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Rapid Screening of Fluoroquinolone Resistance Determinants in *Streptococcus pneumoniae* by PCR-Restriction Fragment Length Polymorphism and Single-Strand Conformational Polymorphism

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A rapid method, using PCR-restriction fragment length and single-strand conformation polymorphism (SSCP), was applied to screen for mutations of the fluoroquinolone resistance determinants in *Streptococcus pneumoniae*. One hundred nonduplicate *Streptococcus pneumoniae* isolates with ciprofloxacin MICs of ≥4.0 μg/ml from the Prince of Wales Hospital, Hong Kong, years 2000 to 2003, were examined. For each isolate, PCR amplicons of quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* genes were digested with AluI, HinfI, Sau3AI, and MspI, respectively, and analyzed by SSCP. Each SSCP pattern was given a number, and each isolate obtained a four-digit code, e.g., 1111, that represented the SSCP profile. The SSCP patterns were correlated to mutations characterized from sequence analyses of PCR amplicons. The most common SSCP profile obtained was no. 5232 (40%), which included strains with two amino acid substitutions in the ParC (Lys-137-Asn) and ParE (Ile-460-Val) genes, followed by the SSCP profile 5223 (17%), which included strains with amino acid substitutions in the ParE (Ile-460-Val) gene only. Ten isolates (10%) with amino acid substitutions at GyrA and ParE (±ParC) genes were resistant to levofloxacin with a MIC of ≥16 μg/ml. Other SSCP profiles were unique in distinguishing the common amino acid substitutions in GyrA (Ser-81-Phe) and ParC (Lys-137-Asn, Ser-79-Phe plus Lys-137-Asn, Asp-83-Asn plus Lys-137-Asn, Ser-79-Phe, and Glu-96-Asp). SSCP analysis of restricted fragments generated patterns that were highly discriminative for mutations present in the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE*. This method provides a database of high resolution profiles on these mutations and allows rapid screening for new mutations of the fluoroquinolone resistance genes.

Antibiotic-resistant *Streptococcus pneumoniae* has evolved to be a worldwide problem in the last decade. Fluoroquinolones are an important class of antibiotics, and agents such as levofloxacin or moxifloxacin are incorporated in guidelines for the empirical treatment of community-acquired pneumonia (17, 18). The prevalence of levofloxacin nonsusceptibility (MIC of ≥4.0 μg/ml) varies by region and has remained low, ranging from 0% in some European countries (24) to 1.3% in the United States (25). Higher rates have been documented in some countries, e.g., South Korea (2.9%) (4), and in Hong Kong, a rate of 13% has been reported in 2001 (9) and the spread hypothesized to be a result of clonal dissemination of the Spanish 23F strain of *S. pneumoniae* (10). However, there is increasing evidence that interspecies transfer of the *parE-parC* gene region arising from other viridans group streptococci occurs (7, 28). It is thus important to identify and monitor the spread of fluoroquinolone resistance determinants among *S. pneumoniae* strains.

Fluoroquinolone resistance in *S. pneumoniae* is primarily due to mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding the A and B subunits of DNA gyrase and topoisomerase IV, in particular, the *parC* and *gyrA* genes (3, 6, 14). Often, the level of resistance increases as more amino acids are substituted for by additional mutations. Mutations in *parE* and *gyrB* have also been reported, but to a lesser extent (2, 23, 30). Reduced susceptibility to fluoroquinolone may also be due to altered accumulation of the drug or efflux, but this plays a less significant role (31).

Screening for mutations in the QRDRs is performed by sequencing of the *gyrA*, *gyrB*, *parC*, and *parE* genes of the fluoroquinolone-resistant strains. Methods for detection of known mutations in the QRDRs have included PCR-restriction fragment length polymorphism (PCR-RF) (1), oligonucleotide probe assay (6), TaqMan assay (8), and single-strand conformational polymorphism (SSCP) (27). Often, these methods are not designed to have, or do not have, a sufficiently high sensitivity or resolution to identify new mutations. Tawata et al. (29) introduced a modified method of using a combination of PCR-restriction fragment length polymorphism and

TABLE 1. PCR primers for QRDRs of the fluoroquinolone resistance genes

Gene	Primer (5′ to 3′)[a]	Amplicon size (bp)	Reference
gyrA	CCG TCG CAT TCT TTA CG AGT TGC TCC ATT AAC CA	382	22
gyrB	TTC TCC GAT TTC CTC ATG AGA AGG GTA CGA ATG TGG	457	19
parC	TGG GTT GAA GCC GGT TCA TGC TGG CAA GAC CGT TGG	366	20
parE	AAG GCG CGT GAT GAG AGC TCT GCT CCA ACA CCC GCA	289	22

[a] For each gene, the first primer listed is forward, and the second is reverse.

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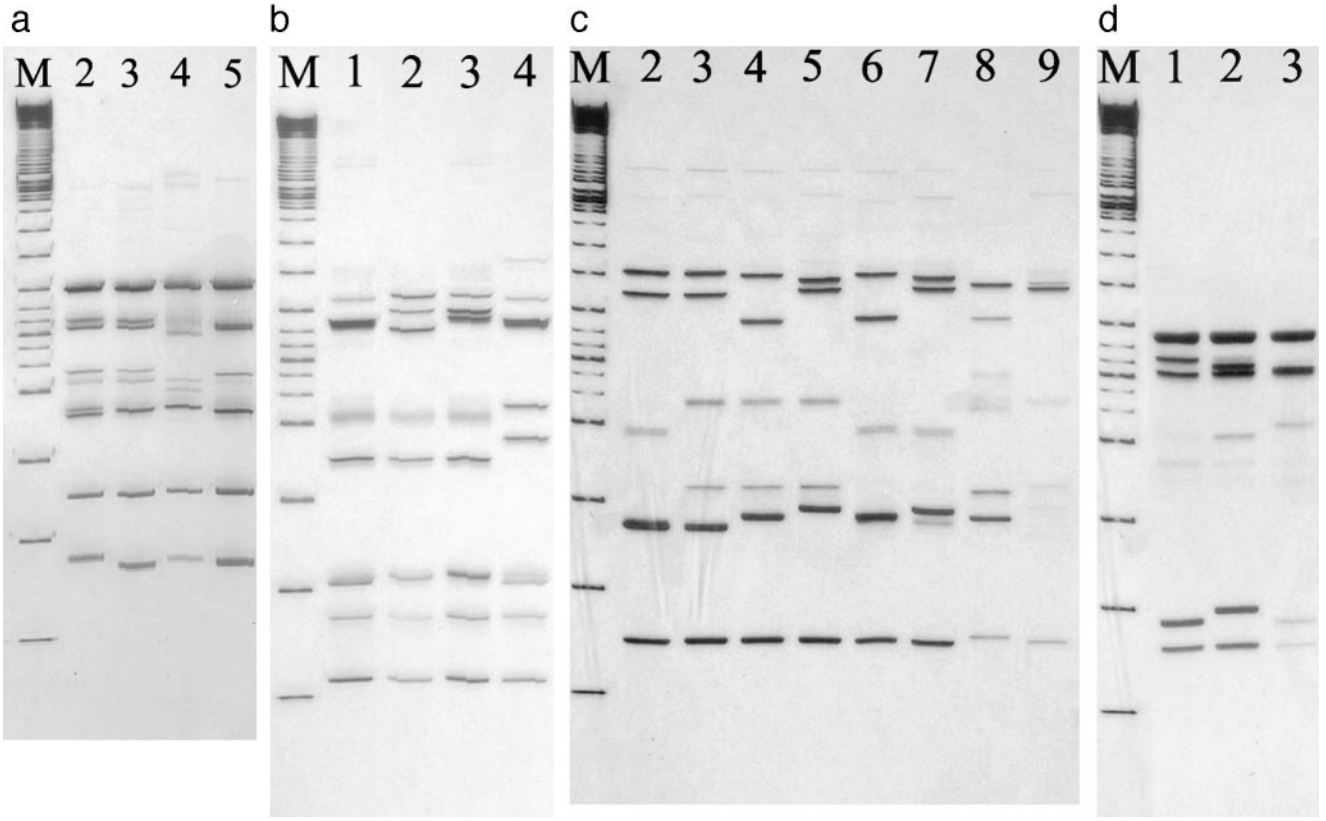

FIG. 1. SSCP patterns of DNA amplicons of *gyrA* (a), *gyrB* (b), *parC* (c), and *parE* (d) after restriction digestion with AluI, HinfI, Sau3AI, and MspI, respectively. (a) SSCP patterns 2 to 5 of the *gyrA* gene after digestion with AluI restriction enzyme. Lane M represents a 100-bp DNA ladder. (b) SSCP patterns 1 to 4 of the *gyrB* gene after restriction digestion using HinfI. Lane M represents a 100-bp DNA ladder. (c) SSCP patterns 2 to 9 of the *parC* gene after restriction digestion using Sau3AI. Lane M represents a 100-bp DNA ladder. (d) SSCP patterns 1 to 3 of the *parE* gene after restriction digestion using MspI. Lane M represents a 100-bp DNA ladder.

single-strand conformational polymorphism (PCR-RF–SSCP) as a tool for mass screening of the genome. We thus sought to identify and monitor the spread of fluoroquinolone resistance determinants in *S. pneumoniae* using a modified method of SSCP to examine and screen for new mutations at the QRDRs of the respective fluoroquinolone resistance genes in *S. pneumoniae.*

MATERIALS AND METHODS

Bacterial isolates and susceptibility tests. One thousand eighty-one nonduplicate *S. pneumoniae* isolates from patients admitted to the Prince of Wales Hospital, a 1,350-bed teaching and tertiary hospital in Hong Kong, from 2000 to 2003 were screened for fluoroquinolone nonsusceptibility. The MICs of ciprofloxacin, levofloxacin, moxifloxacin, and gatifloxacin were determined by the microdilution broth method as described by NCCLS (21). One hundred isolates that were nonsusceptible to ciprofloxacin (CIP) with MICs of ≥4 μg/ml were

TABLE 2. Correlation of SSCP patterns with nucleotide sequences and amino acid substitutions in *gyrA* gene, compared to that of *S. pneumoniae* R6

SSCP pattern	Description of pattern[b]	No. isolates (%)	MIC range (μg/ml)[a]		75[d]		81[d]		83[d]	
			CIP	LEV	nt[e]	aa[f]	nt	aa	nt	aa
1[c]	R6	0			TAC	Tyr	TCC	Ser	ATT	Ile
2	1 sub + 1 var	9 (9)	16–64	>16	--T		-T-	Phe		
3	1 sub	1 (1)	8	16			-T-	Phe		
4	1 var	1 (1)	4	2					--C	
5	1 var	89 (89)	4–>16	0.5–16	--T					

[a] CIP, ciprofloxacin; LEV, levofloxacin.
[b] sub, no. of amino acid substitution; var, no. of base pair variation compared to R6.
[c] *S. pneumoniae* R6 strain.
[d] Numeral indicates amino acid position. nt, nucleotide; aa, amino acid.
[e] Nucleotide sequence changes relative to *S. pneumoniae* R6.
[f] Amino acid substitution corresponding to nt change.

TABLE 3. Correlation of SSCP patterns with nucleotide sequences and amino acid substitutions in *gyrB* compared to that of *S. pneumoniae* R6

SSCP pattern	Description of pattern[b]	No. isolates (%)	MIC range (μg/ml)[a]		381[d]		384[d]		386[d]		461[d]		472[d]	
			CIP	LEV	nt[e]	aa[f]	nt	aa	nt	aa	nt	aa	nt	aa
1[c]	R6	14 (14)	4	1–2	GTA	Vla	GGA	Gly	TTG	Leu	AAC	Asn	GCT	Ala
2	3 var	80 (80)	4–64	0.5–>16	--G		--G		--A					
3	2 var	5 (5)	4–16	1–4			--G		--A					
4	2 var	1 (1)	4	2							--T		--C	

[a] CIP, ciprofloxacin; LEV, levofloxacin.
[b] sub, no. of amino acid substitution; var, no. of base pair variation compared to R6.
[c] *S. pneumoniae* R6 strain.
[d] Numeral indicates amino acid position. nt, nucleotide; aa, amino acid.
[e] Nucleotide sequence changes relative to *S. pneumoniae* R6.
[f] Amino acid substitution corresponding to nt change.

further examined on the mutations and amino acid substitutions at the QRDRs of the respective fluoroquinolone resistance genes using PCR-RF–SSCP.

Analysis by PCR-RF–SSCP. Chromosomal DNA from the isolates was obtained by melting a small piece of DNA plug in 150 μl double-distilled H_2O at 65°C. DNA plug was prepared for pulsed-field gel electrophoresis according to the method previously described (11, 12). For each isolate, the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified by PCR using the primers listed in Table 1 and conditions as previously described (19, 20, 22). For the restriction digestion, the PCR amplicons of *gyrA*, *gyrB*, *parC*, and *parE* genes were digested with AluI, HinfI, Sau3AI, and MspI enzymes (Amersham Biosciences), respectively, each in a 10-μl reaction mixture containing reaction buffer and incubated at 37°C overnight as recommended by the manufacturer. For example, AluI was the restriction enzyme used for the digestion of the PCR amplicons of the *gyrA* gene, HinfI for *gyrB*, and so forth. A quantity of 3.5 μl (approximately 100 to 150 ng) of the digested product was mixed with an equal volume of denaturing solution (94% formamide, 0.05% xylene cyanol solution, 0.4 mg/ml bromophenol blue) according to the manufacturer's instructions (ExcelGel DNA Analysis Kit; Amersham Biosciences), the mixture was denatured at 95°C for 8 min in a thermocycler, and the mixture was placed on ice immediately. The denatured mixture was electrophoresed on precast gels for SSCP (ExcelGel DNA Analysis Kit;, Amersham Biosciences) at 600 V, with a current of 50 mA and 30 W power for 90 min at 4°C using the Multiphor II electrophoresis unit (Amersham Biosciences). The precast gels were 12.5% acrylamide gels and contained 48 wells for sample loading. The DNA gel was stained by silver stain according to the manufacturer's instructions (DNA silver staining kit; Amersham Biosciences) to visualize and permanently stain the discrete DNA bands.

Analysis of SSCP patterns. Each SSCP pattern was given a number and each isolate obtained a four-digit code that represented the SSCP profile. For example, 1234 represented the SSCP profile of pattern 1 for *gyrA*, pattern 2 for *gyrB*, pattern 3 for *parC*, and pattern 4 for the *parE* gene. The SSCP patterns were correlated to the mutations characterized from the sequence analyses of the respective PCR amplicons, and any amino acid substitutions were noted. At least three pairs of forward and reverse sequences of the PCR amplicons, if available, for each corresponding SSCP pattern were sequenced for confirmation. Sequencing was performed with an ABI 310 sequencer and an ABI Prism dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). The sequences were aligned and compared with the corresponding QRDR regions of the four fluoroquinolone resistance genes of the *S. pneumoniae* R6 strain from the GenBank.

RESULTS

The different SSCP patterns obtained from PCR-RF–SSCP of the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes are shown in Fig. 1a to d, respectively. Four SSCP patterns were observed for *gyrA*, four patterns for *gyrB*, eight for *parC*, and three for *parE*. The corresponding nucleotide change and amino acid substitution for each of the SSCP patterns are shown in Tables 2 to 5. The sequencing results confirmed that the same SSCP pattern represented the same sequence of the QRDR of the PCR amplicons of the specific fluoroquinolone-resistant genes.

Four SSCP patterns were obtained for *gyrA* (Fig. 1a). The majority of isolates (90%) belonged to pattern 5 and were found to have one base pair difference from *S. pneumoniae* R6 (Table 2). Only one amino acid substitution (Ser-81-Phe) was

TABLE 4. Correlation of SSCP patterns with nucleotide sequences and amino acid substitutions in *parC* compared to that of *S. pneumoniae* R6

SSCP pattern	Description of pattern[b]	No. isolates (%)	MIC range (μg/ml)[a]		41[d]		79[d]		83[d]		96[d]		128[d]		137[d]	
			CIP	LEV	nt[e]	aa[f]	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
1[c]	R6	0			CAA	Gln	TCT	Ser	GAT	Asp	GAG	Glu	GGC	Gly	AAG	Lys
2	1 var	40 (40)	4–16	1–16	--G											
3	1 sub + 1 var	49 (49)	4–>16	0.5–>16	--G								--T		--T	Asn
4	2 sub + 2 var	2 (2)	8–>16	2–16	--G				A--	Asn			--T		--T	Asn
5	2 sub + 2 var	2 (2)	>16	>16	--G		-T-	Phe					--T		--T	Asn
6	1 sub + 1 var	1 (1)	4	2	--G				T--	Tyr						
7	1 sub + 1 var	4 (4)	8–64	2–16	--G		-T-	Phe								
8	1 sub + 2 var	1 (1)	4	1	--G								--T		--T	Asn
9	3 sub + 2 var	1 (1)	32	16	--G		-T-	Phe			--T	Asp	--T		--T	Asn

[a] CIP, ciprofloxacin; LEV, levofloxacin.
[b] sub, no. of amino acid substitution; var, no. of base pair variation compared to R6.
[c] *S. pneumoniae* R6 strain.
[d] Numeral indicates amino acid position. nt, nucleotide; aa, amino acid.
[e] Nucleotide sequence change relative to *S. pneumoniae* R6.
[f] Amino acid substitution corresponding to nt change.

TABLE 5. Correlation of SSCP patterns with nucleotide sequences and amino acid substitutions in *parE* compared to that of *S. pneumoniae* R6

SSPC pattern	Description of pattern[b]	No. isolates (%)	MIC range (μg/ml)[a]		460[d]		476[d]	
			CIP	LEV	nt[e]	aa[f]	nt	aa
1[c]	R6	4 (4)	4–16	1–2	ATC	Ile	ATC	Ile
2	1 sub + 1 var	54 (54)	4–32	0.5–>16	G--	Val	--T	
3	1 sub	42 (42)	4–64	1–16	G--	Val		

[a] CIP, ciprofloxacin; LEV, levofloxacin.
[b] sub, no. of amino acid substitution; var, no. of base pair variation compared to R6.
[c] *S. pneumoniae* R6 strain.
[d] Numeral indicates amino acid position. nt, nucleotide; aa, amino acid.
[e] Nucleotide sequence change relative to *S. pneumoniae* R6.
[f] Amino acid substitution corresponding to nt change.

found, represented in both SSCP patterns 2 and 3, and was present in 10 (10%) of the isolates. The CIP and levofloxacin (LEV) MICs of these isolates were ≥8 μg/ml and ≥16 μg/ml, respectively. Four SSCP patterns were obtained for *gyrB* (Fig. 1b), and these included a number of silent mutations, but there was no amino acid substitution. Fourteen (14%) of the sequences were identical to that of the wild-type strain, R6, while 86% had two or three nucleotide changes (patterns 2 to 4) (Table 3). Nine SSCP patterns were obtained for *parC* (Fig. 1c). SSCP pattern 2 included 40 (40%) isolates that differed from the R6 strain by a base pair, while the other 60 (60%) isolates had one, two, or three amino acid substitutions, as shown in SSCP patterns 3 to 9. These amino acid substitutions included Ser-79-Phe, Asp-83-Asn/Tyr, Glu-96-Asp, and Lys-137-Asn. The most common substitution is represented by SSCP pattern 3, with 49 (49%) of the isolates with an amino acid substitution of Lys-137-Asn (Table 4). Ninety-six (96%) isolates had an amino acid substitution of Ile-460-Val in *parE*, as represented by SSCP patterns 3 and 4 (Table 5). These isolates have various MICs ranging 4 to 64 μg/ml and 0.5 to ≥16 μg/ml for CIP and LEV, respectively.

The correlation of the different SSCP profiles with the amino acid substitutions at GyrA, ParC, and ParE and the fluoroquinolone MICs are summarized in Table 6. The most common SSCP profile was 5232 (40%), which included strains with two amino acid substitutions in the ParC (Lys-137-Asn) and ParE (Ile-460-Val) genes, followed by the SSCP profile of 5223 (17%), which included strains with amino acid substitutions in the ParE (Ile-460-Val) gene only. Ten isolates (10%) with amino acid substitutions at the GyrA and ParE (±ParC)

TABLE 6. Correlation of different SSCP profiles with amino acid substitutions of GyrA, ParC, and ParE and fluoroquinolone MICs

Gene(s) with amino acid substitution	SSCP profile	Position of amino acid substitution						No. of isolates (*n* = 100)	Fluoroquinolone MIC (μg/ml)								
		GyrA	ParC				ParE		CIP				LEV				
		Ser-81-	Ser-79-	Asp-83-	Glu-96-	Lys-137-	Ile-460-		4	8	16	>16	0.5 to 1	2	4	8	16	>16
No substitution	5121							1	1					1				
	5221							2	2				1	1				
parE	4223						Val	1	1					1				
	5123							11	11				4	7				
	5222							3	3				1	2				
	5223							17	16	1			8	8		1		
	5323							4	3		1		1	2	1			
parC, *parE*	5372		Phe				Val	1		1				1				
	5273							1		1				1				
	5263			Tyr			Val	1	1					1				
	5232					Asn	Val	40	35	4		1	20	18			2	
	5233							2	2					2				
	5132							2	2					2				
	5433							1	1					1				
	5283							1	1				1					
	5242			Asn		Asn	Val	1		1				1				
parC	5231					Asn		1			1			1				
gyrA, *parE*	2223	Phe					Val	1			1						1	
gyrA, *parC*, *parE*	2273	Phe	Phe				Val	2				2					2	
	2232	Phe				Asn	Val	2				2						2
	3232							1		1							1	
	2252	Phe	Phe			Asn	Val	2				2					1	1
	2242	Phe		Asn		Asn	Val	1				1					1	
	2292	Phe			Asp	Asn	Val	1				1					1	

genes are clearly resistant to levofloxacin with MICs of ≥16 μg/ml.

DISCUSSION

PCR-RF–SSCP has been described as a useful tool for mass screening for DNA polymorphism analysis on other genes (15, 26) and is able to detect a nucleotide substitution, deletion, or insertion in up to a 22,000-base-pair amplicon (29). We applied this method for the analysis of the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes of *S. pneumoniae*. The restriction digestion of the PCR amplicons generates a higher degree of polymorphism that could be readily detected by SSCP. Each restriction enzyme was chosen to cut the respective PCR amplicons in regions to produce fragments in the range of 100 to 200 bp. Up to nine bands in the SSCP pattern were observed by using this method. However, using PCR-SSCP alone, only two to three bands were produced, and the method was unable to discriminate single nucleotide changes (results not shown). The SSCP patterns were able to detect a single base difference (for example, patterns 3, 4, and 5 in *gyrA*); therefore, the patterns produced from PCR-RF–SSCP were highly discriminative for mutations present in *gyrA*, *gyrB*, *parC*, and *parE*.

Among the hundred isolates screened, the SSCP profiles distinguished the common amino acid substitutions in GyrA (Ser-81-Phe), ParC (Ser-79-Phe, Asp-83-Asn/Tyr, and Lys-137-Asn) and ParE (Ile-460-Val) (5, 6, 14). Of the 36 isolates that possessed an amino acid substitution (Ile-460-Val) at ParE alone, 34 (94%) strains had a CIP MIC of 4 μg/ml and a LEV MIC of 0.5 to 2 μg/ml, similar to isolates that had no amino acid substitution detected, supporting that the ParE substitution did not contribute to significant fluoroquinolone resistance (14). However, two isolates had higher fluoroquinolone MICs (LEV MIC of 4 or 8 μg/ml) that may be attributed to other mutations in regions that were not studied here or some other mechanism, such as those that alter the drug permeation.

ParC substitution has been reported as contributing to low-level fluoroquinolone resistance (13, 16, 20). Although CIP MICs may be increased, the LEV MIC generally remained low, in the range of 0.5 to 2 μg/ml. Many reports showed that ParC and GyrA substitutions are responsible for high-level fluoroquinolone resistance and ParC substitution was a first-step prerequisite in the development of high-level resistance (13, 16, 20). Our findings supported that all nine of our isolates with ParC and GyrA substitutions had LEV MICs of ≥16 μg/ml. These amino acid substitutions have previously been described to be responsible for high levels of resistance (13, 16, 20). An exception, Glu-96-Asp, is an unusual substitution found in ParC, and its importance remains to be determined.

Our data are similar to the previous findings from Hong Kong in which Ser-81-Phe (9) was the substitution found in GyrA that contributed to high-level resistance to fluoroquinolones. However, an additional number of new substitutions in ParC (Asp-83-Tyr/Asn and Glu-96-Asp) were identified in the present study.

PCR-RF–SSCP provides a database of high-resolution profiles on these mutations and allows rapid screening for any new mutation. The analysis and comparison of the pattern with the known sequences provide an alternative method for detecting mutation in the gene studied other than direct nucleotide sequencing. The four-digit code that represents the SSCP profile for an isolate also provides an objective way of recognizing the QRDR mutations associated with that strain. If the SSCP patterns for a fluoroquinolone-resistant gene, e.g., *gyrA*, exceed 10, then the single-digit number could be replaced by a single alphabet letter, e.g., A to indicate the eleventh pattern, B for the twelfth pattern, etc. The method is sensitive and cheaper and easier to perform compared to direct nucleotide sequencing of all sequences. In addition, this method is rapid, and up to 48 samples could be run per gel. The method could readily be adaptable to a clinical laboratory if the laboratory has a PCR facility. The only additional requirement is an electrophoresis system for SSCP and the gels and reagents are readily available commercially. It is discriminatory and could readily be applied as a screening method to examine for known and new mutations of the fluoroquinolone resistance genes.

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