Methods

### Bacterial isolates

Clinical isolates of *S. pneumoniae* were prospectively collected from January 2011 to July 2015. If multiple isolates were identified from the same patients, only the first isolate was retained for further investigation. All isolates were cultured and identified with standard methods in the Clinical Microbiology Laboratory at Chang Gung Memorial Hospital (CGMH), Taoyuan, Taiwan.

### Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of penicillin and ceftriaxone were analyzed by E-test (AB Biodisk, Solna, Sweden). Susceptibilities to levofloxacin and moxifloxacin were examined by a standard disc-diffusion method. The results were interpreted according to the suggestions of the Clinical Laboratory Standards Institute.[9,10]

### Serotyping and genotyping

The serotypes of pneumococcal isolates were determined by using commercialized antisera (Statens Serum Institut, Copenhagen, Denmark) and polymerase chain reaction methods, as previously described.[11–13] Multilocus sequence typing was further determined by the polymerase chain reaction sequencing of a set of pneumococcal housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* ) with the use of published primers.[14] The sequence data were compared to the multilocus sequence typing database maintained in the public domain (http://spneumoniae.mlst.net). New alleles and allelic profiles were submitted to the database curator for the assignment of sequence type (ST) numbers. For isolates showing fluoroquinolone resistance, the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes were analyzed by a published method.[15]

### Statistical analysis

Poisson test was applied to analyze the isolation rate of FQ-nonsusceptible *S. pneumoniae* between years. A $p$ value $< 0.05$ indicated a significant difference between different years.

## Results

From 2011 to the first 6 months of 2015, 1281 *S. pneumoniae* isolates were collected from CGMH, and 42 levofloxacin or moxifloxacin-nonsusceptible isolates were identified. From 2011 to 2015, the number of FQ-nonsusceptible isolates increased in CGMH. We recorded a 2.9-fold increase in rate from 1.6% (2 of 127) in 2011, 3.6% (13 of 359) in 2012, and 3.5% (11 of 310) in 2013, to 4.6% (13 of 283) in 2014, but it decreased to 1.5% (3 of 202) in the first 6 months of 2015 (Figure 1). The decrease in 2015 reached statistical significance, when compared to data in either 2014 ($p = 0.012$) or 2011–2014 ($p = 0.043$). The mean age of these patients was 58 years with a median of 77 years (range, 3–100 years).

The characteristics of the 42 levofloxacin or moxifloxacin-nonsusceptible *S. pneumoniae* isolates from CGMH are

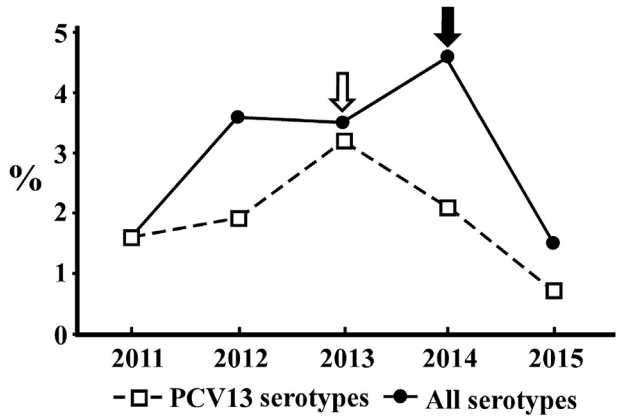


**Figure 1.** Incidence of fluoroquinolone-nonsusceptible *Streptococcus pneumoniae* isolates among different serotypes from January 2011 to July 2015 in Chang Gung Memorial Hospital. The nationwide catch-up program was launched in 2013 (white arrow) and national immunization program with a three-dose schedule in 2015 (black arrow). The decrease in 2015 reached statistical significance, comparing to either 2014 ($p = 0.012$) or 2011–2014 ($p = 0.043$).

shown in Table 1. The distribution of serotypes is shown in Figure 2. The majority of isolates (36 of 42, 85.76%) were from respiratory tract secretions, including six isolates from patients with acute sinusitis. Only one of the six isolates from sinusitis was PCV13 serotype (Serotypes 3, 15A, 15B, and 34). Six of the 42 FQ-nonsusceptible *S. pneumoniae* isolates were cultured from blood. All of these six patients with bloodstream infection were adults aged 49–100 years, and four of them were older than 65 years. Five of the six blood isolates were PCV13 serotypes and the most prevalent was serotype 14 (4 of 6, 66.6%). Seven of the 42 FQ-nonsusceptible *S. pneumoniae* isolates were cultured from pediatric patients (age 3–16 years) and all of the pediatric isolates were derived from respiratory secretions. Four of the six pediatric isolates were pus drained form acute sinusitis, and none of them were PCV13 serotypes. Two were serotype 15A and others were serotype 15B. Other pediatric isolates belonged to PCV13 serotypes (serotypes 19F and 23F). Three FQ-nonsusceptible isolates were collected in the first 6 months of 2015, all from sputum of adult patients. The serotypes of the three isolates were 14, 15B, and 23F.

The following 13 serotypes were found among the FQ-nonsusceptible isolates: 14 ($n = 12$, 33.3%), 15B ($n = 6$, 14.2%), 23F ($n = 7$, 16.6%), 19F ($n = 4$, 9.5%), 23A ($n = 3$, 7.1%), 15A ($n = 3$, 7.1%), 3 ($n = 1$, 2.4%), 6A ($n = 1$, 2.4%), 9V ($n = 1$, 2.4%), 11A ($n = 1$, 2.4%), 13 ($n = 1$, 2.4%), 19A ($n = 1$, 2.4%), and 34 ($n = 1$, 2.4%). The PCV13 serotypes were dominant in these isolates ($n = 27$, 64.3%).

Alterations in GyrA were found in 41 isolates, mostly in S81 ($n = 39$, 92.8%), and 14 (35.8%) of them had another alteration in S114. Another GyrA alteration found in two other isolates was E85. Alterations in ParC were also found frequently ($n = 35$, 83.3%). All of these isolates had S79 alteration, and two of them were combined with both S52 and N91 alterations. In ParE the most frequent alteration was in D435 ($n = 16$, 38.1%); others were A326 ($n = 7$, 16.7%), P454 ($n = 1$, 2.4%), and R447 ($n = 1$, 2.4%). Only one isolate had both D435 and A326 alterations. GyrB alterations were not identified among the FQ-nonsusceptible *S. pneumoniae* isolates in CGMH. Among the 42 isolates, a combination of two or more alterations in QRDR was found in 41 isolates, including GyrA plus ParC ($n = 17$, 40.4%), and Gyr A plus ParE ($n = 6$, 14.3%). Eight isolates (19%) showed alteration in all GyrA, ParC, and ParE. One isolate had ParE alteration only.

We identified 19 genotypes among the 42 FQ-nonsusceptible *S. pneumoniae* isolates. Most ($n = 40$, 95.2%) were international clones or related variants. The most prevalent genotype was ST876 ($n = 10$, 23.8%), a single locus variant of Netherlands$^{15B}$-37, and all were serotype 14. The second largest genotype was ST83 ($n = 5$, 11.9%), a single locus variant of Spain$^{23F}$-1, all were serotype 15B. Other genotypes contained three isolates were ST63 (Sweden$^{15A}$-25), all 15A; ST242 (Taiwan$^{23F}$-15), and ST81 (Spain$^{23F}$-1). Among most of these isolates with similar genotype, we found identical QRDR amino acid alterations. In all ST876 isolates we found S81W with S114G in GyrA and S79F in ParC. D435N was also found in nine of the 10 ST876 isolates. In all ST7128 isolates, we identified S81W in GyrA and S79F in ParC. In all ST81 isolates there were S81W in GyrA, S79F in ParC, and A326V in ParE. Identical multiple QRDR alteration was found in two ST81 (serotype 15B) isolates; both were cultured from pediatric patients with acute sinusitis. These alterations included S81Y with S114G in GyrA; S79I, S52G, and N91D in ParC; and A326V in ParE.

Only seven (16.7%) isolates among the 42 FQ-nonsusceptible *S. pneumoniae* isolates from CGMH were susceptible to ceftriaxone (MIC $\leq$ 0.5 μg/mL). By contrast, there were 35 (83.3%) penicillin-susceptible (MIC $\leq$ 2 μg/mL) isolates found. All the penicillin-nonsusceptible isolates were considered multidrug-resistant because they were also nonsusceptible to ceftriaxone.

## Discussion

According to a previous survey, the overall resistance rates to respiratory fluoroquinolones in pneumococci remained low in most Asian countries, while levofloxacin and moxifloxacin resistance was more frequent in Korea and Taiwan.[16,17] A study from northern Taiwan revealed a significant increase of the prevalence of levofloxacin-nonsusceptible *S. pneumoniae* from 1.2% in 2001 to 4.2% in 2007.[18] The trend was also found in our study from 2011 to 2014 and ceased in the first half of 2015. Prescription of antibiotic for respiratory tract infection was closely related to the development of bacterial resistance to antibiotics.[19,20] The use of FQ in the treatment of tuberculosis was proposed in previous studies to increase the potential for the spread of levofloxacin-nonsusceptible pneumococci.[21] However, levofloxacin treatment for tuberculosis was excluded from the benefit package of Taiwan's National Health Insurance since 2007. Another important event that may promote the prescription of respiratory FQ was the modification of the management guideline on community-acquired pneumonia, in which respiratory FQ was recommended to be the first-line outpatient treatment for those with comorbidities.[22] The record from our antibiotic stewardship program also showed an increase in the

**Table 1** The characteristics of the 42 levofloxacin or moxifloxacin-nonsusceptible *Streptococcus pneumoniae* isolates

| Related international clone | Sex | Age (y) | Source | Sequence type | Serotype | QRDRs | | | | MIC (mg/mL) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | GyrA | GyrB | ParC | ParE | Pen | Cro |
| Netherlands$^{15B}$-37 | F | 91 | Blood | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 0.5 | 1 |
| | M | 73 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 1 | 1 |
| | F | 79 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 1 | 1 |
| | M | 83 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 1 | 1 |
| | M | 60 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 1 | 1 |
| | M | 24 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 2 | 1 |
| | M | 49 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 2 | 1 |
| | M | 73 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 2 | 2 |
| | F | 100 | Blood | 876 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 4 | 1 |
| | F | 94 | Respiratory | 876 (SLV199) | 14 | S81W S114G | — | S79F | — | 1 | 1 |
| | F | 49 | Blood | 5749 (SLV199) | 14 | S81W S114G | — | S79F | D435N | 1 | 1 |
| | M | 60 | Blood | 9635 (SLV199) | 14 | S81W S114G | — | — | D435N | 2 | 2 |
| Spain$^{23F}$-1 | M | 72 | Respiratory | 81 | 23F | S81F | — | S79F | A326V | 1 | 1 |
| | M | 86 | Respiratory | 81 | 19F | S81F | — | S79F | A326V | 1 | 2 |
| | M | 86 | Respiratory | 81 | 19F | S81F | — | S79F | A326V | 2 | 2 |
| | M | 4 | Pus | 83 (SLV81) | 15B | S81Y S114G | — | S52G S79I N91D | A326V | 4 | 2 |
| | M | 5 | Pus | 83 (SLV81) | 15B | S81Y S114G | — | S52G S79I N91D | A326V | 4 | 2 |
| | F | 72 | Respiratory | 83 (SLV81) | 15B | S81F | — | — | A326V D435N | 2 | 2 |
| | F | 48 | Respiratory | 83 (SLV81) | 15B | E85K | — | — | D435N | 2 | 1 |
| | M | 30 | Respiratory | 83 (SLV81) | 15B | S81F | — | S79Y | — | 4 | 2 |
| | M | 81 | Respiratory | 10079 | 15B | S81F | — | S79F | A326V | 2 | 0.5 |
| Taiwan$^{23F}$-15 | M | 12 | Respiratory | 242 | 23F | S81F | — | S79Y | R447C | 0.25 | 0.125 |
| | M | 70 | Respiratory | 242 | 23F | S81F | — | — | D435N | 2 | 2 |
| | F | 7 | Respiratory | 242 | 23F | S81Y | — | S79F | — | 1 | 1 |
| | M | 75 | Pus | 9625 (SLV242) | 3 | S81F | — | — | D435N | 1 | 1 |
| | M | 71 | Blood | 7128 (DLV242) | 23F | S81F | — | S79F | — | 2 | 2 |
| | M | 81 | Respiratory | 7128 (DLV242) | 23F | S81F | — | S79F | — | 8 | 8 |
| | M | 75 | Respiratory | 7128 (DLV242) | 23F | S81F | — | S79F | D435N | 8 | 4 |
| Taiwan$^{19F}$-14 | F | 16 | Respiratory | 236 | 19F | S81F | — | — | D435N | 2 | 1 |
| | M | 71 | Respiratory | 9998 (SLV236) | 19F | S81F | — | S79F | — | 2 | 2 |
| | M | 67 | Respiratory | 320 (DLV236) | 19A | S81F | — | S79Y | — | 4 | 2 |
| Colombia$^{23F}$-26 | M | 66 | Respiratory | 338 | 23A | E85K | — | S79F | — | 0.25 | 0.25 |
| | M | 90 | Respiratory | 338 | 23A | S81F P33H | — | S79F | — | 0.5 | 0.25 |
| | M | 85 | Blood | 8080 (SLV338) | 23A | S81F | — | S79F | — | 0.5 | 0.5 |
| Spain$^{9V}$-3 | F | 40 | Respiratory | 166 (SLV156) | 13 | S81F | — | S79Y | — | 2 | 2 |
| | M | 57 | Respiratory | 166 (SLV156) | 11A | S81F | — | S79F | — | 2 | 2 |
| | M | 76 | Respiratory | 166 (SLV156) | 9V | S81F S114G | — | S79Y | — | 2 | 2 |

**Table 1** (*continued*)

| Related international clone | Sex | Age (y) | Source | Sequence type | Serotype | QRDRs | | | | MIC (mg/mL) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | GyrA | GyrB | ParC | ParE | Pen | Cro |
| Sweden$^{15A}$-25 | M | 3 | Pus | 63 | 15A | S81F | — | S79F | — | 1 | 1 |
| | M | 7 | Pus | 63 | 15A | S81F | — | S79F | — | 2 | 1 |
| | M | 67 | Respiratory | 63 | 15A | S81F | — | S79F | — | 2 | 1 |
| | M | 33 | Pus | 6823 | 34 | — | — | — | P454S | 0.01 | 0.01 |
| | M | 71 | Respiratory | 855 | 6A | S81F | — | S79F | — | 0.5 | 0.5 |

Cro = ceftriaxone; MIC = minimum inhibitory concentration; Pen = penicillin; QRDR = quinolone resistance-determining region; single locus variant (SLV).

consumption of FQ, especially in 2015 when the annual consumption was significantly higher than the average amount from 2010 to 2014 (data not shown). The increasing trend of FQ prescription in Taiwan was described in a retrospective study.[23] An appropriate antibiotic stewardship program that promotes the judicious use of FQ from the treatment of respiratory tract infections should be emphasized to stop the increasing trend of FQ resistance in respiratory pathogens.

Clinical evidence indicates that use of PCV7 had changed the course of an upward trend in fluoroquinolone resistance.[24–26] PCV7 was first available in 2005 in Taiwan, followed by PCV10 in 2010 and PCV13 in 2011.[8] The PCV7 vaccination rate among children aged 5 years or younger reached 45.5% in 2010. In 2013, a national catch-up immunization program providing one dose of PCV13 for children aged 2–5 years was launched, which raised the proportion of PCV13 immunization with the recommended schedules rapidly from 31.9% to 64.2% in 2013.[27]

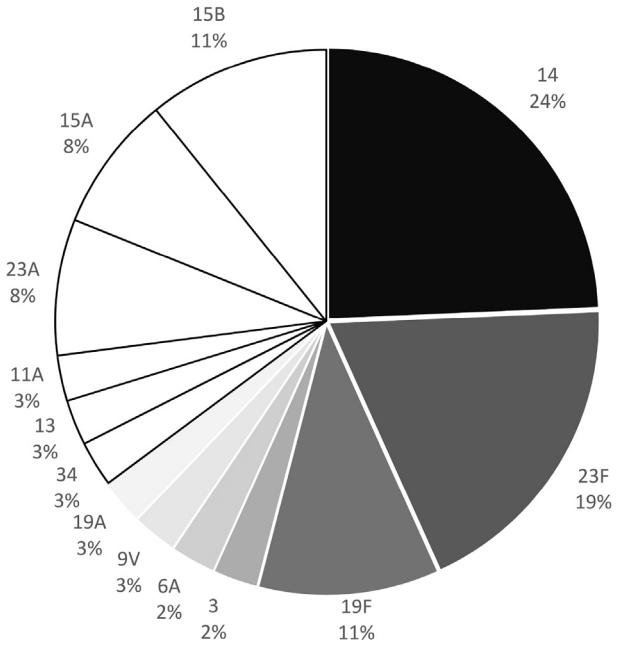


**Figure 2.** Distribution of serotypes among the 42 fluoroquinolone-nonsusceptible *Streptococcus pneumoniae* isolates.

In our observation, the prevalence of FQ-nonsusceptible *S. pneumoniae* isolates belonging to PCV13 serotypes decreased from 3.2% in 2013, 2.1% in 2014, to 0.7% in 2015. The proportion of PCV13 serotypes among FQ-nonsusceptible *S. pneumoniae* isolates also decreased from 90.9% in 2013 to 46.1% in 2014 and 66.7% in 2015. In 2015, the national immunization program of PCV13 was promoted to offer a three-dose schedule. The promotion, which was documented to provide protection as the standard schedule, appears to be related to the decrease of FQ-nonsusceptible rate we observed in the first half of 2015.[28] The PCV13 serotypes were reported to be the major serotypes of FQ-nonsusceptible isolates in previous studies.[24,25] The observation suggested that the use of PCV13 contributed to the reduction of FQ-nonsusceptible pneumococcal infection.[24,25] Since the proportion of FQ-nonsusceptible *S. pneumoniae* isolates with PCV13 serotypes remains high, the nationwide vaccination policy is important in minimizing the emergence of FQ resistance in Taiwan.

Despite the decrease of PCV13 serotypes, the prevalence of FQ-nonsusceptible *S. pneumoniae* in CGMH has still been increasing from 2011 to 2014. The proportion of the non-PCV13 serotypes among FQ-nonsusceptible isolates has increased. A prospective survey in Hong Kong 2 years after PCV13 use demonstrated that serogroup 15 was the most common among nasopharyngeal carriage isolates in children aged 5 years or younger, probably due to expansion of preexisting clones.[29] The increasing proportion of serogroup 15 was also reported in a cross-sectional survey of children aged 3–40 months within a French population with 82.7% of PCV13 coverage rate.[24]Among our 42 *S. pneumoniae* isolates, all the isolates from patients aged 5 years or younger and those from pus of sinusitis belonged to serogroup 15. Moreover, two isolates of serotype 15B with identical sequence type and QRDR alterations were identified, suggesting an important role of serogroup 15 in PCV13 era. Close monitoring is necessary.

Evidence of clonal dissemination attributable to fluoroquinolone resistance has been reported in Hong Kong, Spain, and USA.[25,30–32] Variation of QRDR was demonstrated in previous studies from USA and a cluster of isolates that shared the same sequence was found to have identical QRDR mutation.[25,32] Moreover, all isolates in the cluster were from one state. This was considered strong

evidence of clonal spread. In Taiwan, a prospective survey for levofloxacin-nonsusceptible *S. pneumoniae* was carried out from 2004 to 2006 in CGMH and another medical center.[16] The international clones or related variants were identified in the study but the QRDR mutations were heterogeneous among isolates with a similar genotype, indicating that part of the dissemination may originate from independent selection. In our study, most of the FQ-nonsusceptible *S. pneumoniae* isolates belonged to international clones or their variants. The QRDR analysis found that many isolates, cultured in different years from patients with different age, sex, and specimens, shared both the same sequence type and identical QRDR mutation. This included two multidrug-resistant isolates of ST83 that had eight different amino acid alterations among three QRDRs. Since our isolates were cultured from a single medical institution, the homogeneity of QRDR mutations indicated a growing burden of clonal spread among FQ-nonsusceptible strains.

In conclusion, our observations demonstrate a rising trend of FQ resistance among *S. pneumoniae*, which seemingly ceased after the introduction of PCV13 into the national immunization program. We also found the transition of the dominance by PCV13 serotypes to that by non-PCV13 serotypes. Due to the homogeneity of QRDR in different isolates, clonal spreading among FQ-nonsusceptible strains was proposed. Strategies including antimicrobial stewardship, nationwide vaccination policy, and continuous surveillance are necessary to reduce the dissemination of FQ-nonsusceptible *S. pneumoniae*.

## Conflicts of interest

All authors declare no conflicts of interest.

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