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Melting Curve Analysis for Rapid Detection of Topoisomerase Gene Mutations in *Haemophilus influenzae*▽

Shigeki Nakamura,[1] Katsunori Yanagihara,[1,2]* Yoshitomo Morinaga,[1,2] Koichi Izumikawa,[1] Masafumi Seki,[1] Hiroshi Kakeya,[1] Yoshihiro Yamamoto,[1] Shimeru Kamihira,[2] and Shigeru Kohno[1,3]

Second Department of Internal Medicine,[1] Department of Laboratory Medicine,[2] and Division of Molecular and Clinical Microbiology, Department of Molecular Microbiology and Immunology,[3] Nagasaki University Graduate School of Medical Science, Nagasaki, Japan

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We established a real-time PCR assay with melting curve analysis to rapidly genotype quinolone resistance-determining regions (QRDRs) of gyrase A and topoisomerase IV genes in *Haemophilus influenzae*. This assay is a useful tool for the detection of fluoroquinolone resistance and for the early detection of preexisting QRDR mutations.

Haemophilus influenzae is a major causative pathogen isolated from infections, including acute and chronic respiratory infections, acute otitis media, sinusitis, and meningitis in pediatric patients. Recent reports have noted the prevalence of fluoroquinolone (FQ)-resistant *H. influenzae* (1, 3, 4, 9, 11, 14, 20, 22, 26). FQ-resistant *H. influenzae* often carries mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and the *parC* genes, which encode subunits of DNA gyrase and topoisomerase IV, respectively (9, 11, 16, 18, 23). A combination of real-time PCR methods and melting curve analysis (PCR-MCA) is a useful tool for the rapid detection of key gene mutations associated with drug resistance in various microorganisms (13, 24, 25), but there are no reports about *H. influenzae*. The aim of this study was to develop a PCR-MCA method for detecting *H. influenzae* strains by targeting a total of four QRDR positions in the *gyrA* (codons 84 and 88) and the *parC* (codons 84 and 88) genes that are frequently associated with FQ resistance (9, 11, 16, 23). This current method could simultaneously identify the *gyrA* and the *parC* mutations by using only one PCR performance.

Seventeen *H. influenzae* clinical isolates were used. Ten of the strains were susceptible to FQ, and seven of the strains had low susceptibility or were resistant to FQ. The seven FQ-resistant/low-susceptibility strains consisted of one strain (NUH-1) from Nagasaki University Hospital, one strain (BY-1) from Bayer (Osaka, Japan), two strains (DR-1 and DS-2) from Daiichi-Sankyo (Tokyo, Japan), and three strains (MSC24060, MSC27995, and MSC11438) kindly provided by Meiji-Seika Kaisha (Tokyo, Japan) (21). The 10 FQ-susceptible strains were isolated from patients at Nagasaki University Hospital. Identification of *H. influenzae* was confirmed by colony morphology, Gram staining, growth on chocolate agar, and the X and V factor requirements. The MICs of ciprofloxacin (CPFX), sparfloxacin, levofloxacin (LVFX), gatifloxacin, moxifloxacin, garenoxacin, and sitafloxacin were determined by a broth dilution method using *Haemophilus* test medium according to the recommendations of the Clinical and Laboratory Standards Institute (5). *H. influenzae* ATCC 51907 was used for quality control.

DNA was extracted from each strain by using a QIAamp DNA mini-kit (Qiagen, Hilden, Germany). Sequences of the oligonucleotides and probes are shown in Tables 1 and 2. The sequences are from the known sequences of the *parC* and *gyrA* genes, which were derived from GenBank accession no. NP439678 and NP439419, respectively. To identify mutations in the QRDRs of *gyrA* and *parC* in these strains, we performed PCR and direct DNA sequencing according to the method described by Vila et al. (26).

Real-time PCR-MCA was performed with a total volume of 10 μl containing 2 μl of DNA template, 5 μl of LightCycler 480 Probe Master mixture (Roche Diagnostics, Basel, Switzerland), 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 5 min at 95°C, followed by 30 cycles of 10 s at 95°C, 10 s at 58°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 it to 90°C at a rate of 0.2°C/s. The PCR-MCA was performed by using LightCycler 480 Basic software

TABLE 1. Characteristics of specific primers used for sequencing *gyrA* and *parC*

Target gene	Primer	Sequence	Nucleotide positions	Amplicon size (bp)
gyrA	Forward	5′-TGGATCGCGAAGG CAATAC-3′	158–176	197
	Reverse	5′-TGGCGCATCACCAT CAAT-3′	354–337	
parC	Forward	5′-GTATTGTATATGCG ATGTCTGAAC-3′	143–166	
	Reverse	5′-CCATCTACAAGTGG ATAACGA-3′	317–297	175

* Corresponding author. Mailing address: Department of Laboratory Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Phone: 81-95-819-7276. Fax: 81-95-849-7285. E-mail: k-yanagi@nagasaki-u.ac.jp.

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TABLE 2. Characteristics of specific probes used to detect amino acid mutations in QRDRs

Target	Probe	Sequence[a]
Codon 84 of GyrA	Anchor	5′AGCGCGTGTTGTGGGTG ATGTAATCGGTAAATAT CAC3′-FITC
Codon 84 of GyrA	Sensor	5′-LCRed610-CGCATGGTGA CTCCGC-3′-P
Codon 88 of GyrA	Anchor	5′CCATCAACCAACATATA GCGAAGTGAGAAGGGT TGT3′-FITC
Codon 88 of GyrA	Sensor	5′-Cy5-CCATACGAACGATG GTATCGTACA-3′-P
Codon 84 of ParC	Anchor	5′TCGGTGATGTACTCGGT AAATTCCATCCACATGG T3′-FITC
Codon 88 of ParC	Sensor	5′-LCRed610-ACAGTGCTTG TTATGAAGCTAT-3′-P

[a] FITC, fluorescein isothiocyanate; P, phosphorylated.

(Roche Diagnostics, Basel, Switzerland). The total assay time was approximately 1 h. The resulting QRDR DNA sequences were compared with the sequence of strain Rd (GenBank accession no. NC000907), which was used as the wild-type standard strain.

Using specific probes for the wild-type strain, we showed that all of the mutant strains had characteristic melting peaks with distinct melting temperature (T_m) values, as shown in Fig. 1. The minimum T_m shifts for mutant strains compared to that of the wild-type strain were 6.13°C for *parC* codons 84 and 88, 11.58°C for *gyrA* codon 84, and 7.9°C for *gyrA* codon 88. The PCR-MCA correctly detected seven LVFX low-susceptibility/resistant strains, as determined by a comparison with sequencing results (Table 3). From the sequencing results, all seven strains with low susceptibility/resistance to FQ had at least two single-amino-acid substitutions at four QRDR positions (Table 4). All LVFX-susceptible *H. influenzae* strains had the same T_m values as that of the wild-type strain, and sequencing results confirmed that these were in fact wild-type strains (data not shown).

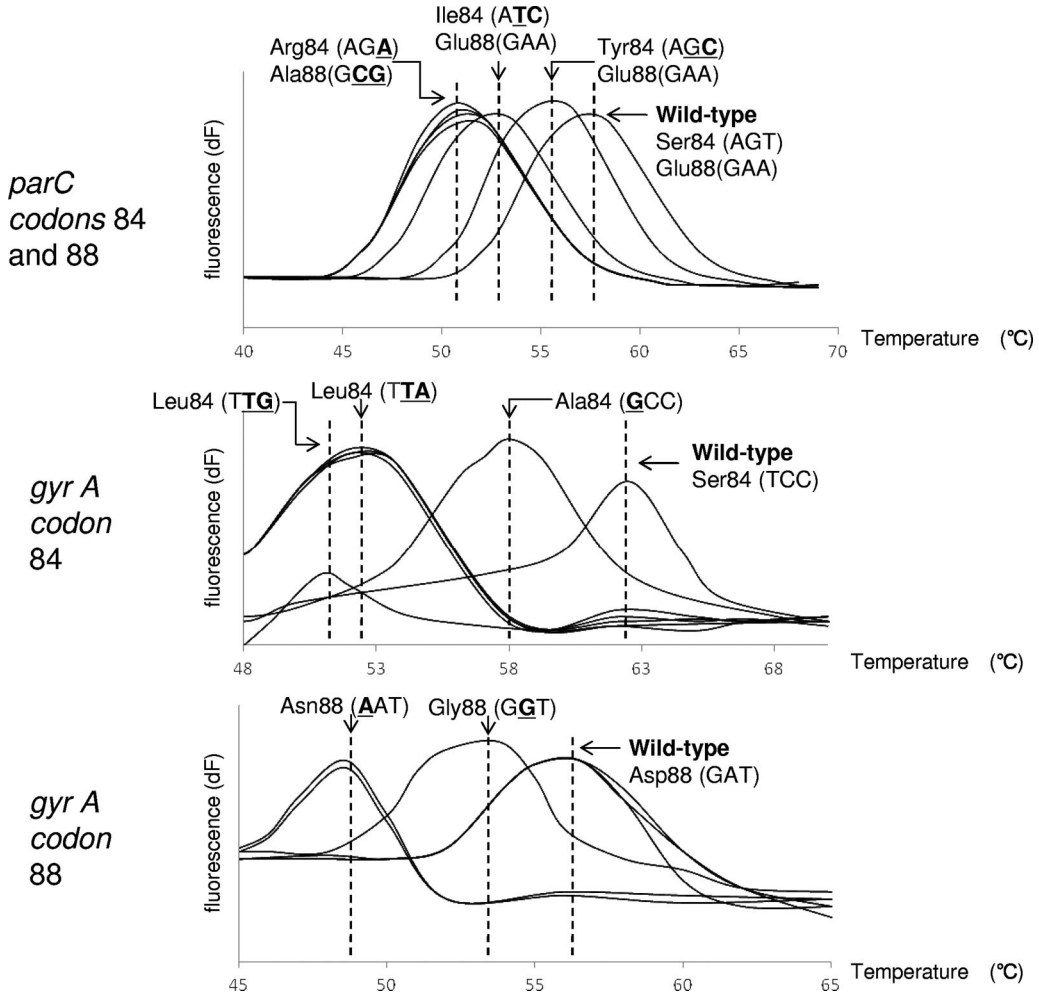

FIG. 1. Melting peak patterns for QRDR mutants with substitutions in *parC* and *gyrA*. MCA was performed with the 175-bp amplicon of the *parC* gene and the 197-bp amplicon of the *gyrA* gene. Top, middle, and bottom panels show melting peak patterns for codons 84 and 88 of the *parC* gene, codon 84 of the *gyrA* gene, and codon 88 of the *gyrA* gene, respectively. Each value on the *y* axis reveals the ratio of the first negative derivative of the change in fluorescence (dF) to the variation in temperature.

TABLE 3. Seven LVFX low-susceptibility/resistant strains detected by PCR-MCA

Strain	MIC (μg/ml) to LVFX	Susceptibility at QRDRs[a]						
		parC codon 84		*parC* codon 88	*gyrA* codon 84		*gyrA* codon 88	
		T_m (°C)	Sequence/aa	Sequence/aa	T_m (°C)	Sequence/aa	T_m (°C)	Sequence/aa
Rd (wild type)	0.003	56.86	AGT/Ser	GAA/Glu	62.44	TCC/Ser	56.49	GAT/Asp
DS-1	8	55.05	AGC/Thr	WT	52.04	TTA/Leu	53.81	GGT/Gly
NUH-1	16	50.73	AGA/Arg	GCG/Ala	52.04	TTA/Leu	53.81	AAT/Asn
BY-1	8	50.73	AGA/Arg	WT	50.86	TTG/Leu	48.59	AAT/Asn
MS24060	1	50.73	AGA/Arg	GCG/Ala	57.93	GCC/Ala	56.49	WT
MS27995	1	52.11	ATC/Ile	WT	52.04	TTA/Leu	56.49	WT
MS11438	4	50.73	AGA/Arg	GCG/Ala	52.04	TTA/Leu	48.59	TAT/Tyr
DS-2	4	50.82	AGA/Arg	GAT/Asp	53.24	TAC/Thr	49.70	TAT/Tyr

[a] aa, amino acid. WT, wild type.

We compared the ability of the present PCR-MCA to detect FQ susceptibility in 17 *H. influenzae* strains with that of the conventional phenotypic method. All LVFX-susceptible strains that had no mutation in codons 84 and 88 of *gyrA* and *parC* were classified as susceptible according to Clinical and Laboratory Standards Institute criteria (6) (data not shown). As shown in Table 4, the mutation profiles for the QRDRs in the *gyrA* and *parC* genes revealed a close relationship between the MIC level and the number of QRDR mutations. Previous studies have found that the conventional phenotypic method failed to detect strains that have a single QRDR mutation; these strains have the potential to develop into a highly resistant pathogen (8). Several reports have noted that a significant number of *Streptococcus pneumoniae* isolates already have a single-step mutation and are prone to acquiring a second-step mutation (19). Unfortunately, our study lacks a collection of single mutant strains. It was reported that the selection window for CPFX with wild-type cells was below serum drug concentrations in human volunteers receiving twice-daily doses of 500 mg (10). However, Odoul et al. reported that the median area under the inhibitory curve was decreased by about half the proposed target value for CPFX in a cystic fibrosis patient receiving an oral regimen of 15 mg of CPFX/kg of body weight twice a day (15). Increasing the times that the drug concentrations fall in the mutant selection window causes the mutation (7). Actually, the previously reported strains that failed treatment had a double mutation in the *gyrA* and the *parC* genes (2). Furthermore, Pérez-Vázquez et al. reported that hypermutability is a risk condition for the development of FQ-resistant *H. influenzae* infection (17), and Li et al. reported that stepwise selection induced high-level resistance in *H. influenzae* (12). We emphasize the clinical importance of the detection of first-step QRDR mutations in either *gyrA* or *parC* for attempting to predict a strain's evolution into FQ resistance. In addition, we should consider the FQ dosage carefully to avoid the low FQ concentration when we treat patients who have chronic lung disease.

In conclusion, the PCR-MCA was easily and quickly performed and had an accuracy that was at least as satisfactory as that of the conventional phenotypic method. FQ-resistant *H. influenzae* is expected to become a more important pathogen in the future, because FQ is the most effective antibiotic against *H. influenzae* infection, and the number of FQ-resistant strains may rise further, along with the recent increase in FQ prescription. Although additional studies are needed, we anticipate that the PCR-MCA used in this study may be a useful tool for surveillance studies in the screening of FQ resistance as an alternative to DNA nucleotide sequencing, because this PCR-MCA can recognize the *gyrA* and the *parC* mutations more clearly, easily, and rapidly than sequencing.

TABLE 4. Relationship between FQ susceptibility, amino acid changes in *gyrA* and *parC*, and results of PCR-MCA

Strain	MCA[a]	Amino acid change encoded by:		MIC (μg/ml) of[b]:						
		gyrA	*parC*	LVFX	SPFX	GFLX	MFLX	CPFX	GRNX	STFX
Rd	WT			0.003	0.003	0.003	0.003	0.003	0.0015	0.0006
DS-1	MT	Ser84→Leu Asp88→Gly	Gly82→Asp	8	4	2	4	4	4	0.25
NUH-1	MT	Ser84→Leu Asp88→Asn	Ser84→Arg Glu88→Ala	16	16	8	16	16	8	1
BY-1	MT	Ser84→Leu Asp88→Asn	Ser84→Arg Gly82→Asp	8	8	8	32	16	>32	2
MSC24060	MT	Ser84→Ala	Ser84→Arg Glu88→Ala	1	1	0.5	2	2	0.5	0.12
MSC27995	MT	Ser84→Leu	Ser84→Ile	1	1	0.5	0.5	1	1	0.03
MSC11438	MT	Ser84→Leu Asp88→Tyr	Ser84→Arg Glu88→Ala	4	4	4	8	4	8	0.25
DS-2	MT	Ser84→Leu Asp88→Tyr	Ser84→Arg Glu88→Ala	4	8	2	2	8	4	0.12

[a] Mutants (MT) were defined as the strains with a T_m value shift compared to that of the wild type (WT), detected by using *H. influenza* Rd.
[b] SPFX, sparfloxacin; GTFX, gatifloxacin; MXFX, moxifloxacin; GRNX, garenoxacin; STFX, sitafloxacin.

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