Melting curve analysis for rapid detection of topoisomerase gene mutations in *Haemophilus influenzae*

Running title: Melting curve assay of quinolone-resistant *H. 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)]

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

[*]**Corresponding author:** Katsunori Yanagihara

Department of Laboratory Medicine, Nagasaki University School of Medicine

1–7–1 Sakamoto, Nagasaki 852-8501, Japan

Tel: +81 – 95 – 819 – 7276; Fax: +81 – 95 – 849 – 7285

E-mail: k-yanagi@nagasaki-u.ac.jp

Abstract

We combined a real time PCR assay with melting curve analysis to rapidly genotyping 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 the early detection of preexisting QRDR mutations.

H. 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*, which encode subunits of DNA gyrase and topoisomerase IV, respectively (9, 11, 16, 18, 23). Real-time PCR methods combined with melting curve analysis (PCR-MCA) are 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 *H. influenzae* strains, targeting a total of four QRDR positions in the *gyrA* (codons 84 and 88) and *the parC* (codons 84 and 88) that are frequently associated with FQ

resistance (9, 11, 16, 23). This current method could identify simultaneously both the *gyrA* and the *parC* mutations by 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 were: 1 strain (NUH-1) from Nagasaki University Hospital, 1 strain (BY-1) from Bayer (Osaka, Japan), 2 strains (DR-1, DS-2) from Daiichi-Sankyo (Tokyo, Japan), and 3 strains (MSC24060, MSC27995, MSC11438) kindly provided by Meiji-Seika Kaisha (Tokyo, Japan) (21). The 10 FQ-susceptible strains were isolated 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 requirement. The minimum inhibitory concentrations (MICs) of ciprofloxacin (CPFX), sparfloxacin (SPFX), levofloxacin (LVFX), gatifloxacin (GTFX), moxifloxacin (MXFX), garenoxacin (GRNX), and sitafloxacin (STFX) were determined by a broth dilution method using *Haemophilus* test medium according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (5). *H. influenzae* ATCC51907 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 1A and 1B. 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 of Vila *et al.* (26).

Real-time PCR methods combined with melting curve analysis (MCA-PCR) was performed in 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 to 90°C at a rate of 0.2°C/s. The PCR-MCA assay was performed using LightCycler 480 Basic software (Roche Diagnostics, Basel, Switzerland). The total assay time was approximately 1 h. The QRDR DNA sequencing results 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, all of the mutant strains showed characteristic melting peaks with a distinct T_m value, as shown in Figure 1. The minimum T_m shifts for mutant strains compared to 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 assay correctly detected seven LVFX low susceptibility/resistant strains, as determined by a comparison with sequencing results (Table 2). From sequencing results, all seven FQ low susceptibility/resistant strains had at least two single amino acid substitutions at four QRDR positions (Table 3). All LVFX-susceptible *H. influenzae* strains had the same T_m value as the wild-type strain, and sequencing results confirmed that these were in fact wild-type strains (data not shown).

We compared the ability of the present PCR-MCA assay to detect FQ susceptibility in seventeen *H. influenzae* strains with that of the conventional phenotypic method. All LVFX-susceptible strains which had no mutation in condons 84 and 88 of *gyrA* and *parC* classified susceptible according to CLSI criteria (6) (data not shown). As shown in Table 3, the mutation profiles for the QRDRs in the *gyrA* and *parC* revealed a close relationship between the MIC level and the number of QRDR mutations. Previous studied 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 (7). Several reports have noted that a significant number of *Streptococcus pneumoniae* isolates already have a single step mutation, and prone to acquiring second step mutation (19). Unfortunately, our study lacks a collection of single-mutant strains. It was reported that the selection window for ciprofloxacin with wild-type cells was below serum drug concentrations in human volunteers administered twice-daily of 500mg (10). But Odoul *et al.* reported the median area under the inhibitory curve was decreased about half the proposed target value for ciprofloxacin in the cystic fibrosis patient receiving 15 mg/kg twice a day regimen of oral ciprofloxacin (15). Furthermore Pérez-Vázquez *et al.* reported that hypermutability is a risk condition for the development of fluoroquinolone-resistant *H. influenzae* (17) and Li *et al*. also reported the stepwise selection of ciprofloxacin-resistant *H. influenzae* mutants and could be the high level resistant strain (12). Increasing the time that drug concentration fall into the mutant selection window causes the mutation (8). Actually, the previously reported strains that failed treatment had a double mutant in the *gyrA* and the *parC* (2). 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. In addition, we should consider the FQ dosage carefully to avoid the low FQ concentration when we treat the patients who have chronic lung disease.

In conclusion, the PCR-MCA assay 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*, and the number of FQ-resistant strain may arise further along with the recent increase of FQ prescription. Although additional studies are need, we anticipate that this PCR-MCA assay 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 assay can recognize the *gyrA* and the *parC* mutants more clearly, easily and rapidly than sequencing.

References

1. Barriere, S. L., and J. Hindler. 1993. Ciprofloxacin resistant *Haemophilus influenzae* in a patient with chronic lung disease. Ann. Pharmacother. **27**:309-310.
2. Bastida, T., M. Perez-Vazquez, J. Campos, Cortés-Lletget MC, Román F, Tubau F, de la Campa AG, Alonso-Tarrés C. Levofloxacin treatment failure in *Haemophilus influenzae* pneumonia. Emerg. Infect. Dis. 2003; **9**: 1475-1478.
3. Biedenbach, D. J., and R. N. Jones. 2003. Five-year analysis of *Haemophilus influenzae* isolates with reduced susceptibility to fluoroquinolones: prevalence results from the SENTRY antimicrobial surveillance program. Diagn. Microbiol. Infect. Dis. **46**:55-61.
4. Campos, J., F. Román, M. Georgiou, C. García, R. Gómez-Lus, R. Cantón, H. Escobar, and F. Baquero. 1996. Long-term persistence of ciprofloxacin-resistant *Haemophilus influenzae* in patients with cystic fibrosis. J. Infect. Dis. **174**:1345-1347.
5. Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M07-A7, 7th ed. Clinical and Laboratory Standards Institute, Wayne, PA.

6. Clinical and Laboratory Standards Institute. 2007. Performance standards for antimicrobial susceptibility testing, 17th ed. Approved standard M100-S17. CLSI, Wayne, PA.

7. Fukushima, K.Y., Y. Hirakata, K. Sugahara, Yanagihara K, Kondo A, Kohno S, Kamihira S. 2006. Rapid screening of topoisomerase gene mutations by a novel melting curve analysis method for early warning of fluoroquinolone-resistant Streptococcus pneumoniae emergence. J. Clin. Microbiol. **44**: 4553-4558.

8. Drlica, K. 2003. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother. **52**:11-7.

9. Georgiou, M., R. Munoz, F. Roman, R. Canton, R. Gomez-Lus, J. Campos, and A. G. De La Campa. 1996. Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of GyrA and ParC. Antimicrob. Agents Chemother. **40**:1741-1744.

10. Gonzalez, M.A., AH. Moranchel, S. Duran, A. Pichardo, JL. Magana, B. Painter, A. Forrest, GL. Drusano. 1985. Multiple-dose pharmacokinetics of ciprofloxacin administered intravenously to normal volunteers. Antimicrob Agents Chemother. **28**:235-9.

11. Li, X., N. Mariano, J. J. Rahal, C. M. Urban, and K. Drlica. 2004. Quinolone-resistant *Haemophilus influenzae* in a long-term-care facility: nucleotide sequence characterization of alterations in the genes encoding DNA gyrase and DNA topoisomerase IV. Antimicrob. Agents Chemother. **48**:3570-3572.

12. Li, X., N. Mariano, J. J. Rahal, C. M. Urban, and K. Drlica. 2004. Quinolone-resistant *Haemophilus influenzae*: determination of mutant selection window for ciprofloxacin, garenoxacin, levofloxacin, and moxifloxacin. Antimicrob. Agents Chemother. 2004; **48**:4460-4462.

13. Motoshima, M., K. Yanagihara, K. Fukushima, Matsuda J, Sugahara K, Hirakata Y, Yamada Y, Kohno S, Kamihira S. 2007. Rapid and accurate detection of *Pseudomonas aeruginosa* by real-time polymerase chain reaction with melting curve analysis targeting gyrB gene. Diagn. Microbiol. Infect. Dis. **58**:53-58.

14. Nazir, J., C. Urban, N. Mariano, J. Burns, B. Tommasulo, C. Rosenberg, S. Segal-Maurer, and J. J. Rahal. 2004. Quinolone-resistant *Haemophilus influenzae* in a long-term care facility: clinical and molecular epidemiology. Clin. Infect. Dis. **38**:1564-1569.

15. Odoul, F., LC. Guellec, C. Giraut, DC. Gialluly, S. Marchand, G. Paintaud, MC. Saux, JC. Rolland, E. Autret-Leca. 2001. Ciprofloxacin pharmacokinetics in young cystic fibrosis patients after repeated oral doses. Therapie. **56**:519-24.

16. Pérez-Vázquez, M., F. Román, B. Aracil, R. Cantón, and J. Campos. 2004. Laboratory detection of *Haemophilus influenzae* with decreased susceptibility to nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin due to *gyrA* and *parC* mutations. J. Clin. Microbiol. **42**:1185-1191.

17. Pérez-Vázquez, M., F. Román, S. García-Cobos, and J. Campos. 2007. Fluoroquinolone resistance in *Haemophilus influenzae* is associated with hypermutability. Antimicrob. Agents Chemother. **51**:1566-1569.

18. Pérez-Vázquez, M., F. Román, M. C. Varela, R. Cantón, and J. Campos. 2003. Activities of thirteen quinolones by three susceptibility testing methods against a collection of *Haemophilus influenzae* isolates with different levels of susceptibility to ciprofloxacin: evidence for cross-resistance. J. Antimicrob. Chemother. **51**:147-151.

19. 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.

20. Rodríguez-Martínez, J. M., L. López, I. García, and A. Pascual. 2006. Characterization of a clinical isolate of *Haemophilus influenzae* with a high level of fluoroquinolone resistance. J. Antimicrob. Chemother. **57**:577-578.

21. Sanbongi, Y., T. Suzuki, Y. Osaki, N. Senju, T. Ida, and K. Ubukata. 2006. Molecular evolution of beta-lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. Antimicrob. Agents Chemother. **50**(7):2487-2492.

22. Yokota, S., Y. Ohkoshi, K. Sato, and N. Fujii. 2008. Emergence of fluoroquinolone-resistant *Haemophilus influenzae* strains among elderly patients but not among children. J. Clin. Microbiol. **46**:361-365.

23. Yoshizumi, S., Y. Takahashi, Y. Watanabe, E. Okezaki, Y. Ishii, and K. Tateda. 2004. In vitro antibacterial activities of new fluoroquinolones against clinical isolates of *Haemophilus influenzae* with ciprofloxacin-resistance-associated alterations in GyrA and ParC. Chemotherapy. **50**:265-275.

24. 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.

25. Vernel-Pauillac, F., V. Falcot, D. Whiley, and F. Merien. 2006. Rapid detection of a chromosomally mediated penicillin resistance-associated *ponA* mutation in *Neisseria gonorrhoeae* using a real-time PCR assay. FEMS Microbiol. Lett. **255**:66-74.

26. Vila, J., J. Ruiz, F. Sánchez, F. Navarro, B. Mirelis, M. T. Jiménez de Anta, and G. Prats. 1999. Increase in quinolone resistance in *Haemophilus influenzae* strain isolated from a patient with recurrent respiratory infections treated with ofloxacin. Antimicrob. Agents Chemother. **43**:161-162.

Figure legend

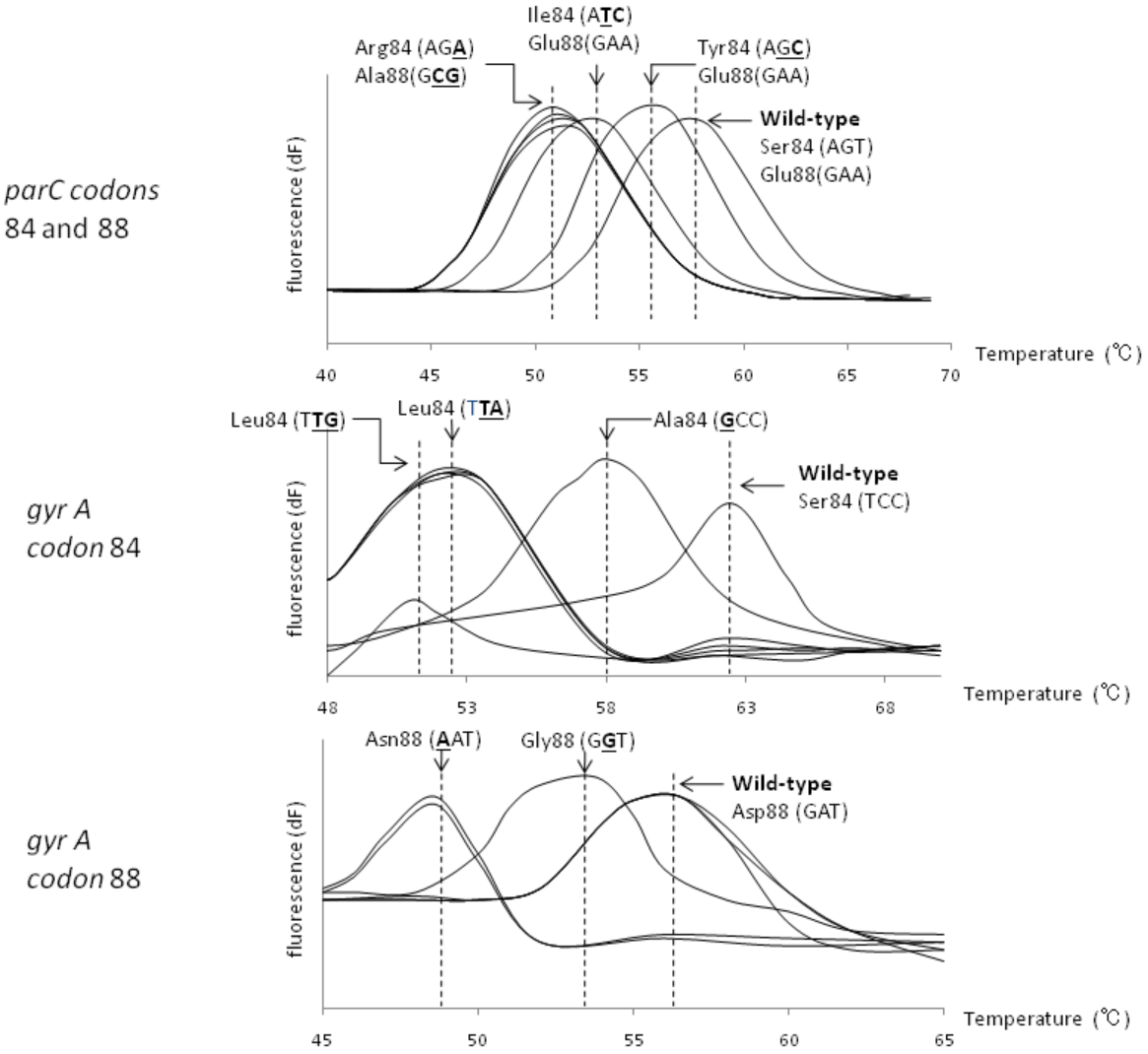

Figure 1. Melting peak patterns for QRDR mutants with substitutions in *parC* and *gyrA*. Melting curve analysis was performed with the 175-bp amplicon of the *parC* gene and the 197-bp amplicon of the *gyrA* gene. Panels A, B and C show melting peak patterns for codons 84 and 88 of the *parC* gene, 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.

Target gene	Primer	Sequence	Position (nt)	Amplicon Size (bp)
GyrA	Forward Reverse	5'-TGGATCGCGAAGGCAATAC-3' 5'-TGGCGCATCACCATCAAT-3'	158-176 354-337	197
ParC	Forward Reverse	5'-GTATTGTATATGCGATGTCTGAAC-3' 5'-CCATCTACAAGTGGATAACGA-3'	143-166 317-297	175

Table 1A.

Characteristics of specific primers used for sequencing *gyrA* and *parC*

Target *AA	Probe	Sequence
codon 84 of *GyrA*	Anchor	5'AGCGCGTGTTGTGGGTGATGTAATCGGTAAATATCAC3'**-FITC**
	Sensor	5'-**LCRed610**-CGCATGGTGACTCCGC-3'-P
codon 88 of *GyrA*	Anchor	5'CCATCAACCAACATATAGCGAAGTGAGAAGGGTTGT3'**-FITC**
	Sensor	5'-**Cy5**-CCATACGAACGATGGTATCGTACA-3'-P
codon 88&84 of *ParC*	Anchor	5'TCGGTGATGTACTCGGTAAATTCCATCCACATGGT3'**-FITC**
	Sensor	5'-**LCRed610**-ACAGTGCTTGTTATGAAGCTAT-3'-P

Table 1B.

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

Strain	MIC to LVFX (μg/ml)	nucleotide[c]/amino acid at position						
		parC 84		*parC* 88	*gyrA* 84		*gyrA* 88	
		T_m(°C)	Sq	Sq	T_m(°C)	Sq	T_m(°C)	Sq
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

Table 2.

		Amino acid change		*MIC of quinolones* (μg/ml)						
Strain	***MCA***	***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**

•MT (mutant) was defined as the strains with a T_m shift compared to wild-type.

** WT (wild-type; *H. influenzae Rd*)

Table 3.

The relationship between fluoroquinolones susceptibility, amino acid changes in gyrA and parC, and the results of PCR-MCA analysis.