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Rapid Screening of Topoisomerase Gene Mutations using a Novel Melting Curve Analysis for Early-Warning of Fluoroquinolone-Reistant *Streptococcus pnuemoniae*

Running title-

Genetic screening of quinolone-resistant *S. pneumoniae*

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Abstract

We developed a real-time PCR assay combined with melting curve analysis for rapidly genotyping quinolone-resistant 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 of detecting pre-existent single QRDR mutations.

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia (CAP) and is also responsible for substantial morbidity and mortality worldwide. Anti-pneumococcal fluoroquinolones (FQs), such as levofloxacin, gatifloxacin, gemifloxacin, and moxifloxacin, have greater activity against *S. pneumoniae*, and several are now approved for empirical treatment of CAP[2, 18]. However, recent reports have already noted an increase in the prevalence of FQ-resistant pneumococci[4, 11, 27].

FQ resistance in *S. pneumoniae* often involves mutation in the quinolone resistance-determining regions (QRDR) of the *parC* gene and the *gyrA* gene, which encode subunits of topoisomerase IV and DNA gyrase[9, 14, 15, 23]. 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 QRDR stepwise-mutations[7, 10, 24]. Rapid detection of QRDR mutations is required and it may constitute a more reliable approach than the current phenotypic method, which can represent susceptibility but cannot detect the potential of *S. pneumoniae* to harbor resistance. PCR-based techniques, such as PCR-restriction fragment length polymorphism[1, 16, 20], single-strand comformational polymorphism[16], and PCR-oligonucleotide ligation[3] 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 method 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[13, 25, 26].

The aim of the present study was to develop and to validate a rapid single-step PCR-MCA assay for genotyping *S. pneumoniae* strains, which 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[4, 8, 21].

Seventy-two *S. pneumoniae* clinical isolates were used; they consisted of 22 levofloxacin -resistant strains with minimum inhibitory concentration (MIC) of ≥ 8 μg/ml and 50 LVX-susceptible strains with MIC of ≤ 2 μg/ml[6]. Twenty-two resistant strains were collected from Nagasaki University Hospital (7), Sapporo Medical University School of Medicine (5)[28, 29], and the Levofloxacin Surveillance Group (10)[27] in Japan. Fifty susceptible strains were isolated at Nagasaki University Hospital. Identification of *S. pneumoniae* was confirmed by optochin susceptibility[12] and by the autolysine gene[22]. The MICs of ciprofloxacin (CIP), levofloxacin (LVX), gatifloxacin (GAT), and moxifloxacin (MXF) were determined by a broth dilution method[5, 6]. *S. pneumoniae* ATCC 49619 was used for quality control.

DNA was extracted from each strain using a QIA amp® DNA Mini kit (QIAGEN, Hilden, Germany). All of the designed oligonucleotides are shown in Table 1. These refer to the known sequence of the *parC* and *gyrA* genes, which were derived from GenBank accession no. AF170996 and no. 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), 3mM $MgCl_2$, 0.2 μM each probes, and 0.5 μM each primers. Thermal cycling was performed with an initial hold for 10 min at 95ºC, followed by 35 cycles of 5 sec at 95ºC, 9 sec at 55ºC and 12 sec at 72ºC. Melting curve was generated by cooling to 35ºC for 10 sec, followed by heating to 70ºC at a rate of 0.2ºC/s. The PCR-MCA assay was performed using LightCycler® analysis software 3.5 (Roche Diagnostics, Basel, Switzerland). The total assay time was approximately 1 hr. PCR amplification products of 72 strains were all directly sequenced at the nucleotide level using the BigDye® Terminator ver.3.1 Sequencing Standard Kit and an ABI PRISM^{TM} 310 Genetic Analyzer (both by Applied Biosystems, CA, USA). The QRDR DNA sequencing results were compared with the 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.

MCA for LVX-resistant S. pneumoniae and sequence analysis of QRDRs.

From sequencing results, all of the 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 for genotyping QRDRs, ATCC49619 was selected as the wild-type strain. For the mutant-control strains, DR22 ($TTT_{237-239}$;Ser79Phe of *parC* and $GGA_{255-257}$;Gly85Glu of *gyrA*), L007 ($TAT_{237-239}$;Ser79Tyr of *parC*), SR68

($AGA_{237\text{-}239}$;Ser79Arg of *parC* and $TTC_{243\text{-}245}$;Ser81Phe of *gyrA*), SR27 ($TAT_{249\text{-}251}$;Asp83Tyr of *parC*), SR248 ($AAT_{249\text{-}251}$;Asp83Asn of *parC* and $GCC_{243\text{-}245}$;Ser81Ala of *gyrA*), L001 ($TAC_{243\text{-}245}$;Ser81Tyr of *gyrA*), and L003 ($AAA_{255\text{-}257}$;Gly85Lys of *gyrA*) were chosen. Using probes specific for the wild-type strain, all of control strains showed characteristic melting peaks with a distinct T_m value corresponding to each mutant, as shown in Fig 1. The MCA at *parC* codon 79 produced four different peaks, T_m values of: $TCT_{237\text{-}239}$ wild-type, 60.4ºC; $TTT_{237\text{-}239}$ mutant, 50.0ºC; $TAT_{237\text{-}239}$ mutant, 49.3ºC; and $AGA_{237\text{-}239}$ mutant, 36.8ºC (Figure 1-A). Similarly, the MCA at *parC* codon 83 produced three different peaks (Figure 1-B), four different peaks at *gyrA* codon 81 (Figure 1-C), and three different peaks at *gyrA* codon 85 (Figure 1-D). The minimum T_m shift for mutant from wild-type strains was 10.0ºC at *parC* codon 79, 7.7ºC at *parC* codon 83, 4.5ºC at *gyrA* codon 81, and 6.2ºC at *gyrA* codon 85, with an acceptable T_m reproducibility of <0.8% CV (MCA was performed 5 times for each strain). The difference in T_m value between $TTT_{237\text{-}239}$ (Ser79Phe) and $TAT_{237\text{-}239}$ (Ser79Tyr) of the *parC* codon79 (Figure1-A) and between $TAT_{249\text{-}251}$ (Asp83Tyr) and $AAT_{249\text{-}251}$ (Asp83Asn) of the *parC* codon83 (Figure 1-B) was both very similar, thus these mutations are impossible to differentiate but can be detected as being present. The PCR-MCA assay correctly genotyped 22 LVX-resistant strains, compared with sequencing results (Table 2).

MCA of LVX-susceptible S. pneumoniae 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_ms of the 47 strains were 60.4ºC (CV: 0.41%) at *parC* codon79, 56.7ºC (CV: 0.52%) at *parC* codon83, 56.5ºC (CV: 0.87%) at *gyrA* codon81, and 56.7ºC (CV: 0.34%) at *gyrA* codon85, and sequencing results comfirmed that these are in fact wild-type strains. Two of the remaining three strains had T_ms of 49.8ºC and 50.0ºC, which were lower than that (60.4ºC) of the wild-type strain at *parC* codon79, while the other strain had a T_m of 48.8ºC, which differed from the wild-type strain (56.7ºC) at *parC* codon83. Indeed, direct sequencing revealed that the former two strains had a $TTT_{237\text{-}239}$ (Ser79Phe) mutation, while the latter strain had a $AAT_{249\text{-}251}$ (Asp83Asn) mutation in the *parC* gene.

Comparison of the 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 conventional phenotypic method, as shown in Figure 2 and Table 3. All of the 47 LVX-susceptible strains with no mutations had susceptibility MICs of ≤ 2μg/ml, while 22 of the remaining 25 mutation-containing strains (88%) had resistance MICs of ≥ 8 μg/ml (Figure 2-B, Table 3-B). Interestingly, the mutation profiles of the QRDRs in the *parC* and *gyrA* genes revealed a close relationship between MIC level and the number of QRDR mutations. We observed that 6 out of the 9 strains with a single QRDR mutation

were highly resistant to LVX (Figure 2-B, Table 3-B). This can be explained by the fact that 3 out of the 6 strains harbored additional *parE* mutation and an active efflux, 2 out of the 6 strains harbored additional *parE* mutation, and the remaining strain harbored additional *gyrB* mutation (data not shown). Compared with the conventional phenotypic method, the diagnostic sensitivity and specificity of the PCR-MCA assay to detect FQ non-susceptible strains was 100% (25/25) and 100% (47/47) for CIP, 100% (22/22) and 94% (47/50) for LVX, 100% (21/21) and 92% (47/51) for GAT, and 100% (15/15) and 82% (47/57) for MXF. Assuming QRDR mutation provides a positive diagnosis of the presence of FQ-nonsusceptible *S. pneumoniae*, this assay is particularly useful because it has a negative predictive value of 100% for all four FQs, and a positive predictive value of 100% for CIP, LVX, and GAT, and 93.7% for MXF (Figure2, 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 which already have a single mutation but are still considered fully susceptible[8, 21]. Although our study lacks a collection of moderately FQ-resistant (LVX MIC of 2μg/ml or 4μg/ml) *S. pneumoniae*, Lim et al.[17] have reported that about 60% of *S. pneumoniae* isolates with a LVX MIC of 2 μg/ml carry pre-existent *parC* mutations. Typically or generally, stepwise mutation starts with the *parC* gene, which frequently leads to secondary mutations in the *gyrA* gene,

eventually resulting in highly resistance to all FQs[19]. This has been also supported by clinical reports of a FQ-susceptible *S. pneumoniae* strain carrying a first-step mutation that evolved into a second-step QRDR mutation during FQ treatment, resulting in treatment failure[7]. We emphasize the clinical importance of the detection of first-step QRDR mutations either in *gyrA* or *parC* when 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 of the sensor probe would be undetectable. Resistance as a result of other mechanisms (such as *parE* and *gyrB* mutation, and efflux) can also not be detected. However, these mechanisms provide resistance potential but are not conclusive indicators of highly resistance to FQ compared with *parC* and *gyrA* mutation [8, 21].

In conclusion, the PCR-MCA assay was easily and quickly performed and had an accuracy at least as satisfactory as that of the conventional phenotypic method. Moreover, single QRDR mutations which harbored potential of 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 to achieve rapid screening of QRDR mutations and as an early warning system for the emergence of FQ-resistant *S. pneumoniae*.

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

Target gene	Primer	Sequence	Position (nt)	Amplicon Size (bp)
parC	Forward	5'-GTTCAACGCCGTATTCTT-3'	138-155	246
	Reverse	5'-TGCCTCAGTATAACGCATAG-3'	364-383	
gyrA	Forward	5'-GAATGAATTGGGTGTGAC-3'	282-299	225
	Reverse	5'-ATACGTGCCTCGGTATAA-3'	489-506	

Target *AA	Probe	Sequence	Position (nt)
codon 79 of parC	Anchor	5'-GTCGGCCAAGTCAGTCGGGAACATCATGGGGAATTTCCACCC-3'- FITC	203-244
	Sensor	5'- LCRed640-CACGGGGATTCTTCTATC-3'- P	246-264
codon 83 of parC	Anchor	5'-TATCTATGATGCCATGGT-3'-FITC	260-277
	Sensor	5'-LCRed640-CGTATGTCACAGAACTGGAAAAATCGTGAGATTCTAGTTGAAATGCACGG-3'-P	279-328
codon 81 of gyrA	Anchor	5'-TAAATAGAGGAATCCCC-3'-FITC	367- 383
	Sensor	5'-LCRed640-TGTGGGTGGTATTTACCCATGACATCCCCTGTA ATACGAGCAGATT-3'-P	320- 365
codon 85 of gyrA	Anchor	5'-TCTATTTATGAAGCCATG-3'-FITC	376- 394
	Sensor	5'-LCRed640-CCGTATGGCTCAATGGTGGAGCTACCGTTACATGCTTGTAGATGGTCATG-3'-P	396- 445

.Abbreviations: *AA: amino acid, 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.

Table 2. Melting peak (*Tm*) and direct DNA sequencing results (Sq) of four QRDR positions in 22 LVX-resistant *S. pneumoniae* strains

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

a Strains N001-N007 were isolated at Nagasaki University Hospital, and L001-L010 were strains supplied from Levofloxacin Surveillance Group.
b Abbreviations: LVX, levofloxacin. c changed nucletides are in boldface. d strains source from reference 21), supplied from Sapporo Medical University.
e strains source from reference 22) supplied from Sapporo Medical University. f WT, identical to the nucleotide distribution of the wild-type, ATCC49619. g MC, mutant control

Table 3. Relationship between QRDR mutation detected by MCA and MICs of FQs in 72 *S. pneumoniae* strains

A

CIP

	MIC		
QRDR mutation	S	I	R
none	47	0	0
single *parC* or *gyrA*	0	3	6
both *parC* and *gyrA*	0	0	16

B

LVX

	MIC		
QRDR mutation	S	I	R
none	47	0	0
single *parC* or *gyrA*	3	0	6
both *parC* and *gyrA*	0	0	16

C

GAT

	MIC		
QRDR mutation	S	I	R
none	47	0	0
single *parC* or *gyrA*	4	4	1
both *parC* and *gyrA*	0	0	16

D

MXF

	MIC		
QRDR mutation	S	I	R
none	47	0	0
single *parC* or *gyrA*	9	0	0
both *parC* and *gyrA*	1	8	7

A, **B**, **C**, and **D** showed results with ciprofloxacin (CIP), levofloxacin (LVX), gatifloxacin (GAT), and moxifloxacin (MXF), respectively. Each number in tables represents number of strains. Abbreviations: S; susceptible, I; intermediate, and R; resistant. The CLSI MIC breakpoints are as follows: LVX (susceptible $\leq$2μg/ml, intermediate 4μg/ml, and resistant $\geq$8μg/ml), GAT (susceptible $\leq$1μg/ml, intermediate 2μg/ml, and resistant $\geq$4μg/ml), and MXF (susceptible $\leq$1μg/ml, intermediate 2μg/ml, and resistant $\geq$4μg/ml). The breakpoint standard for CIP was obtained from interpretive guideline supplied by the Japanese Society of Chemotherapy (susceptible $\leq$1μg/ml, intermediate 2μg/ml, and resistant $\geq$4μg/ml).

Figure legends

Figure 1. Melting peak patterns of QRDR mutations in *parC* and *gyrA*.

Melting curve analysis of the 246-bp amplicon of the *parC* gene, and 225-bp amplicon of the *gyrA* gene obtained with real-time PCR for wild-type and mutant-control strains. **A**, **B**, **C**, and **D** showed melting peak patterns at codon 79 of the *parC* gene, codon 83 at the *parC* gene, codon 81 of the *gyrA* gene, and codon 85 of the *gyrA* gene, respectively.

Values of the *y* axis represent the ration of the first negative derivative of the change in fluorescence (dF) with the variation in temperature. A point mismatch in QRDR resulted in a lower T_ms of mutant strains relative to wild-type strain.

Figure2. Comparison of outcomes between conventional FQ susceptibility testing and the PCR-MCA assay for genotyping QRDR mutations.

A, **B**, **C**, and **D** showed results with ciprofloxacin (CIP), levofloxacin (LVX), gatifloxacin (GAT), and moxifloxacin (MXF), respectively.

Vertical white square represent wild-type, and black square represent mutant type of the four position of QRDR in *parC* gene and *gyrA* gene results from the PCR-MCA assay. White circle represent wild-type strains and black circle represent mutant containing strains. Horizontal axis represents MICs of each strains. Dark mesh area represent resistant, and light mesh area represent intermediate susceptibility to each FQ. The CLSI MIC breakpoints[26] are as follows: LVX (susceptible ≤2μg/ml, intermediate 4μg/ml, and resistant ≥8μg/ml), GAT (susceptible ≤1μg/ml, intermediate 2μg/ml, and resistant ≥4μg/ml), and MXF (susceptible ≤1μg/ml, intermediate 2μg/ml, and resistant ≥4μg/ml). The breakpoints standard for CIP was obtained from interpretive guideline supplied by the Japanese Society of Chemotherapy (susceptible ≤1μg/ml, intermediate 2μg/ml, and resistant ≥4μg/ml).

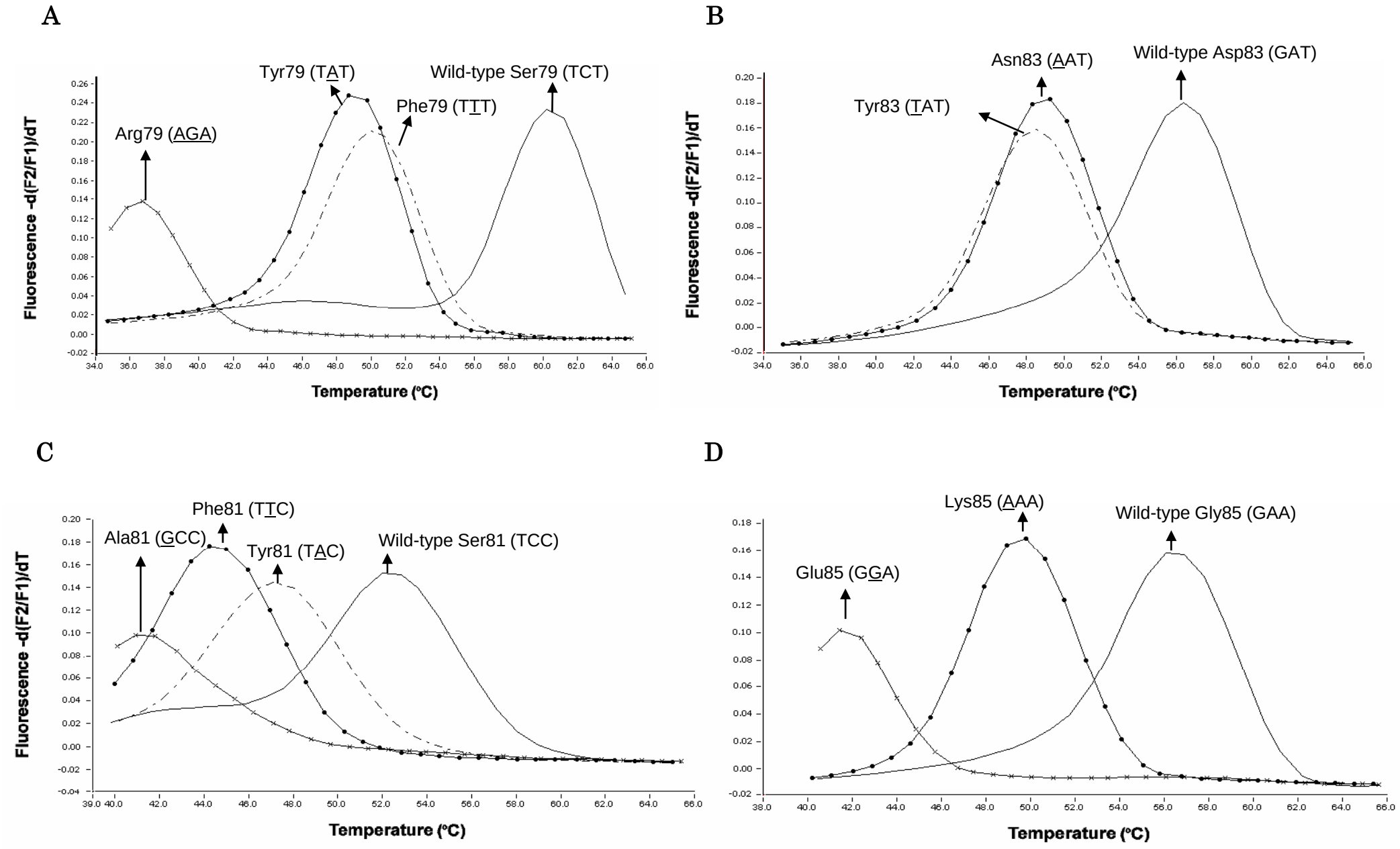
A
Arg79 (AGA)
Tyr79 (TAT)
Phe79 (TTT)
Wild-type Ser79 (TCT)
Fluorescence -d(F2/F1)/dT
Temperature (°C)
B
Tyr83 (TAT)
Asn83 (AAT)
Wild-type Asp83 (GAT)
Fluorescence -d(F2/F1)/dT
Temperature (°C)
C
Ala81 (GCC)
Phe81 (TTC)
Tyr81 (TAC)
Wild-type Ser81 (TCC)
Fluorescence -d(F2/F1)/dT
Temperature (°C)
D
Glu85 (GGA)
Lys85 (AAA)
Wild-type Gly85 (GAA)
Temperature (°C)

FIG. 1

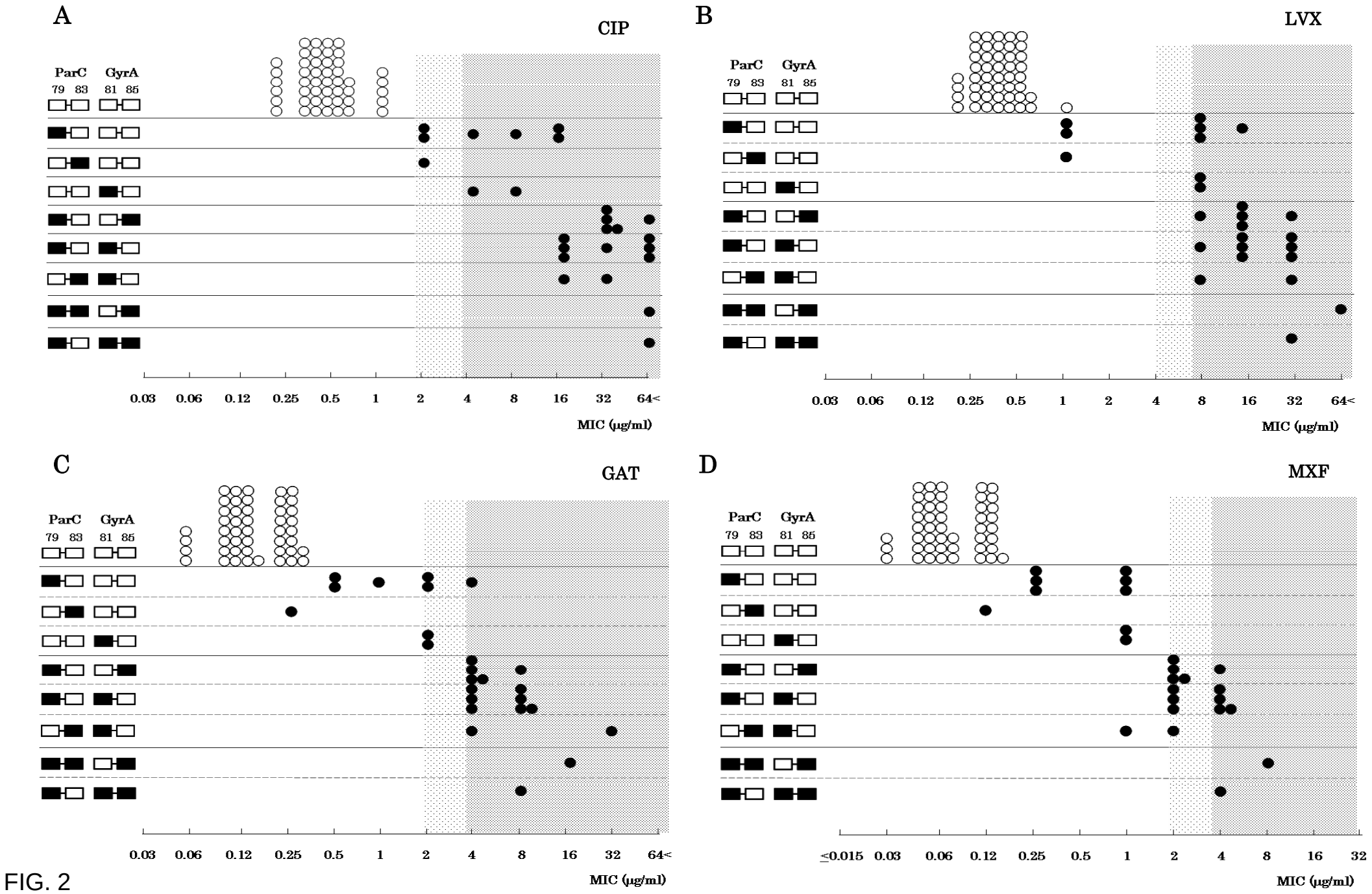
A
CIP
ParC
GyrA
79 83
81 85
0.03 0.06 0.12 0.25 0.5 1 2 4 8 16 32 64<
MIC (μg/ml)
B
LVX
ParC
GyrA
79 83
81 85
0.03 0.06 0.12 0.25 0.5 1 2 4 8 16 32 64<
MIC (μg/ml)
C
GAT
ParC
GyrA
79 83
81 85
0.03 0.06 0.12 0.25 0.5 1 2 4 8 16 32 64<
MIC (μg/ml)
D
MXF
ParC
GyrA
79 83
81 85
≤0.015 0.03 0.06 0.12 0.25 0.5 1 2 4 8 16 32
MIC (μg/ml)

FIG. 2