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Original

Molecular Analysis of Levofloxacin-resistant *Streptococcus pneumoniae* in Japan

Hisashi SHOJI*, Takahiro TAKUMA and Yoshihito NIKI

Abstract: Quinolone resistance has been attributed to amino acid mutations in the type II topoisomerases of pathogens. To better understand this mechanism of resistance, we analyzed the molecular epidemiology of levofloxacin-resistant *Streptococcus pneumoniae* in Japan. We measured the quinolone susceptibility of 668 strains of *S. pneumoniae* obtained through nationwide surveillance from 2006 to 2008. We also sequenced the quinolone resistance-determining regions (QRDRs) in type II topoisomerases and analyzed the relationship between minimum inhibitory concentration (MIC) and six specific mutations (Ser81 or Glu85 in GyrA, Asp435 in GyrB, Ser79 or Asp83 in ParC, and Asp435 in ParE). Eighteen of the isolated strains (2.7%) showed intermediate susceptibility and resistance to levofloxacin (MIC > 4 μg/ml), with no significant difference among years. However, garenoxacin and sitafloxacin showed excellent activity against these strains. Of the 18 strains, 17 (94.4%) showed mutation in QRDRs, while in 12 out of 14 levofloxacin-resistant strains (85.7%) two or more mutations were identified. A single QRDR mutation was found in 3 of 60 levofloxacin-susceptible strains (5%), all of which had an MIC of 2 μg/ml. We therefore found a high isolation frequency of levofloxacin-resistant *S. pneumonia* in Japan over a 2-year period. Furthermore, QRDR mutations were present in 5% of susceptible strains; these were thought to be in the early stages of resistance. In the future, the increasing use of levofloxacin might result in more strain resistance. We therefore suggest that strong quinolones such as garenoxacin and sitafloxacin could be proactively administered to high-risk patients.

Key words: *Streptococcus pneumoniae*, GyrA, ParC, quinolone resistance-determining regions

Introduction

Streptococcus pneumoniae is a gram-positive bacterial pathogen that causes respiratory and otolaryngological infections such as pneumonia, tympanitis and sinusitis, as well as serious invasive diseases, such as meningitis and septicemia. *S. pneumoniae* is also a major cause of community-acquired pneumonia, similar to *Haemophilus influenzae*[1]. Penicillin and other β-lactams are frequently used to treat *S. pneumoniae* infections; however, the increasing number of penicillin intermediate-resistant *S. pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) infections that are resistant to β-lactams has become a serious clinical problem[1-3].

Department of Medicine, Division of Clinical Infectious Diseases, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
* To whom corresponding should be addressed.

The most effective antibiotics for community-acquired pneumonia, including PRSP, in the outpatient setting is a group of drugs called 'Respiratory Quinolones'. In Japan, tosufloxacin (TFLX), levofloxacin (LVFX), moxifloxacin (MFLX), garenoxacin (GRNX), and sitafloxacin (STFX) are included in this group. These drugs have an excellent transfer rate to the lungs and show strong antibacterial activity against most community-acquired pneumonia-causing pathogens, including *H. influenzae* and *Mycoplasma pneumoniae*[1]. The guidelines for community-acquired pneumonia in Japan recognize the effectiveness of 'Respiratory Quinolones' and recommend them for use in elderly patients or patients with underlying disease[1]. However, quinolone-resistant *S. pneumoniae* have now been reported worldwide[4, 5].

Quinolone inhibits bacterial growth by disrupting the DNA replication of type II topoisomerase[6]. Type II topoisomerases are currently recognized to include DNA gyrase, which is responsible for the formation and elimination of supercoiled structures in DNA strands, and topoisomerase IV, which cuts and re-ligates tangled DNA during DNA replication[6]. Each of these enzymes is composed of two dimers of subunit types A and B, which together form a tetramer. DNA gyrase is composed of GyrA and GyrB, while topoisomerase IV comprises ParC and ParE; subunit A (GyrA and ParC) possesses DNA cutting and ligating activity, and subunit B (GyrB and ParE) possesses ATPase activity. Quinolones bind to the exposed double-stranded DNA and form a DNA−DNA gyrase-quinolone antibiotic complex, thereby preventing the re-ligation of DNA and subsequent replication[7]. Amino acid substitutions in either enzyme may lead to the inhibition of such complex formations, and in particular, mutations in the quinolone resistance-determining regions (QRDRs) within subunit A and B are closely related to resistance[7]. The mechanism of quinolone resistance by *S. pneumoniae* is thus attributed to mutations in the QRDRs and acceleration of drug efflux pumps, but high-level resistance is believed to solely involve mutations in the QRDRs[8]. Previously, quinolone-resistant *S. pneumoniae* with substitutions at serine 81 and glutamic acid 85 in GyrA, at aspartic acid 435 in GyrB, at serine 79 and aspartic acid 83 in ParC, and at aspartic acid 435 in ParE have been reported[7].

In this study, we investigated the frequency of LVFX-resistant *S. pneumoniae* in Japan and analyzed the six mutations in QRDRs identified in quinolone-resistant strains. In addition, we performed a similar analysis of quinolone-susceptible *S. pneumoniae* to assess the tendency for quinolone resistance in Japan.

Materials and methods

Strains

We obtained 668 *S. pneumoniae* strains as part of a nationwide surveillance program in Japan conducted from 2006 to 2008[9–11]. *S. pneumoniae* identification was confirmed by detection of the *lytA* gene. LVFX susceptibility was defined in accordance with Clinical Laboratory Standard Institute criteria, with an LVFX MIC ≤ 2 μg / ml defined as susceptible, 4 μg / ml as intermediate, and ≥8 μg / ml as resistant[12]. A total of 60 strains were examined, comprising 5 susceptible strains with an LVFX MIC of 0.5 μg / ml and 15 strains at 2 μg / ml extracted at random each year. All strains were grown on Sheep Blood Agar® (Nippon Beckton-Dickinson, Tokyo, Japan)

and maintained at 35℃ with 5% CO_2. These strains were stored in a Microbank™ system (IWAKI, Tokyo, Japan) at −80℃.

Susceptibility testing

The bacterial susceptibility to antibiotics was studied by using the broth microdilution method, performed according to Clinical Laboratory Standards Institute guidelines[13]. The test medium was prepared using cation-adjusted Mueller Hinton broth (Eikenkagaku, Tokyo, Japan) with lysed horse blood (Nippon Biotest Laboratory, Tokyo, Japan). The quinolones evaluated were LVFX (Daiichi Sankyo, Tokyo, Japan), MFLX (Bayer Yakuhin, Osaka, Japan), GRNX (Toyama Chemical, Toyama, Japan), TFLX (Toyama Chemical), and STFX (Daiichi Sankyo). MIC_{50} and MIC_{90} were calculated as the concentration to inhibit 50% or 90%, respectively, of growth of the bacterial strains used.

PCR amplification and sequence analysis of QRDRs

We determined the nucleotide sequence of *lytA* and the QRDRs in the *gyrA*, *gyrB*, *parC*, and *parE* genes. PCR was performed in a 50-μl reaction volume containing 50 ng of DNA template and Phusion polymerase (FINNZYMES, Massachusetts, USA) using the GeneAmp® PCR System 9700 (Applied Biosystems, California, USA). The following specific primers were used to obtain DNA fragments: *lytA* (F: 5'-TGAAGCGGATTATCACTGGC-3', R: 5'-GCTAAACTCCCTGTATCAAGCG-3'), *gyrA* (F: 5'-CCGTCGCATTCTTTACG-3', R: 5'-AGTTGCTCCATTAACCA-3'), *gyrB* (F: 5'-CATGGAAAATCCACAGATTG-3', R: 5'-ATCGGCATCGGTCATCAAAA-3'), *parC* (F: 5'-TGGGTTGAAGCCGGTTCA-3', R: 5'-TGCTGGCAAGACCGTTGG-3'), and *parE* (F: 5'-AAGGCGCGTGATGAGAGC-3', R: 5'-TCTGCTCCAACACCCGCA-3')[14].

The PCR conditions were as follows: denaturation at 98℃ for 30 s and 35 amplification cycles of 98℃ for 5 s, annealing at 50℃ for 10 s, and polymerization at 72℃ for 15 s; a final cycle of 72℃ for 5 min was used to fully extend the amplicons.

The PCR products were purified using a GebElute™ PCR purification Kit (SIGMA-ALDRICH, Saint Louis, USA) and both strands of the DNA fragments were sequenced using a Quant-iT™ dsDNA BR Assay Kit (Invitrogen, California, USA). Nucleotide sequences analysis was commissioned from Akita Prefectural University, Biotechnology Center.

Statistical analysis

SPSS 16.0J was used for statistical analysis. Fisher's exact test was used to compare the isolation frequency of LVFX-intermediate and -resistant *S. pneumonia* strains for each year. A significant difference was defined by a *P* value below 0.05.

Results

Frequency and susceptibility of LVFX-intermediate and -resistant S. pneumoniae

Table 1 shows the isolation frequency of LVFX-intermediate and -resistant *S. pneumoniae* for

Table 1. Annual frequency of levofloxacin-intermediate and -resistant *Streptococcus pneumoniae*

LVFX MIC (μg / ml)	2006	2007	2008	Total
	Strains (%)	Strains (%)	Strains (%)	Strains (%)
≤ 2	195 (97.5)	249 (96.9)	206 (97.6)	650 (97.3)
4	0	2 (0.8)	2 (1.0)	4 (0.6)
≥ 8	5 (2.5)	6 (2.3)	3 (1.4)	14 (2.1)
Total	200	257	211	668

Fisher's exact test : N.S.

Table 2. MICs against each quinolone in all strains

Agent	2006		2007		2008		Total	
	MIC (μg / ml)		MIC (μg / ml)		MIC (μg / ml)		MIC (μg / ml)	
	50%	90%	50%	90%	50%	90%	50%	90%
LVFX	1	2	1	2	1	2	1	2
TFLX	0.125	0.25	0.25	0.25	0.125	0.25	0.125	0.25
MFLX	–	–	0.25	0.5	0.25	0.25	0.25	0.25
GRNX	–	–	0.125	0.25	≤ 0.06	0.125	≤ 0.06	0.125
STFX	–	–	–	–	≤ 0.06	0.125	≤ 0.06	0.125

each year. Regarding the LVFX susceptibility assessments, 4 intermediate strains were isolated among a total of 668 (0.6% ; 0 in 2006, and 2 in both 2007 and 2008), and 14 resistant strains were isolated (2.1% ; 5 in 2006, 6 in 2007, and 3 in 2008). Furthermore, the frequency analysis showed no significant difference among the years using Fisher's exact test. The MIC_{50} and MIC_{90} results for each quinolone examined are shown in Table 2, with MFLX, GRNX, and STFX not used in 2006, and STFX not used in 2007. All quinolones used showed good activity against *S. pneumonia*, with a MIC_{50} and MIC_{90} for LVFX of 1 μg / ml and 2 μg / ml, respectively, showing a tendency to higher activity than the other quinolones. The MIC_{50} and MIC_{90} for both GRNX and STFX were $<$ 0.06 μg / ml and 0.125 μg / ml, respectively, and those were the lowest MICs.

Quinolone susceptibilities and amino acid mutations in the QRDRs

Table 3 shows the MIC for each quinolone and the GyrA, GyrB, ParC, and ParE amino acid mutations for each of the 18 LVFX-intermediate and -resistant strains. There were four LVFX intermediate strains, although the other quinolones demonstrated good activity against these strains, and three of the four had one or more mutations in either Ser81 or Glu85 in GyrA, Asp435 in GyrB, Ser79 or Asp83 in ParC, and Asp435 in ParE. However, no mutation was found in the 2008-236 strain.

Of the 14 LVFX-resistant strains, 5 had an LVFX MIC of 8 μg / ml. These 14 strains all had

Table 3. Quinolone susceptibilities and amino acid mutations in QRDRs of the intermediate and resistant strains

Strain		MIC (μg/ml)*					Amino acid changes in each QRDR					
							GyrA		GyrB	ParC		ParE
		LVFX	TFLX	MFLX	GRNX	STFX	Ser81	Glu85	Asp435	Ser79	Asp83	Asp435
2007	26	4	1	2	0.5	0.25	Phe	–	–	–	–	–
2007	229	4	1	1	0.25	0.25	Phe	–	–	–	Asn	–
2008	139	4	0.25	0.5	0.125	0.25	–	–	–	–	–	Asn
2008	236	4	0.5	0.25	0.25	0.5	–	–	–	–	–	–
2006	10	8	2	4	2	1	Phe	–	–	Ile	Asn	–
2006	42	8	8	4	0.25	0.5	Phe	–	–	–	Tyr	–
2006	57	8	4	4	2	0.5	Phe	–	–	Tyr	–	–
2006	114	8	1	4	0.5	0.5	Phe	–	–	Phe	–	–
2006	179	8	0.5	4	0.25	0.5	Phe	–	–	–	–	Asn
2007	135	16	8	4	2	1	Phe	–	–	Phe	–	–
2007	192	16	>16	4	2	1	–	–	–	Phe	–	–
2007	278	16	2	4	1	1	Tyr	–	–	Tyr	–	–
2008	45	16	>16	4	1	0.5	Phe	–	–	–	–	–
2008	165	16	>16	4	1	0.5	Phe	–	–	Phe	–	–
2008	168	16	>16	4	1	0.5	Phe	–	–	Phe	–	–
2007	97	32	0.125	2	0.25	0.25	–	–	Val	Phe	–	–
2007	100	32	>16	4	1	1	Tyr	–	–	Phe	–	–
2007	118	64	>16	8	2	2	–	Lys	–	Tyr	–	–

* MFLX : moxifloxacin, LVFX : levofloxacin, TFLX : tosufloxacin, GRNX : garenoxacin, STFX : sitafloxacin

at least two QRDR mutations, with three mutations in strain 2006-10, and 6 of the 14 strains had an LVFX MIC of 16 μg / ml. Strains 2007-192 and 2008-45 had only a single mutation each (Ser79 in ParC and Ser81 in GyrA, respectively). Two strains had an LVFX MIC of 32 μg / ml, and for one it was 64 μg / ml. Each of these had two mutations in their QRDRs. The GRNX and STFX MICs were ≤ 2 μg / ml in all strains, with strong activity demonstrated against strains with high resistance to LVFX.

In the analysis of LVFX MIC and amino acid mutations in each QRDR for the 60 LVFX-susceptible strains, one strain was found in each year of the surveillance. Strains 2006-2 (Asp83-Val), 2007-274 (Ser79-Tyr), and 2008-41 (Ser79-Phe) had a mutation in ParC, and all had an MIC of 2 μg / ml. No mutation in QRDRs was found among the 15 strains with an LVFX MIC of 0.5 μg / ml (data not shown).

Relationship between the site of mutations in QRDRs and LVFX MIC

The number of strains with mutations in DNAgyrase and topoisomerase IV, and their MIC value distribution, are shown in Fig. 1. Among LVFX-intermediate strains (MIC = 4 μg / ml), one strain was found with each of the following, respectively : only DNAgyrase mutations, only topoisomerase IV mutations, and mutations in both. Only one strain had no mutation in any

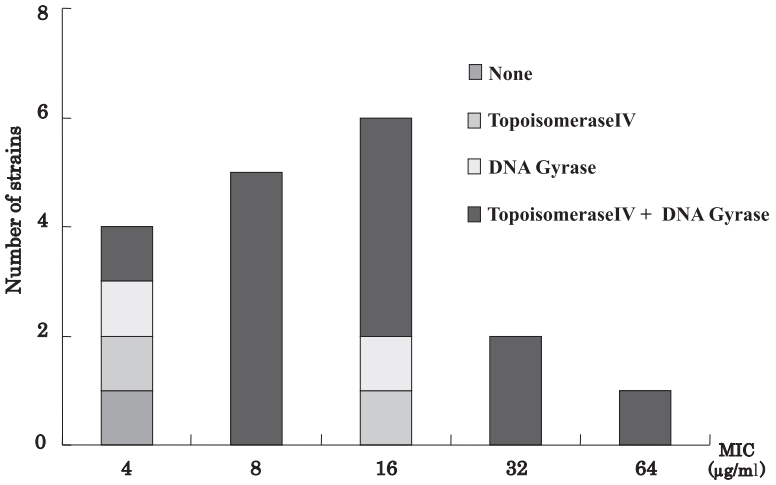

Fig. 1. As LVFX MIC increases, a greater proportion of strains appear to show mutation in both DNA Gyrase and topoisomerase IV.

QRDRs. Among 14 LVFX-resistant strains (MIC ≥ 8 μg / ml), 12 strains (85.7%) had mutations in both DNAgyrase and topoisomerase IV. As the LVFX MIC value tended to increase, the proportion of strains with mutations in both enzymes increased.

Discussion

In this study, we performed an epidemiological analysis of quinolone susceptibility and a molecular analysis of the quinolone-resistant genes in *S. pneumoniae* strains clinically isolated in Japan. An analysis of quinolone susceptibility showed that 2.7% of all isolated strains demonstrated an increased LVFX MIC (>4 μg / ml). This result was higher than previous reports of global surveillance by Canton *et al*, Sahm *et al*, and Morrissey *et al*[4, 15, 16]. Furthermore, no significant difference in isolation frequency was found among years, indicating that LVFX-resistant strains are increasing domestically.

There was almost no change in quinolone susceptibility across the surveillance years, with respiratory quinolones generally showing good activity against *S. pneumoniae* (Table 2). We attributed the differences in antibacterial activity among the drugs to differences in inhibitory activity against the target enzymes. In particular, the 50% inhibitory concentrations (IC_{50}) of GRNX and STFX against DNAgyrase and topoisomerase IV are 4-10 times lower than that of LVFX[17, 18].

Quinolone resistance was previously described to occur mechanistically via step-wise amino acid mutations in type II topoisomerases as follows: 1) 1st-Step mutation of either DNAgyrase or topoisomerase IV; followed by 2) 2nd-Step mutation of both DNAgyrase and topoisomerase IV[7]. Almost all strains with 1st-Step mutations remain susceptible, but strains with 2nd-Step mutations have been reported as highly resistant, since both the quinolone target enzymes, DNAgyrase and topoisomerase IV, are inhibited[7]. In this study, 17 out of 18 LVFX-intermediate and -resistant strains (94.4%) demonstrated amino acid mutations in their QRDRs; 12 out of

14 (85.7%) LVFX-resistant strains demonstrated 2nd-Step mutations and 2 out of 4 LVFX-intermediate strains (2007-26 and 2008-139) showed 1st-Step mutations. It is thought that the cell populations with 1st-Step mutations also contain a minority of cells with 2nd-Step mutations (hetero-resistance), and that quinolone used on these cell populations could select for those cells with 2nd-Step mutations, thereby creating a resistant strain. Furthermore, strains with 1st-Step mutations might become highly resistant if a mutation occurs newly in the other target enzyme. If another mutation is induced by quinolones, then they may become highly resistant [19].

Out of 60 LVFX-susceptible strains tested herein, we detected 3 (5%) 1st-Step strains, all of which showed topoisomerase IV mutations. Similar to the above-mentioned intermediate strains, it is likely that high-level resistance will induce the occurrence of mutations in the other target enzyme, DNAgyrase. In Japan, MIC values are not normally recorded when carrying out antimicrobial susceptibility tests, with only "S", "I", or "R" recorded for each antibiotic. Therefore, even in cases for which the antimicrobial susceptibility test is recorded as "S", the administration of quinolone might lead to an increased number of resistant strains.

In this study, 3 of the 60 LVFX-susceptible strains examined (5%) were 1st-Step strains, which is a significantly higher rate than previously reported, such as only 4 out of 1106 susceptible strains (0.4%) in a USA study [20]. In addition, the isolation frequency of LVFX-intermediate and -resistant strains in this study was 2.7%, which is also higher than that reported in other countries [4, 15, 16]. A similar phenomenon has seen in *Haemophilus influenza* [21], and these findings suggest that the environment in Japan is associated with a higher rate of quinolone resistance in comparison with other countries. The reason for this may be the super-aging society in Japan. In this study, the resistant and intermediate strains were all isolated from adults aged 60 and over (data not shown), and as reported previously, LVFX-resistant *S. pneumoniae* is more commonly isolated from elderly than younger patients [22]. Since elderly people generally have an insufficient immunological response to pathogens in comparison to younger people, they tend to be administered antibiotics for longer periods. For this reason, the selection pressure of antibiotics is more severe than in younger groups, setting up an environment more likely to favor quinolone resistance.

Another factor may be the dose of quinolones. Recently, the pharmacokinetics / pharmacodynamics (PK / PD) theory has played an important role in the administration of antibiotics. According to this theory, an important parameter regarding the prevention of quinolone resistance is the ratio between the maximum drug concentration (C_{MAX}) and MIC (C_{MAX} / MIC) [23]. In Japan, it is becoming more common for LVFX to be administered as a single, 500-mg dose, which is a smaller dosage than that used in the USA (750 mg per dose), and perhaps as a result not sufficient to prevent quinolone resistance.

Ideally, studies such as ours should also consider the history of antibiotics administration when analyzing quinolone-resistant strains. However, we could not investigate all the strains in this study in this regard. We analyzed only the six regions considered relevant to quinolone resistance in domestically isolated *S. pneumoniae* (Ser81 or Glu85 in GyrA, Asp435 in GyrB, Ser79 or Asp83 in ParC, and Asp435 in ParE). However, one strain (2008-236) among the

LVFX-intermediate strains did not demonstrate any of these mutations, while two strains (2007-192, 2008-45) among the highly resistant strains (LVFX MIC＞16 μg/ml) had mutations to either DNAgyrase or Topoisomerase IV. Previously, *S. pneumoniae* with a similar pattern of mutations were reported, and this was attributed to resistance mechanisms other than that involving type II topoisomerase amino acid mutations[4, 24]. QRDR mutations other than the six mutations investigated in this study included Lys137Asn in ParC, which was detected in 6 out of 60 susceptible strains (10%) and in 7 out of 18 intermediate and resistant strains (38.9%). Similarly, Ile460Val in ParE was detected in 50 out of 60 susceptible strains (83.3%) and in 14 out of 18 intermediate and resistant strains (77.8%). These mutations have been reported as not contributing to quinolone resistance, and our results supported this assumption by showing no relationship to the LVFX MIC[25, 26]. In this study, mutations related to quinolone resistance were frequently identified in QRDR regions, but we suggest that both of these other mutations, and the active promotion of quinolone drug efflux, may contribute to resistance.

This study demonstrated that various factors contribute to increasing quinolone resistance in *S. pneumoniae*. QRDR mutations were present in 5% of even susceptible strains, which are thought to be in the early stages of resistance. In the future, an increasing use of quinolones is likely to result in more resistant strains; however, GRNX and STFX still demonstrate good activity against the quinolone-resistant *S. pneumoniae* strains isolated in Japan. Our findings suggest that these strong quinolones should be proactively administered to elderly pneumonia patients and others similar patient with underlying disease.

Conflict of interest

Our laboratory is an endowed chair and funded by Shionogi & Co., Ltd., Meiji Seika Pharma Co., Ltd., and Toyama Chemical Co., Ltd.. We receive a donation for this research from Takeda Pharmaceutical Co., Ltd., Pfizer Japan Inc., Daiichi Sankyo Co., Ltd., Bayer Yakuhin, Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Dainippon Sumitomo Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Kyorin Pharmaceutical Co., Ltd., Astellas Pharma Inc., Chugai Pharmaceutical Co., Ltd., and GlaxoSmithKline K.K.

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