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ORIGINAL ARTICLE

Comparison of drug sensitivity and genotypes of clinically isolated strains of levofloxacin-resistant *Streptococcus pneumoniae* obtained from Okinawa Island, the Japanese main island and Hong Kong

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The prevalence of fluoroquinolone-resistant *Streptococcus pneumoniae* is increasing worldwide. In the present study, a comparison of drug sensitivity and genotypes of clinically isolated strains of levofloxacin (LVFX)-resistant *S. pneumoniae* obtained from Hong Kong, Okinawa Island and the Japanese main island (Honshu) was performed. MICs of quinolones (LVFX, tosufloxacin, ciprofloxacin, gatifloxacin and sitafloxacin (STFX)) and other antibiotics (penicillin G, cefcapene, cefditoren, clarithromycin and azithromycin) were determined by a microdilution broth method according to the Clinical and Laboratory Standards Institute Standards. The quinolone-resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* of these strains were analyzed by PCR-based sequencing. All 40 strains tested had more than one amino-acid substitution in the QRDRs of *gyrA*, *gyrB*, *parC* or *parE*. Although there seemed to be some clonality in strains obtained from Hong Kong, there was no clonality in strains obtained from Okinawa and Japan. Strains obtained from Hong Kong, Okinawa Island and the Japanese main island were genetically different by pulsed-field gel electrophoresis analysis. The range of MIC values of STFX against isolates resistant to LVFX (MIC 4–32 mg l^{-1}) was 0.12–0.5 mg l^{-1}, and MIC_{80} values of STFX against LVFX-resistant isolates were 0.25 mg l^{-1}. This study suggests that LVFX-resistant *S. pneumoniae* is similar in all three locations and STFX is potent against LVFX-resistant *S. pneumoniae* with multiple mutations in QRDRs of gyrase A and topoisomerase IV.
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Keywords: gyrase; levofloxacin-resistant *S. pneumoniae*; PFGE; sitafloxacin; topoisomerase IV

INTRODUCTION

Streptococcus pneumoniae is a major cause of respiratory tract infections, bacteremia and bacterial meningitis. Although penicillin has been the most effective drug for the treatment of infections caused by *S. pneumoniae*, the incidence of multiple drug-resistant *S. pneumoniae* is currently increasing throughout the world.[1] In particular, the rapid spread of pneumococcal clones resistant to β-lactams and macrolides has promoted the use of selected fluoroquinolones for the treatment of pneumococcal infections. Therefore, fluoroquinolones with anti-pneumococcal activity, such as levofloxacin (LVFX), tosufloxacin (TSFX), ciprofloxacin (CPFX), sparfloxacin (SPFX), gatifloxacin (GTFX) and sitafloxacin (STFX), may have an important role in the management of pneumococcal disease.[2]

The reported increase in *S. pneumoniae* resistance to fluoroquinolones is of great concern. So far, two mechanisms responsible for the reduced susceptibility to fluoroquinolones have been identified in clinical isolates: target alteration and/or reduced drug accumulation due to drug efflux.[3] The targets of fluoroquinolones are DNA gyrase and topoisomerase IV, which are encoded by *gyrA*, *gyrB*, *parC* and *parE*, and fluoroquinolone-resistant strains show amino-acid substitutions in the quinolone-resistance determinant regions (QRDRs) of DNA gyrase and topoisomerase IV. Multiple mutations within the QRDRs of both *gyrA* and *parC* result in high-level resistance against LVFX.[4] We hypothesized that the increase of fluoroquinolone-resistant *S. pneumoniae* strains might be related to the selection of resistant clones in restricted areas. Therefore, in the present study, a comparison of the drug sensitivities and genotypes of clinically isolated strains of LVFX-resistant *S. pneumoniae* obtained from Hong Kong, Okinawa Island and the Japanese main island (Honshu) was performed.

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MATERIALS AND METHODS

Bacterial strains

Forty clinical isolates of *S. pneumoniae* with resistance to LVFX (MIC $\geqslant 4\,mg\,l^{-1}$) were used in this study, including 19 from Hong Kong, 9 from Okinawa Island and 12 from the Japanese main island (Honshu). Strains were collected from various specimens submitted to the clinical laboratory. Only one isolate per patient was considered. The isolates were confirmed to be *S. pneumoniae* by colony morphology, optochin susceptibility and bile solubility, and the presence of the autolysin gene *lytA* was confirmed by PCR (Wakunaga Pharmaceuticals, Hiroshima, Japan). Bacteria were grown on 5% sheep blood agar (Kyokuto, Tokyo, Japan) at 37 °C in an atmosphere of 5% CO_2. LVFX-susceptible clinical strains, *S. pneumoniae* ATCC 700669 and ATCC 700671, were used as quality control strains for sequence analysis and MIC determination.

Antimicrobial susceptibility testing

The MICs of quinolones (LVFX, TSFX, CPFX, SPFX, GTFX and STFX) and other antibiotics (penicillin G (PCG), cefcapene (CFPN), cefditoren (CDTR), clarithromycin (CAM) and azithromycin (AZM)) were determined by the twofold broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards).[5] Cation-adjusted Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA) was supplemented with 3% lysed horse blood. Microdilution trays (final volume, 100 µl per well) were inoculated using an automatic MIC-2000 inoculator (Dynatech Laboratories, Alexandria, VA, USA). Final inocula contained $\sim 5\times10^4$ CFU per well. The MIC of each drug was defined as the lowest concentration resulting in the complete inhibition of visible growth after 18 h incubation.

Sequencing and analysis of DNA-related drug sensitivity

Quinolone-resistance gene. Mutations in the QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes of LVFX-resistant strains were investigated by a method involving PCR. The primer sequences used in this study have been previously described.[6] Bacterial genomic DNA was prepared from several colonies of *S. pneumoniae* grown on a blood agar plate by boiling with Chelex-100 (Bio-Rad, Hercules, CA, USA). Subsequently, 5 µl of the extract was added to 50 µl of a PCR solution (1×PCR buffer, 200 µM of each dNTP, 2.5 U of Taq polymerase (Takara Biomedical, Kyoto, Japan) and 0.5 µM of sense and reverse primers). PCR conditions were as follows: 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The PCR products were electrophoresed on an agarose gel to confirm the presence of the product, and were then purified with a PCR purification kit (Qiagen Sciences, Germantown, MD, USA) to prepare the sequencing template. The sequencing reaction was conducted with a Rhodamine Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Biosystems, Foster city, CA, USA). The reaction mixtures were placed in a thermal cycler and denatured at 94 °C for 2 min. Reactions were then subjected to 25 cycles (94 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min). The nucleotide sequences were determined using an ABI PRISM3100 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA).

Penicillin-binding protein gene alterations. We evaluated alterations in penicillin-binding protein (PBP) genes of *S. pneumoniae* by PCR, according to Ubukata *et al.*,[7] with some modifications described by Harimaya *et al.*[8] The system contains primer sets for unaltered PBP genes (*pbp1a*, *pbp2x* and *pbp2b*). Extraction of DNA from a colony on a blood agar plate and PCR was performed according to Ubukata *et al.*[9] The reaction profile used was 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min. PCR products were separated in 3% NuSieve 3:1 agarose (Cambrex, East Rutherford, NJ, USA) and visualized by UV light illumination. The bands of PBP genes were detected on the agarose gel when the PBP genes were not altered, and the bands were not detected when the genes were altered.

Macrolide-resistance gene. Two sets of oligonucleotide primers designed on the basis of our sequence results were as follows: ermB1, 5′-721CGTACCTTGG ATATTCACCG740-3′ and ermB2, 5′-944GTAAACAGTTGACGATATTCTCG 922-3′ for the *ermB* gene; and mefA1, 5′-288CTGTATGGAGCTACCTGT CTGG309-3′ and mefA2, 5′-581CCCAGCTTAGGTATACGTAC562-3′ for the *mefA* gene. The DNA fragments amplified by these primers were 224 bp for the *ermB* and 294 bp for the *mefA* gene. A single colony on the blood agar medium was suspended in a microtube containing 30 µl lysis solution.[7] The tube was set in a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer, Norwalk, CT, USA), and bacterial cells were lysed by incubation at 60 °C for 10 min followed by 94 °C for 5 min. Next, 2 µl of the lysed bacterial solution was placed in a PCR tube containing 25 µl of a reaction mixture. One milliliter of the reaction mixture consisted of 60 ng of a primer for each of *ermB* and *mefA*, 80 µl 25 mM dNTP mixture, 25 U *Taq* DNA polymerase and 100 µl of 10×PCR buffer. The PCR conditions were 94 °C for 20 s, 52 °C for 20 s and 72 °C for 15 s, with 30 cycles in total.

Molecular typing

Isolates were characterized by pulsed-field gel electrophoresis (PFGE) by previously described methods.[10,11] Fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad) as previously described, and were compared using the Fingerprinting software (Bio-Rad). The PFGE profiles were visually examined, and clusters were assigned on the basis of three or fewer band differences,[12] corresponding to a similarity index of ~80%. Therefore, a cluster was defined as three or more isolates sharing $\geqslant$80% similarity on the dendrogram.

RESULTS

A list of LVFX-resistant strains obtained from Hong Kong, Okinawa Island and the Japanese main island and susceptibilities of several antibiotics against these strains are shown in Table 1. The MICs for LVFX, TFLX, CPFX, SPFX, GFLX, STFX, PCG, CFPN, CDTR, CAM and AZM against the control strain, ATCC 700669, were 1, 0.12, 2, 0.25, 0.25, 0.06, 2, 1, 1, <0.06 and $<0.06\,mg\,l^{-1}$, respectively (Table 1). The MICs for LVFX, TFLX, CPFX, SPFX, GFLX, STFX, PCG, CFPN, CDTR, CAM and AZM against the second control strain, ATCC 700671, were 1, 0.12, 1, 0.25, 0.25, <0.03, 2, 1, 0.5, <0.06 and <0.06 mg l, respectively (Table 1).

The ranges of MICs for PCG, CFPN, CDTR, CAM and AZM against isolates resistant to LVFX (MIC 4–32 $mg\,l^{-1}$) are shown in Figure 1. Strains obtained from Okinawa had relatively high susceptibilities to β-lactams and macrolides (Table 1; Figure 1). In addition, the ranges of MICs for LVFX, TFLX, CPFX, SPFX, GFLX and STFX against isolates resistant to LVFX (MIC 4–32 $mg\,l^{-1}$) are shown in Figure 2. STFX demonstrated the lowest MICs against LVFX-resistant strains (Figure 2). The range of MIC for STFX against isolates resistant to LVFX (MIC 4–32 $mg\,l^{-1}$) was 0.125–0.5 $mg\,l^{-1}$ and the MIC_{80} for STFX against LVFX-resistant isolates was 0.25 $mg\,l^{-1}$ (Table 1; Figure 2).

Nucleotide sequencing of the region encoding the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* was carried out to investigate the involvement of gene mutations in LVFX-resistant clinical isolates. The results of sequencing analysis were reproducible. Table 2 summarizes the substitutions of deduced amino-acid sequences within QRDRs of *gyrA*, *gyrB*, *parC* and *parE* of the 42 LVFX-resistant strains. All 40 LVFX-resistant strains had more than one amino-acid substitution in the QRDRs of *gyrA*, *gyrB*, *parC* or *parE*, which included Ser81Tyr/Phe, Glu85Lys, Ser114Gly and Ala115His in *gyrA*, Ser79Phe/Ile/Tyr, Asp83Tyr, Asn91Asp, Ser107Phe, Lys137Asn and Ala142Ser in *parC* and Asp435Asn and Ile460Val in *parE*. Among these, 34 strains had amino-acid substitutions in both QRDRs of *gyrA* and *parC*. Most isolates had Ser81Tyr/Phe in *gyrA*, and Ile460Val substitutions in *parE*. Only one strain (#19) had the amino-acid substitution Arg477His in *gyrB* (Table 2).

PBP gene mutations

Among 42 isolates, 40 had mutations in the PBP genes. Only two strains obtained from Okinawa Island (#27 and #28) had no mutations in PBP genes (Table 3). Thirty-one strains had *pbp1a+2x+2b*, two strains (#22 and #29) had *pbp2x+2b*, three strains

Table 1 Levofloxacin-resistant strains obtained from Hong Kong, Okinawa Island and Japan main island and sensitivities to several drugs

Number of strains	*Name of strains*	*Strains obtained from*	*LVFX*	*TFLX*	*CPFX*	*SPFX*	*GFLX*	*STFX*	*PCG*	*CFPN*	*CDTR*	*CAM*	*AZM*
1	ATCC 700669	Standard strain	1	0.12	2	0.25	0.25	0.06	2	1	1	<0.06	<0.06
2	ATCC 700671	Standard strain	1	0.12	1	0.25	0.25	<0.03	2	1	0.5	<0.06	<0.06
3	06M092601	Hong Kong	16	4	32	8	4	0.25	2	2	1	>64	>16
4	06M092603	Hong Kong	16	4	32	8	4	0.25	2	2	1	>64	>16
5	06M092604	Hong Kong	16	4	32	8	4	0.25	2	2	1	>64	>16
6	06M092605	Hong Kong	16	4	16	8	4	0.25	2	1	1	4	8
7	06M092606	Hong Kong	16	4	16	8	4	0.25	2	2	1	>64	>16
8	06M092607	Hong Kong	8	0.5	4	1	2	0.25	4	2	2	0.5	0.5
9	06M092608	Hong Kong	8	4	16	4	4	0.25	4	2	2	>64	>16
10	06M092609	Hong Kong	16	4	32	8	8	0.25	2	2	1	1	2
11	06M092610	Hong Kong	16	4	32	8	8	0.25	2	1	1	0.5	1
12	06M092611	Hong Kong	16	4	32	8	8	0.25	2	2	1	0.5	1
13	06M092612	Hong Kong	16	2	32	16	8	0.5	2	4	2	0.5	2
14	06M092613	Hong Kong	4	0.25	2	0.5	1	0.25	2	0.5	0.5	>64	>16
15	06M092614	Hong Kong	4	0.25	2	0.5	2	0.25	1	0.5	0.5	>64	>16
16	06M092615	Hong Kong	4	0.25	2	0.5	2	0.25	2	0.5	0.5	>64	>16
17	06M092616	Hong Kong	16	4	32	8	4	0.25	4	2	1	16	>16
18	06M092617	Hong Kong	16	4	32	8	8	0.5	2	2	1	0.5	1
19	06M092618	Hong Kong	16	8	32	8	4	0.25	2	2	1	>64	>16
20	06M092619	Hong Kong	16	4	32	8	4	0.25	2	2	1	>64	>16
21	06M092620	Hong Kong	8	4	16	4	4	0.25	2	2	1	1	2
22	2528	Okinawa	8	1	4	1	2	0.25	0.5	0.5	0.5	>64	>16
23	AO184	Okinawa	16	8	32	16	4	0.25	1	0.25	0.25	1	2
24	E67	Okinawa	16	8	32	16	8	0.25	0.12	<0.06	<0.06	>64	>16
25	2E54	Okinawa	16	8	64	8	4	0.25	<0.03	<0.06	<0.06	<0.06	<0.06
26	98	Okinawa	16	8	64	8	8	0.25	2	2	1	>64	>16
27	V7	Okinawa	16	8	64	16	8	0.25	<0.03	<0.06	<0.06	<0.06	<0.06
28	HC37	Okinawa	16	8	64	8	8	0.25	<0.03	<0.06	<0.06	<0.06	<0.06
29	YE4	Okinawa	32	8	32	32	8	0.5	0.25	<0.06	<0.06	<0.06	<0.06
30	TZ2-7	Okinawa	32	8	32	16	8	0.5	0.25	0.5	0.25	1	2
31	H35	Okayama	16	4	16	8	4	0.25	<0.03	0.5	0.25	<0.06	<0.06
32	H41	Nagasaki	32	4	32	8	8	0.25	<0.03	<0.06	<0.06	>64	>16
33	H91	Kanagawa	8	1	8	2	2	0.125	2	1	1	2	4
34	H26	Tokyo	16	2	16	8	8	0.5	1	0.5	0.5	1	2
35	H364	Tokyo	16	8	32	8	4	0.25	1	0.5	0.5	1	2
36	H1344	Fukuoka	32	4	32	8	8	0.25	0.06	0.25	0.12	>64	>16
37	H1565	Miyagi	16	8	32	8	4	0.25	2	1	0.5	2	4
38	H358	Tokyo	8	0.5	8	1	2	0.25	1	0.5	0.5	0.25	0.5
39	H2360	Hokkaido	16	8	32	8	4	0.25	2	8	4	4	8
40	H1881	Fukuoka	8	2	16	8	2	0.125	0.25	0.5	0.5	>64	>16
41	H1892	Fukuoka	8	2	32	4	2	0.125	1	2	1	>64	>16
42	H2364	Hokkaido	8	0.5	4	2	4	0.125	<0.03	1	0.5	2	2

Abbreviations: LVFX, levofloxacin; TFLX, tosufloxacin; CPFX, ciprofloxacin; SPFX, sparfloxacin; GFLX, gatifloxacin; STFX, sitafloxacin; PCG, penicillin G; CFPN, cefcapene; CDTR, cefditoren; CAM, clarithromycin; AZM, azithromycin.

(#24, #32 and #36) had *pbp2x*, one strain (#25) had *pbp2b*, and three strains (#31, #40 and #42) had *pbp1a+2x* (Table 3). In particular, all 19 strains obtained from Hong Kong had *pbp1a+2x+2b.* In contrast, strains obtained from Okinawa Island and the Japanese main island demonstrated heterogeneous mutation patterns in PBP genes (Table 3).

Expressions of macrolide-resistant genes

Among 42 strains, 18 strains had *mefA* gene expression and 18 strains had *ermB* gene expression. Only one strain (#26) expressed both *mefA* gene and *ermB* gene.

Patterns of PFGE

Two ATCC strains (ATCC 700669 and ATCC 70671) and 40 LVFX-resistant strains were classified in 25 different PFGE patterns (Figure 3; Table 3). Two strains obtained from Okinawa Island showed similar PFGE patterns (as shown G1 and G2 in Table 3). In particular, 15 of 19 strains obtained from Hong Kong had a similar PFGE pattern (as shown C in Table 3). In addition, three strains among the remaining four strains obtained from Hong Kong had a similar PFGE pattern (as shown E in Table 3). In contrast, strains obtained from Okinawa Island and the Japanese main island demonstrated heterogeneous patterns by PFGE (Table 3).

DISCUSSION

The emergence of quinolone-resistant *S. pneumoniae* is an important clinical issue. In the PROTEKT study, it has been demonstrated that the frequency of quinolone-resistant *S. pneumoniae* strains in Asia is

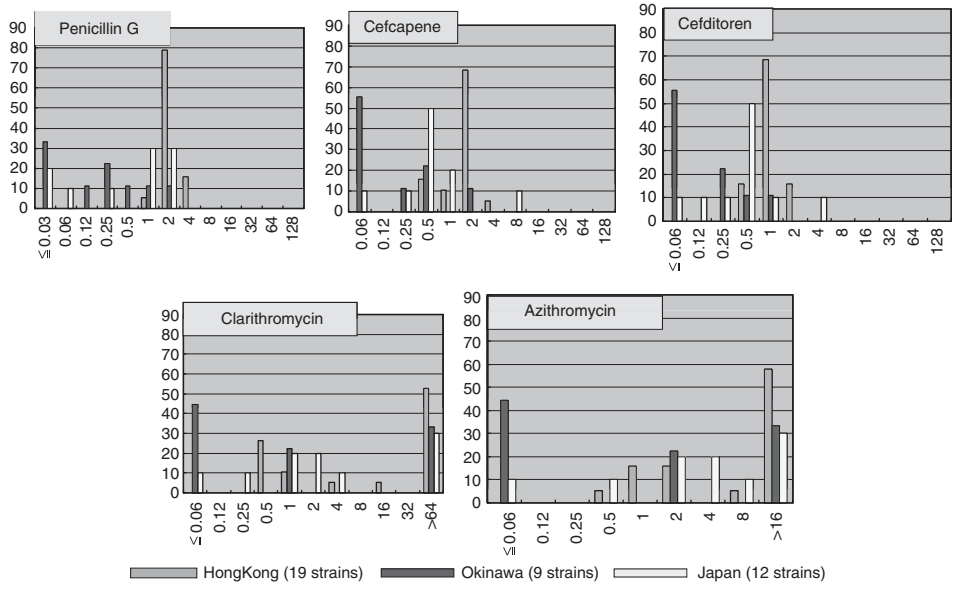

Figure 1 Susceptibilities for several antibiotics of clinically isolated strains of levofloxacin (LVFX)-resistant *S. pneumoniae.* Susceptibilities to penicillin G, cefcapene, cefditoren, clarithromycin and azithromycin were evaluated in 19 strains obtained from Hong Kong, 9 strains obtained from Okinawa and 12 strains obtained from the Japanese main island. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

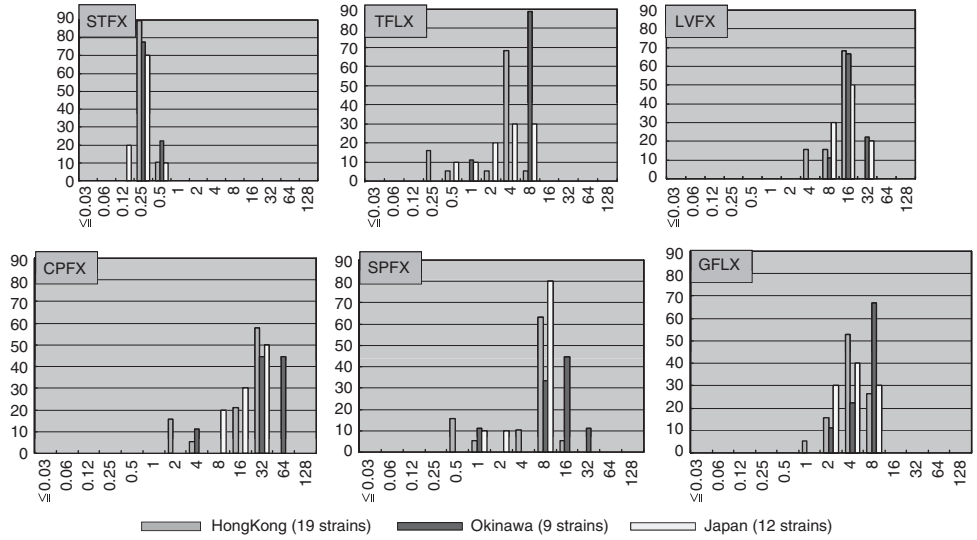

Figure 2 Susceptibilities of clinically isolated strains of levofloxacin (LVFX)-resistant *S. pneumoniae* to several fluoroquinolones. Susceptibilities to sitafloxacin (STFX), tosufloxacin (TFLX), levofloxacin (LVFX), ciprofloxacin (CPFX), sparfloxacin (SPFX) and gatifloxacin (GFLX) were evaluated in 19 strains obtained from Hong Kong, 9 strains obtained from Okinawa and 12 strains obtained from the Japanese main island. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

~3.5%, and the prevalence rate of quinolone-resistant *S. pneumoniae* has been indicated as high in Hong Kong.[13]

Given this information, the purpose of the present study was to compare LVFX-resistant strains obtained from Hong Kong, Okinawa Island and the Japanese main island (Honshu) by analyzing the heterogeneity of strains, drug sensitivities, amino-acid substitutions at QRDRs, PBP alterations, expressions of *mefA* and *ermB* genes and PFGE patterns. Based on the data of drug sensitivity tests, it was suggested that strains obtained from Okinawa Island have relatively high susceptibilities to β-lactams and macrolides. Interestingly, based on the data of PBP gene mutations, all 19 strains obtained from Hong Kong had *pbp1a+2x+2b.* In contrast, strains obtained from Okinawa Island and the Japanese main island demonstrated heterogeneous mutation patterns in the PBP genes. Based on the data of PFGE patterns, 15 of 19 strains obtained from Hong Kong had similar migration patterns. In addition, three strains among the remaining four strains obtained from Hong Kong had similar PFGE patterns. In contrast, strains obtained from Okinawa Island and the Japanese main island demonstrated heterogeneous patterns by PFGE. These data suggested that the same or closely related strains of bacteria had been transmitted in Hong Kong, since strains obtained from Hong Kong were derived from the relatively small restricted area. In addition, it is well known that nosocomial, familial or social droplet transmission was possible in *S. pneumoniae* infection. Therefore, the rate of LVFX

Table 2 Levofloxacin-resistant strains obtained from Hong Kong, Okinawa Island and Japan main island and patterns of mutation of genes related to the quinolone resistance

			GyrA								*GyrB*		*ParC*												*ParE*			
Number	*Name of*	*Strains obtained*	*81*		*85*		*114*		*115*		*477*		*79*		*83*		*91*		*107*		*137*		*142*		*435*		*460*	
of strains	*strains*	*from*	*Ser*	*TCC*	*Glu*	*GAA*	*Ser*	*AGT*	*Ala*	*GCT*	*Arg*	*CGT*	*Ser*	*TCT*	*Asp*	*GAT*	*Asn*	*AAC*	*Ser*	*TCT*	*Lys*	*AAG*	*Ala*	*GCA*	*Asp*	*GAC*	*Ile*	*ATC*
1	ATCC 700669	Standard strain	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
2	ATCC 700671	Standard strain	—	—	—	—	—	—	—	—	—	—	—	—	Gly	GGT	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
3	06M092601	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
4	06M092603	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
5	06M092604	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
6	06M092605	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
7	06M092606	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
8	06M092607	Hong Kong	Tyr	TAC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAT	—	—	Asn	AAC	Val	GTC
9	06M092608	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
10	06M092609	Hong Kong	Tyr	TAC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	Asn	AAC	Val	GTC
11	06M092610	Hong Kong	Tyr	TAC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	Asn	AAC	Val	GTC
12	06M092611	Hong Kong	Tyr	TAC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	Asn	AAC	Val	GTC
13	06M092612	Hong Kong	—	—	Lys	AAA	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
14	06M092613	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAC	—	—
15	06M092614	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAC	—	—
16	06M092615	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAC	—	—
17	06M092616	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
18	06M092617	Hong Kong	Tyr	TAC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	Asn	AAC	Val	GTC
19	06M092618	Hong Kong	Phe	TTC	—	—	—	—	—	—	His	CAT	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
20	06M092619	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
21	06M092620	Hong Kong	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
22	2528	Okinawa	Phe	TTC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	Asn	AAC	Val	GTC
23	AO184	Okinawa	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	—	—	Val	GTC
24	E67	Okinawa	Phe	TTC	—	—	—	—	—	—	—	—	—	—	Tyr	TAT	—	—	—	—	—	—	—	—	Asn	AAC	Val	GTC
25	2E54	Okinawa	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	Asn	AAC	Val	GTC
26	98	Okinawa	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	Asn	AAC	Val	GTC
27	V7	Okinawa	Tyr	TAC	—	—	—	—	—	—	—	—	Ile	ATT	—	—	Asp	GAC	—	—	—	—	Ser	TCT	—	—	Val	GTC
28	HC37	Okinawa	Tyr	TAC	—	—	—	—	—	—	—	—	Ile	ATT	—	—	Asp	GAC	—	—	—	—	Ser	TCT	—	—	Val	GTC
29	YE4	Okinawa	—	—	Lys	AAA	—	—	—	—	—	—	Tyr	TAT	—	—	—	—	—	—	Asn	AAT	—	—	—	—	Val	GTC
30	TZ2-7	Okinawa	—	—	Lys	AAA	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	—	—	Val	GTC
31	H35	Okayama	Phe	TTC	—	—	—	—	—	—	—	—	Tyr	TAT	—	—	—	—	—	—	—	—	—	—	—	—	—	—
32	H41	Nagasaki	Tyr	TAC	—	—	—	—	—	—	—	—	Tyr	TAT	—	—	—	—	—	—	—	—	—	—	—	—	Val	GTC
33	H91	Kanagawa	Phe	TTC	—	—	—	—	Val	GTT	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAC	Val	GTC
34	H26	Tokyo	Tyr	TAC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAC	Val	GTC
35	H364	Tokyo	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	—	—	Val	GTC
36	H1344	Fukuoka	Tyr	TAC	—	—	—	—	—	—	—	—	Tyr	TAT	—	—	—	—	—	—	—	—	—	—	—	—	Val	GTC
37	H1565	Miyagi	Phe	TTC	—	—	—	—	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	—	—	Val	GTC
38	H358	Tokyo	Phe	TTC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asn	AAC	Val	GTC
39	H2360	Hokkaido	Phe	TTC	—	—	Gly	GGT	—	—	—	—	Phe	TTT	—	—	—	—	—	—	—	—	—	—	—	—	—	—
40	H1881	Fukuoka	Phe	TTC	—	—	—	—	—	—	—	—	—	—	Tyr	TAT	—	—	—	—	—	—	—	—	—	—	—	—
41	H1892	Fukuoka	Tyr	TAC	—	—	Gly	GGT	—	—	—	—	Tyr	TAT	—	—	Asp	GAC	—	—	—	—	—	—	—	—	—	—
42	H2364	Hokkaido	Phe	TTC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

resistance was high and PBP as well as PFGE pattern were similar in Hong Kong. In contrast, strains obtained from Okinawa Island as well as Japan were derived from relatively wide geographic areas.

Recently, Hong Kong investigators demonstrated high rates of resistance of *S. pneumoniae* isolates to various fluoroquinolones and the presence of the Spanish 23F clone that had acquired fluoroquinolone resistance while circulating in Hong Kong.[14–16] Our data confirmed a prevalence of similar strains of LVFX-resistant *S. pneumoniae* in Hong Kong. Although the overall rate of strains resistant to fluoroquinolones remains low in most Asian countries, the emergence of highly resistant strains to fluoroquinolones will likely be a concern in the future with regard to the treatment of pneumococcal pneumonia. In fact, clinical failures of LVFX for the treatment of pneumococcal pneumonia have already been documented.[17] To overcome this problem, STFX, a recently developed quinolone, is potent against Gram-positive cocci as well as Gram-negative rods, and excellent activities of these drugs against *S. pneumoniae* have been reported.[18] In the present study, STFX had significantly lower MICs against *S. pneumoniae* with resistance to LVFX, compared with other quinolones tested, and these findings support previous reports that show the potency of this drug against LVFX-resistant *S. pneumoniae* with defined multiple mutations within both the gyrase A and topoisomerase IV genes.[19,20] Interestingly, three (#13, #29 and #30) of five strains (#13, #18, #29, #30 and #35) that showed a higher MIC ($0.5\,mg\,l^{-1}$) against LVFX-resistant strains possessed Glu85Lys substitutions in *gyrA*. This suggests that the primary target of STFX may be *gyrA*, and Glu85Lys substitution in *gyrA* reflected the higher MIC for STFX.

The reason why STFX was potent compared with other quinolones based on mutations in *gyrA* should be discussed. In general, quinolones are known to interact with two related but distinct targets: DNA

Table 3 Levofloxacin-resistant strains obtained from Hong Kong, Okinawa Island and Japan main island, patterns of mutation of genes related to β-lactams and macrolides, and genotyping by pulsed-field gel electrophoresis

Number of strains	*Name of strains*	*Strains obtained from*	*Mutations of penicillin-binding protein* *Patterns of mutation*	*Expressions of macrolide-resistance genes* *mefA*	*ermB*	*Patterns of PFGE*
1	ATCC 700669	Standard strain	pbp1a+2x+2b	–	–	A
2	ATCC 700671	Standard strain	pbp1a+2x+2b	–	–	B
3	06M092601	Hong Kong	pbp1a+2x+2b	–	+	C
4	06M092603	Hong Kong	pbp1a+2x+2b	–	+	C
5	06M092604	Hong Kong	pbp1a+2x+2b	–	+	C
6	06M092605	Hong Kong	pbp1a+2x+2b	+	–	D
7	06M092606	Hong Kong	pbp1a+2x+2b	–	+	C
8	06M092607	Hong Kong	pbp1a+2x+2b	+	–	C
9	06M092608	Hong Kong	pbp1a+2x+2b	–	+	C
10	06M092609	Hong Kong	pbp1a+2x+2b	+	–	C
11	06M092610	Hong Kong	pbp1a+2x+2b	+	–	C
12	06M092611	Hong Kong	pbp1a+2x+2b	+	–	C
13	06M092612	Hong Kong	pbp1a+2x+2b	+	–	C
14	06M092613	Hong Kong	pbp1a+2x+2b	–	+	E
15	06M092614	Hong Kong	pbp1a+2x+2b	–	+	E
16	06M092615	Hong Kong	pbp1a+2x+2b	–	+	E
17	06M092616	Hong Kong	pbp1a+2x+2b	–	+	C
18	06M092617	Hong Kong	pbp1a+2x+2b	+	–	C
19	06M092618	Hong Kong	pbp1a+2x+2b	–	+	C
20	06M092619	Hong Kong	pbp1a+2x+2b	–	+	C
21	06M092620	Hong Kong	pbp1a+2x+2b	+	–	C
22	2528	Okinawa	pbp2x+2b	–	+	F
23	AO184	Okinawa	pbp1a+2x+2b	+	–	G1
24	E67	Okinawa	pbp2x	–	+	H
25	2E54	Okinawa	pbp2b	–	–	I
26	98	Okinawa	pbp1a+2x+2b	+	+	G2
27	V7	Okinawa	no mutation	-	–	J
28	HC37	Okinawa	no mutation	-	–	J
29	YE4	Okinawa	pbp2x+2b	-	–	K
30	TZ2-7	Okinawa	pbp1a+2x+2b	+	–	M
31	H35	Okayama	pbp1a+2x	–	–	N
32	H41	Nagasaki	pbp2x	–	+	O
33	H91	Kanagawa	pbp1a+2x+2b	+	–	P
34	H26	Tokyo	pbp1a+2x+2b	+	–	Q
35	H364	Tokyo	pbp1a+2x+2b	+	–	R
36	H1344	Fukuoka	pbp2x	–	+	S
37	H1565	Miyagi	pbp1a+2x+2b	+	–	T
38	H358	Tokyo	pbp1a+2x+2b	+	–	U
39	H2360	Hokkaido	pbp1a+2x+2b	+	–	V
40	H1881	Fukuoka	pbp1a+2x	–	+	W
41	H1892	Fukuoka	pbp1a+2x+2b	–	+	X
42	H2364	Hokkaido	pbp1a+2x	+	–	Y

Abbreviation: PFGE, pulsed-field gel electrophoresis.

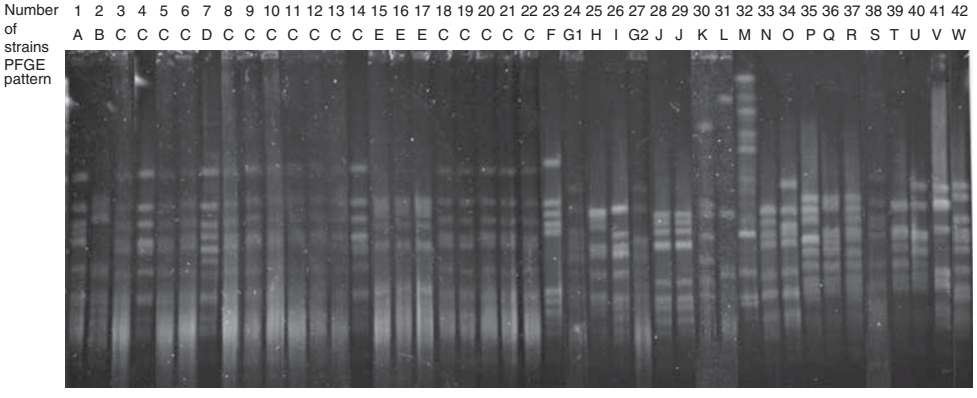

Figure 3 Genomic analysis of two ATCC strains and 40 clinically isolated strains of levofloxacin (LVFX)-resistant *S. pneumoniae* by pulsed-field gel electrophoresis (PFGE).

gyrase and topoisomerase IV. The recognition of dual drug target enzymes also has important implications for the development of resistance. For resistance mutations in the primary target enzyme, the increment in resistance may be limited by the level of sensitivity of the unmutated secondary target enzyme, which becomes the more sensitive enzyme when the primary target is resistant.[21] This scheme implies that concurrent dual mutations in both target enzymes would be required for any quinolone that had equal potency against DNA gyrase and topoisomerase IV.[21] In the present study, as STFX has strong activity against LVFX-resistant *S. pneumoniae*, it was suggested that STFX might have equal strong inhibitory activity against DNA gyrase as well as topoisomerase IV.

There may be some limitations in interpreting the data from this study. Since pneumococcal isolates were collected from one or two referral centers, mainly in urban areas, and the number of isolates was relatively small, data from this study may not reflect the overall resistance status in these populations.

In summary, LVFX-resistant *S. pneumoniae* isolated in Hong Kong showed similarities to strains from Okinawa Island and the Japanese main island. In addition, STFX was potent against LVFX-resistant *S. pneumoniae* with multiple mutations in QRDRs of gyrase A and topoisomerase IV. Further clinical studies are warranted to evaluate the usefulness of STFX against infections caused by LVFX-resistant *S. pneumoniae*.

CONFLICT OF INTEREST

JF has served on speaker's bureaus for GlaxoSmithKline, Abbott Japan, Boehringer Ingelheim, Pfizer, Astellas, Daiichi Sankyo and Taisho Toyama.

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