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Rapid Screening of Topoisomerase Gene Mutations by a Novel Melting Curve Analysis Method for Early Warning of Fluoroquinolone-Resistant *Streptococcus pneumoniae* Emergence▽

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We developed a real-time PCR assay combined with melting curve analysis for rapidly genotyping quinolone resistance-determining regions (QRDR) of topoisomerase genes in *Streptococcus pneumoniae*. This assay was not only accurate for the screening of fluoroquinolone (FQ) resistance but also relevant as an early warning system for detecting preexisting single QRDR mutations.

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia and is also responsible for substantial morbidity and mortality worldwide. Antipneumococcal fluoroquinolones (FQs), such as levofloxacin, gatifloxacin, gemifloxacin, and moxifloxacin, have greater activity against *S. pneumoniae* than do other drugs, and several are now approved for empirical treatment of community-acquired pneumonia (17, 19). However, recent reports have already noted an increase in the prevalence of FQ-resistant pneumococci (3, 10, 27).

FQ resistance in *S. pneumoniae* often involves mutation in the quinolone resistance-determining regions (QRDRs) of the *parC* gene and the *gyrA* gene, which encode subunits of topoisomerase IV and DNA gyrase, respectively (8, 13, 14, 24). Recent clinical treatment failures of FQ-resistant pneumococcal pneumonia have been reported to be due to strains which acquired FQ resistance as a result of stepwise QRDR mutations (6, 9, 21). Rapid detection of QRDR mutations is required and may constitute a more reliable approach than the current phenotypic method, which can represent susceptibility but cannot detect the potential of *S. pneumoniae* strains to harbor resistance. PCR-based techniques, such as PCR-restriction fragment length polymorphism analysis (1, 15, 20), single-stranded conformational polymorphism analysis (15), and PCR-oligonucleotide ligation (2), have been developed to detect QRDR mutations; however, these techniques are not appropriate for practical use, as they are complicated and time-consuming. More recently, real-time PCR methods combined with melting curve analysis (PCR-MCA) have been reported to be successful in the detection of key gene mutations associated with drug resistance in various microorganisms (12, 25, 26).

The aim of the present study was to develop and validate a rapid single-step PCR-MCA assay for genotyping *S. pneumoniae* strains, targeting four QRDR positions (Ser79 and Asp83 of the *parC* gene and also Ser81 and Gly85 of the *gyrA* gene) that are most frequently associated with FQ resistance (3, 7, 22).

Seventy-two *S. pneumoniae* clinical isolates were used. They consisted of 22 levofloxacin (LVX)-resistant strains, with MICs of ≥8 μg/ml, and 50 LVX-susceptible strains, with MICs of ≤2 μg/ml (5). Twenty-two resistant strains were collected from Nagasaki University Hospital (7 strains), Sapporo Medical University School of Medicine (5 strains) (28, 29), and the Levofloxacin Surveillance Group (10 strains) (27) in Japan. Fifty susceptible strains were isolated at Nagasaki University Hospital. Identification of *S. pneumoniae* was confirmed by optochin susceptibility (11) and by the presence of the autolysine gene (23). The MICs of ciprofloxacin (CIP), LVX, gatifloxacin (GAT), and moxifloxacin (MXF) were determined by a broth dilution method (4, 5). *S. pneumoniae* ATCC 49619 was used for quality control.

DNA was extracted from each strain by using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). All of the designed oligonucleotides are shown in Table 1. These refer to the known sequences of the *parC* and *gyrA* genes, which were derived from GenBank accession no. AF170996 and AF053121, respectively. PCR was performed in a total volume of 20 μl containing 5 μl of DNA template (average, 5 ng/reaction), 2 μl of LightCycler FastStart reaction mixture (Roche Diagnostics, Basel, Switzerland), 3 mM $MgCl_2$, a 0.2 μM concentration of each probe, and a 0.5 μM concentration of each primer. Thermal cycling was performed with an initial hold for 10 min at 95°C, followed by 35 cycles of 5 s at 95°C, 9 s at 55°C, and 12 s at 72°C. A melting curve was generated by cooling the reaction mixture to 35°C for 10 s, followed by heating to 70°C at a rate of 0.2°C/s. The PCR-MCA assay was performed using LightCycler analysis software 3.5 (Roche Diagnostics, Basel, Switzerland). The total assay time was approximately 1 h. PCR amplification products for all 72 strains were sequenced directly at the nucleotide level, using a BigDye Terminator v.3.1 standard sequencing kit and an ABI PRISM 310 genetic analyzer (both by Applied Biosystems, CA). The QRDR DNA sequencing

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TABLE 1. Oligonucleotides used for QRDR mutation detection with PCR-MCA assay

Target gene or codon	Primer or probe[b]	Sequence(5′–3′)[a]	Position(s) (nt)	Amplicon size (bp)
Genes				
parC	Forward	GTTCAACGCCGTATTCTT	138–155	246
	Reverse	TGCCTCAGTATAACGCATAG	364–383	
gyrA	Forward	GAATGAATTGGGTGTGAC	282–299	225
	Reverse	ATACGTGCCTCGGTATAA	489–506	
Codons				
Codon 79 of *parC*	Anchor	GTCGGCCAAGTCAGTCGGGAACATCATGGGGAAT TTCCACCC-FITC	203–244	
	Sensor	LCRed640-CACGGGGATTCTTCTATC-P	246–264	
Codon 83 of *parC*	Anchor	TATCTATGATGCCATGGT-FITC	260–277	
	Sensor	LCRed640-CGTATGTCACAGAACTGGAAAAATCGTG AGATTCTAGTTGAAATGCACGG-P	279–328	
Codon 81 of *gyrA*	Anchor	TAAATAGAGGAATCCCC-FITC	367–383	
	Sensor	LCRed640-TGTGGGTGGTATTTACCCATGACATCCCC TGTAATACGAGCAGATT-P	320–365	
Codon 85 of *gyrA*	Anchor	TCTATTTATGAAGCCATG-FITC	376–394	
	Sensor	LCRed640-CCGTATGGCTCAATGGTGGAGCTACCGT TACATGCTTGTAGATGGTCATG-P	396–445	

[a] Abbreviations: FITC, fluorescein isothiocyanate; LCRed 640, LightCyclerRed 640. LCRed 640 is a fluorophore. P, the 3′ end of the probe was phosphorylated to prevent probe elongation by Taq polymerase during PCR.
[b] Primers were used to target genes, and probes were used to target amino acids.

results were compared with the sequence of strain R6 (GenBank accession no. NC_003098), which was used as the wild-type standard strain.

Establishment of MCA for genotyping mutant and wild-type strains. (i) MCA for LVX-resistant *S. pneumoniae* strains and sequence analysis of QRDRs. From sequencing results, all 22 LVX-resistant strains had at least one single amino acid substitution at four QRDR positions (Table 2). No silent mutations were detected in any of these 22 strains. Initially, to establish the PCR-MCA method for genotyping QRDRs, ATCC 49619 was selected as the wild-type strain. Strains DR22 ($TTT_{237–239}$ [Ser79Phe] in *parC* and $GGA_{255–257}$ [Gly85Glu] in *gyrA*), L007 ($TAT_{237–239}$ [Ser79Tyr] in *parC*), SR68 ($AGA_{237–239}$ [Ser79Arg] in *parC* and $TTC_{243–245}$ [Ser81Phe]

TABLE 2. Melting peak (T_m) and direct DNA sequencing results for four QRDR positions in 22 LVX-resistant *S. pneumoniae* strains

Strain[a]	LVX MIC (μg/ml)	ParC codon 79		ParC codon 83		GyrA codon 81		GyrA codon 85	
		T_m (°C)	Sequence[b]	T_m (°C)	Sequence[b]	T_m (°C)	Sequence[b]	T_m (°C)	Sequence[b]
ATCC 49619	0.5	60.4	TCT/Ser	56.7	GAT/Asp	52.3	TCC/Ser	56.8	GAA/Glu
N001	8	50.2	**TTT**/Phe	56.6	WT[f]	52.3	WT	56.8	WT
N002	8	61.0	WT	56.8	WT	45.4	**TTC**/Phe	56.0	WT
N003	8	50.1	**TTT**/Phe	56.7	WT	52.3	WT	50.2	**A**AA/Lys
N004	16	50.8	**TTT**/Phe	56.4	WT	51.4	WT	49.7	**A**AA/Lys
N005	16	50.1	**TTT**/Phe	56.8	WT	52.1	WT	50.2	**A**AA/Lys
N006	8	50.1	**TTT**/Phe	56.8	WT	52.1	WT	56.4	WT
N007	16	50.4	**TTT**/Phe	56.8	WT	52.1	WT	50.3	**A**AA/Lys[MC]
L001	32	50.1	**TTT**/Phe	56.7	WT	47.7	**TA**C/Tyr[MC]	56.6	WT
L002	8	50.0	**TTT**/Phe	56.4	WT	52.2	WT	56.8	WT
L003	≥64	50.1	**TTT**/Phe	49.0	**A**AT/Asn	52.2	WT	50.3	**A**AA/Lys
L004	16	50.0	**TTT**/Phe	56.6	WT	44.9	**TTC**/Phe	56.8	WT
L005	32	49.5	**TA**T/Tyr	56.6	WT	52.2	WT	50.4	**A**AA/Lys
L006	16	50.1	**TTT**/Phe	56.8	WT	45.1	**TTC**/Phe	56.8	WT
L007	16	49.3	**TA**T/Tyr[MC]	56.7	WT	52.3	WT	56.8	WT
L008	8	60.4	WT	56.7	WT	45.1	**TTC**/Phe	56.8	WT
L009	16	49.9	**TTT**/Phe	56.8	WT	45.0	**TTC**/Phe	56.8	WT
L010	32	49.9	**TTT**/Phe	56.7	WT	45.1	**TTC**/Phe	56.8	WT
DR22[d]	32	50.0	**TTT**/Phe[MC]	56.5	WT	41.3	**G**CC/Phe	41.7	**GGA**/Glu[MC]
SR27[c]	32	60.7	WT	48.5	**T**AT/Tyr[MC]	44.5	**TTC**/Phe	56.6	WT
SR68[c]	32	36.8	**AGA**/Arg[MC]	56.5	WT	44.4	**TTC**/Phe[MC]	56.8	WT
SR179[c]	8	36.6	**AGA**/Arg	56.6	WT	44.5	**TTC**/Phe	56.8	WT
SR248[d]	8	60.5	WT	48.7	**AA**T/Asn[MC]	41.2	**G**CC/Ala[MC]	56.6	WT

[a] Strains N001 to N007 were isolated at Nagasaki University Hospital, and strains L001 to L010 were supplied by the Levofloxacin Surveillance Group.
[b] Nucleotide sequence/amino acid sequence. Changed nucletides are shown in bold. WT, identical to the nucleotide distribution of the wild type, ATCC 49619. MC, mutant control.
[c] Strains sourced from reference 22, supplied by Sapporo Medical University.
[d] Strains sourced from reference 23, supplied by Sapporo Medical University.

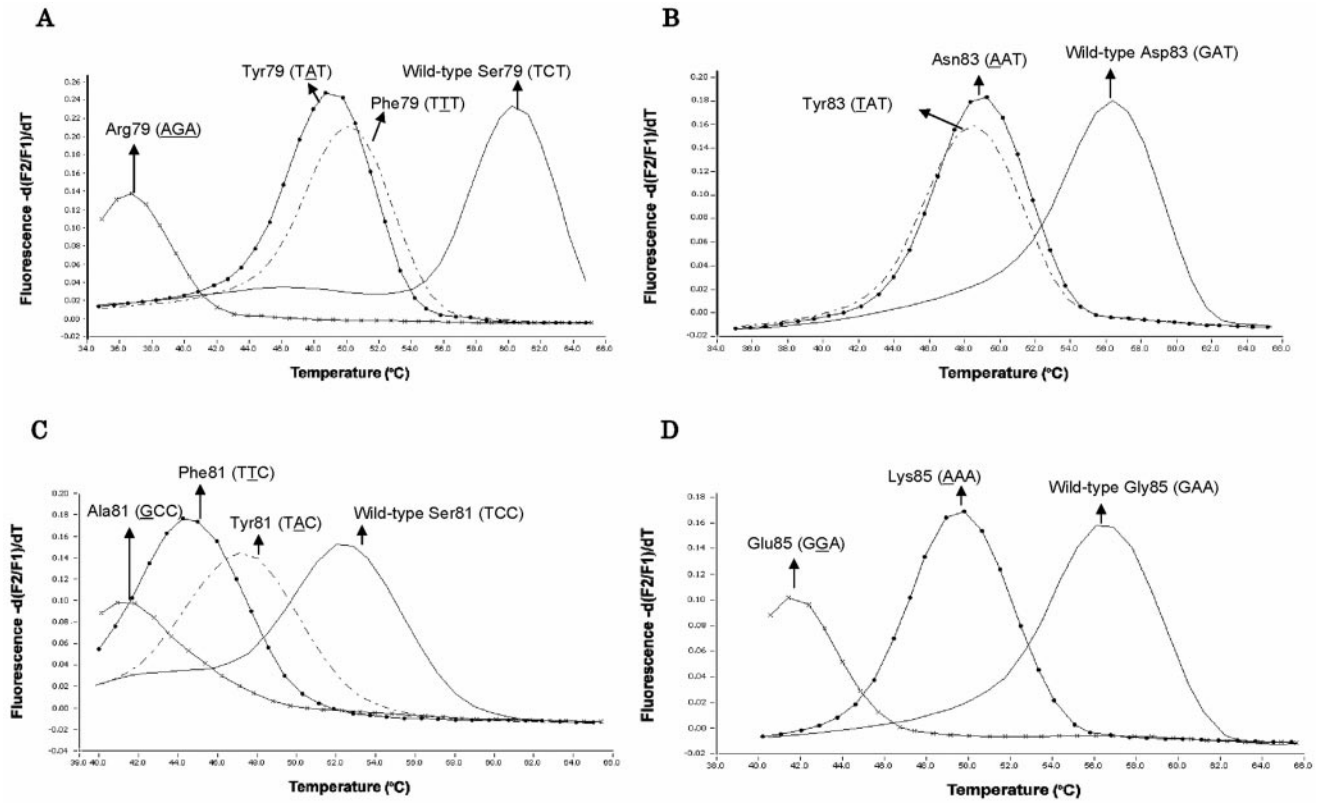

FIG. 1. Melting peak patterns for QRDR mutants with substitutions in *parC* and *gyrA*. Melting curve analysis was performed with the 246-bp amplicon of the *parC* gene and the 225-bp amplicon of the *gyrA* gene obtained by real-time PCR from wild-type and mutant control strains. Panels A, B, C, and D show melting peak patterns for codon 79 of the *parC* gene, codon 83 of the *parC* gene, codon 81 of the *gyrA* gene, and codon 85 of the *gyrA* gene, respectively. Each value on the *y* axis represents the ratio of the first negative derivative of the change in fluorescence (dF) to the variation in temperature. Point mismatches in the QRDRs resulted in lower T_m values for mutant strains than for the wild-type strain.

in *gyrA*), SR27 ($TAT_{249–251}$ [Asp83Tyr] in *parC*), SR248 ($AAT_{249–251}$ [Asp83Asn] in *parC* and $GCC_{243–245}$ [Ser81Ala] in *gyrA*), L001 ($TAC_{243–245}$ [Ser81Tyr] in *gyrA*), and L003 ($AAA_{255–257}$ [Gly85Lys] in *gyrA*) were chosen as mutant control strains. Using probes specific for the wild-type strain, all of the control strains showed characteristic melting peaks, with a distinct T_m value corresponding to each mutant, as shown in Fig. 1. The MCA for *parC* codon 79 produced four different peaks, with the following T_m values: $TCT_{237–239}$ (wild type), 60.4°C; $TTT_{237–239}$ mutant, 50.0°C; $TAT_{237–239}$ mutant, 49.3°C; and $AGA_{237–239}$ mutant, 36.8°C (Fig. 1A). Similarly, MCA produced three different peaks for *parC* codon 83 (Fig. 1B), four different peaks for *gyrA* codon 81 (Fig. 1C), and three different peaks for *gyrA* codon 85 (Fig. 1D). The minimum T_m shifts for mutant strains relative to the wild-type strain were 10.0°C for *parC* codon 79, 7.7°C for *parC* codon 83, 4.5°C for *gyrA* codon 81, and 6.2°C for *gyrA* codon 85, with an acceptable T_m reproducibility with a <0.8% coefficient of variation (CV) (MCA was performed five times for each strain). The differences in T_m values between the mutants with $TTT_{237–239}$ (Ser79Phe) and $TAT_{237–239}$ (Ser79Tyr) at *parC* codon 79 (Fig. 1A) and between those with $TAT_{249–251}$ (Asp83Tyr) and $AAT_{249–251}$ (Asp83Asn) at *parC* codon 83 (Fig. 1B) were both very similar, and thus these mutations are impossible to differentiate but can be detected as being present. The PCR-MCA assay correctly genotyped 22 LVX-resistant strains, as determined by comparison with sequencing results (Table 2).

(ii) MCA of LVX-susceptible *S. pneumoniae* strains and sequence analysis of QRDRs. Forty-seven of the 50 LVX-susceptible *S. pneumoniae* strains had similar T_m values to that of the wild-type strain. The mean T_m values for the 47 strains were 60.4°C (CV, 0.41%) for *parC* codon 79, 56.7°C (CV, 0.52%) for *parC* codon 83, 56.5°C (CV, 0.87%) for *gyrA* codon 81, and 56.7°C (CV, 0.34%) for *gyrA* codon 85, and sequencing results confirmed that these were in fact wild-type strains. Two of the remaining three strains had T_m values of 49.8°C and 50.0°C, which were lower than that (60.4°C) of the wild-type strain for *parC* codon 79, while the other strain had a T_m of 48.8°C, which differed from that of the wild-type strain (56.7°C) for *parC* codon 83. Indeed, direct sequencing revealed that the former two strains had a $TTT_{237–239}$ (Ser79Phe) mutation, while the latter strain had an $AAT_{249–251}$ (Asp83Asn) mutation in the *parC* gene.

Comparison of outcomes between conventional phenotyping and MCA genotyping. We compared the ability of the present PCR-MCA assay to detect FQ susceptibility in 72 *S. pneumoniae* strains with that of the conventional phenotypic method, as shown in Fig. 2 and Table 3. All 47 LVX-susceptible strains with no mutations had susceptibility MICs of ≤2 μg/ml, while 22 of the remaining 25 mutation-containing

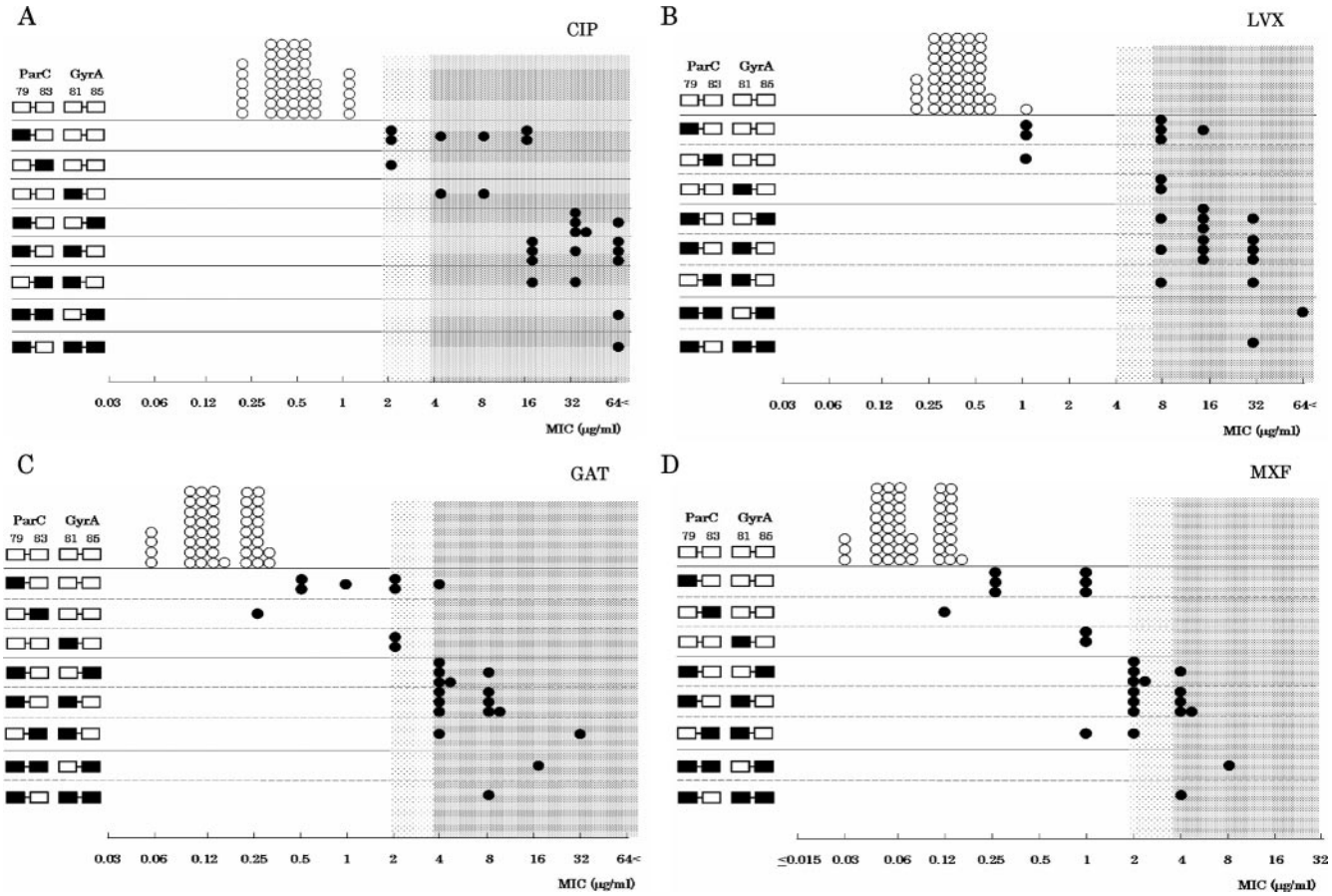

FIG. 2. Comparison of outcomes between conventional FQ susceptibility testing and the PCR-MCA assay for genotyping QRDR mutants. Panels A, B, C, and D show results with CIP, LVX, GAT, and MXF, respectively. White squares represent the wild type, and black squares represent the mutant types for four positions of the QRDRs in the *parC* gene and *gyrA* gene obtained by the PCR-MCA assay. White circles represent wild-type strains, and black circles represent mutation-containing strains. The horizontal axis represents the MIC of each strain. The dark mesh area represents resistance, and the light mesh area represents intermediate susceptibility to each FQ. The CLSI MIC breakpoints (26) were used for the following drugs: LVX (susceptible, ≤2 μg/ml; intermediate, 4 μg/ml; and resistant, ≥8 μg/ml), GAT (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; and resistant, ≥4 μg/ml), and MXF (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; and resistant, ≥4 μg/ml). The breakpoint standard for CIP was obtained from the interpretive guideline supplied by the Japanese Society of Chemotherapy (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; and resistant, ≥4 μg/ml).

strains (88%) had resistance MICs of ≥8 μg/ml (Fig. 2B; Table 3). Interestingly, the mutation profiles for the QRDRs in the *parC* and *gyrA* genes revealed a close relationship between MIC level and the number of QRDR mutations. We observed that six of the nine strains with a single QRDR mutation were highly resistant to LVX (Fig. 2B; Table 3). This can be explained by the fact that three of the six strains harbored additional *parE* mutations and performed active efflux, two of the six strains harbored additional *parE* mutations, and the remaining strain harbored an additional *gyrB* mutation (data not shown). Compared with the conventional phenotypic method, the diagnostic sensitivity and specificity of the PCR-MCA assay for detecting non-FQ-susceptible strains were 100% (25/25) and 100% (47/47), respectively, for CIP, 100% (22/22) and 94% (47/50), respectively, for LVX, 100% (21/21) and 92% (47/51), respectively, for GAT, and 100% (15/15) and 82% (47/57), respectively, for MXF. Assuming that QRDR mutation provides a positive diagnosis of the presence of non-FQ-susceptible *S. pneumoniae*, this assay is particularly useful because it has a negative predictive value of 100% for all four FQs and positive predictive values of 100% for CIP, LVX, and GAT and 93.7% for MXF (Fig. 2; Table 3).

The discrepancies observed in the specificity of the assay were instructive because the conventional phenotypic method failed to pick up strains which have high proportions of a single QRDR mutation. Several reports have noted that a significant number of isolates already have a single mutation but are still considered fully susceptible (7, 22). Although our study lacks a collection of moderately FQ-resistant (LVX MIC of 2 μg/ml or 4 μg/ml) *S. pneumoniae* strains, Lim et al. (16) reported that about 60% of *S. pneumoniae* isolates with an LVX MIC of 2 μg/ml carry preexisting *parC* mutations. Typically, stepwise mutation starts with the *parC* gene, which frequently leads to secondary mutations in the *gyrA* gene, eventually resulting in high-level resistance to all FQs (18). This has also been supported by clinical reports of an FQ-susceptible *S. pneumoniae* strain carrying a first-step mutation that evolved into a second-step QRDR mutation during FQ treatment, resulting in treat-

TABLE 3. Relationship between QRDR mutations detected by MCA and MICs of FQs for 72 *S. pneumoniae* strains

FQ and QRDR mutation	No. of isolates with MIC[a]		
	S	I	R
CIP			
None	47	0	0
Single *parC* or *gyrA* mutation	0	3	6
parC and *gyrA* mutations	0	0	16
LVX			
None	47	0	0
Single *parC* or *gyrA* mutation	3	0	6
parC and *gyrA* mutations	0	0	16
GAT			
None	47	0	0
Single *parC* or *gyrA* mutation	4	4	1
parC and *gyrA* mutations	0	0	16
MXF			
None	47	0	0
Single *parC* or *gyrA* mutation	9	0	0
parC and *gyrA* mutations	1	8	7

[a] Abbreviations: S, susceptible; I, intermediate; and R, resistant. The CLSI MIC breakpoints were used for LVX (susceptible, ≤2 μg/ml; intermediate, 4 μg/ml; and resistant, ≥8 μg/ml), GAT (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; and resistant, ≥4 μg/ml), and MXF (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; and resistant, ≥4 μg/ml). The breakpoint standard for CIP was obtained from the interpretive guidelines supplied by the Japanese Society of Chemotherapy (susceptible, ≤1 μg/ml; intermediate, 2 μg/ml; and resistant, ≥4 μg/ml).

ment failure (6). We emphasize the clinical importance of the detection of first-step QRDR mutations in either *gyrA* or *parC* for attempting to predict evolution into FQ resistance. There are some limitations of this assay that remain to be ironed out; for instance, any mutations outside the sensor probe would be undetectable. Resistance as a result of other mechanisms (such as *parE* or *gyrB* mutation and efflux) also cannot be detected. However, these mechanisms provide resistance potential but are not conclusive indicators of high-level resistance to FQs compared with *parC* and *gyrA* mutations (7, 22).

In conclusion, the PCR-MCA assay was easily and quickly performed and had an accuracy which was at least as satisfactory as that of the conventional phenotypic method. Moreover, single QRDR mutations which harbored potential for FQ resistance could be detected. This assay is also useful for surveillance studies in the screening of FQ resistance as an alternative to DNA nucleotide sequencing. The application of this novel method would be a valuable tool for achieving rapid screening of QRDR mutations and as an early warning system for the emergence of FQ-resistant *S. pneumoniae*.

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REFERENCES

1. **Alonso, R., M. Galimand, and P. Courvalin.** 2004. An extended PCR-RFLP assay for detection of *parC*, *parE*, and *gyrA* mutations in fluoroquinolone-resistant *Streptococcus pneumoniae*. J. Antimicrob. Chemother. **53:**682–683.
2. **Bui, M. H., G. G. Stone, A. M. Nilius, L. Almer, and R. K. Flamm.** 2003. PCR-oligonucleotide ligation assay for detection of point mutations associated with quinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **47:**1456–1459.
3. **Canton, R., M. Morosini, M. C. Enright, and I. Morrissey.** 2003. Worldwide incidence, molecular epidemiology and mutations implicated in fluoroquinolone-resistant *Streptococcus pneumoniae*: data from the global PROTEKT surveillance programme. J. Antimicrob. Chemother. **52:**944–952.
4. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th edition. CLSI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, Pa.
5. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial susceptibility testing; 16th informational supplement. CLSI document M100-S16. Clinical and Laboratory Standards Institute, Wayne, Pa.
6. **Davidson, R., R. Cavalcanti, J. L. Brunton, D. J. Bast, J. C. Azavedo, P. Kibsey, C. Fleming, and D. E. Low.** 2002. Resistance to levofloxacin and failure of treatment of pneumococcal pneumonia. N. Engl. J. Med. **346:**747–750.
7. **Doern, G. V., S. S. Richter, A. Miller, N. Miller, C. Rise, K. Heilmann, and S. Beekmann.** 2005. Antimicrobial resistance among *Streptococcus pneumoniae* in the United States: have we begun to turn the corner on resistance to certain antimicrobial classes? Clin. Infect. Dis. **41:**139–148.
8. **Eliopoulos, G. M.** 2004. Quinolone resistance mechanisms in pnemococci. Clin. Infect. Dis. **38**(Suppl. 4)**:**S350–S356.
9. **Endimiani, A., G. Brigante, A. A. Bettaccini, F. Luzzaro, P. Grossi, and A. Q. Toniolo.** 2005. Failure of levofloxacin treatment in community-acquired pneumococcal pneumonia. BMC Infect. Dis. **5:**106.
10. **Felmingham, D., R. R. Reinert, Y. Hirakata, and A. Rodloff.** 2002. Increasing prevalence of antimicrobial resistance among isolates of *Streptococcus pneumoniae* from PROTEKT surveillance study, and comparative *in vitro* activity of the ketolide, telithromycin. J. Antimicrob. Chemother. **50**(Suppl. S1)**:**25–37.
11. **Gardam, M. A., and M. A. Miller.** 1998. Optochin revisited: defining the optimal type of blood agar for presumptive identification of *Streptococcus pneumoniae*. J. Clin. Microbiol. **36:**833–834.
12. **Glocker, E., M. Berning, M. M. Gerrits, J. G. Kusters, and M. Kist.** 2005. Real-time PCR screening for 16S rRNA mutations associated with resistance to tetracycline in *Helicobacter pylori*. Antimicrob. Agents Chemother. **49:** 3166–3170.
13. **Hooper, D. C.** 2002. Fluoroquinolone resistance among gram positive cocci. Lancet Infect. Dis. **2:**530–538.
14. **Hooper, D. C.** 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updat. **2:**38–55.
15. **Ip, M., S. S. Chau, F. Chi, A. Qi, and R. W. Lai.** 2006. Rapid screening of fluoroquinolone resistance determinants in *Streptococcus pneumoniae* by PCR-restriction fragment length polymorphism and single-strand conformational polymorphism. J. Clin. Microbiol. **44:**970–975.
16. **Lim, S., D. Bast, A. McGeer, J. de Azavedo, and D. E. Low.** 2003. Antimicrobial susceptibility breakpoints and first-step *parC* mutations in *Streptococcus pneumoniae*: redefining fluoroquinolone resistance. Emerg. Infect. Dis. **9:**833–837.
17. **Mandell, L. A., J. G. Bartlett, S. F. Dowell, T. M. File, Jr., D. M. Musher, and C. Whitney.** 2003. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin. Infect. Dis. **37:**1405–1433.
18. **Muñoz, R., and A. G. de la Campa.** 1996. ParC subunit of DNA topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and cooperates with DNA gyrase A subunit in forming resistance phenotype. Antimicrob. Agents Chemother. **40:**2252–2257.
19. **Niederman, M. S., L. A. Mandell, A. Anzueto, J. B. Bass, W. A. Broughton, G. D. Campbell, N. Dean, T. File, M. J. Fine, P. A. Gross, F. Martinez, T. J. Marrie, J. F. Plouffe, J. Ramirez, G. A. Sarosi, A. Torres, R. Wilson, and V. L. Yu.** 2001. Guidelines for the management of adults with community-acquired pneumonia. Am. J. Respir. Crit. Care Med. **163:**1730–1754.
20. **Pan, X. S., J. Ambler, S. Mehtar, and M. Fisher.** 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **40:**2321–2326.
21. **Perez-Trallero, E., J. M. Marimon, L. Iglesias, and J. Larruskain.** 2003. Fluoroquinolone and macrolide treatment failure in pneumococcal pneumonia and selection of multidrug-resistant isolates. Emerg. Infect. Dis. **9:**1159–1162.
22. **Richter, S. S., K. P. Heilmann, S. E. Beekman, N. J. Miller, C. L. Rice, and G. V. Doern.** 2005. The molecular epidemiology of *Streptococcus pneumoniae* with quinolone resistance mutations. Clin. Infect. Dis. **40:**225–235.
23. **Sheppard, C. L., T. G. Harrison, R. Morris, A. Horgan, and R. C. George.** 2004. Autolysine-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. J. Med. Microbiol. **53:**189–195.
24. **Tankovic, J., B. Perichon, J. Duval, and P. Courvalin.** 1996. Contribution of mutations in *gyrA* and *parC* genes to fluoroquinolone resistance of mutants of *Streptococcus pneumoniae* obtained in vivo and in vitro. Antimicrob. Agents Chemother. **40:**2505–2510.
25. **Vacher, S., A. Menard, E. Bernard, A. Santos, and F. Megraud.** 2005. Detection of mutations associated with macrolide resistance in thermophilic *Campylobacter spp*. by real-time PCR. Microb. Drug Resist. **11:**40–47.
26. **Vernel-Pauillac, F., V. Falcot, D. Whiley, and F. Merien.** 2006. Rapid detec-

tion of a chromosomally mediated penicillin resistance-associated *ponA* mutation in *Neisseria gonorrhoeae* using a real-time PCR assay. FEMS Microbiol. Lett. **255:**66–74.

27. **Yamaguchi, K., and A. Ohno.** 2005. Investigation of the susceptibility trends in Japan to fluoroquinolones and other antimicrobial agents in a nationwide collection of clinical isolates: a longitudinal analysis from 1994 to 2002. Diagn. Microbiol. Infect. Dis. **52:**135–143.
28. **Yokota, S., K. Sato, O. Kuwahara, S. Habadera, N. Tsukamoto, H. Ohuchi, H. Akizawa, T. Himi, and N. Fujii.** 2002. Fluoroquinolone-resistant *Streptococcus pneumoniae* strains occur frequency in elderly patients in Japan. Antimicrob. Agents Chemother. **46:**3311–3315.
29. **Yokota, S., K. Sato, S. Yoshida, and N. Fujii.** 2004. Molecular epidemiology of fluoroquinolone-resistant *Streptococcus pneumoniae* in Japan. Kansenshogaku Zasshi **78:**428–434. (In Japanese.)