INTRODUCTION

Fluoroquinolones (FQs) are widely used to treat various bacterial infections [1, 2]. FQ resistance has increased globally in Acinetobacter species, which are clinically important pathogens that frequently cause infections among intensive care unit patients [2-5].

In A. baumannii, FQ resistance occurs mainly through muta-
tions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (gyrA) and topoisomerase IV (parC), although overexpression of efflux pumps can contribute to FQ resistance [6-10]. The most frequently described mutations in A. baumannii are within the QRDRs at Ser-83 in GyrA and at Ser-80 and Glu-84 in ParC [6-8, 11]. In particular, a double mutation, affecting the Ser-83 of GyrA and Ser-80 or Glu-84 of ParC, renders A. baumannii highly FQ resistant [7, 11]. The single mutation affecting Glu-87 of GyrA, an important mutation associated with FQ resistance in other gram-negative microorganisms [12], has rarely been found in A. baumannii [8]. Detection of these mutations is therefore important for assessing FQ resistance in A. baumannii and epidemiological studies of resistant strains.

Although DNA sequencing is a reliable technique for detecting mutations, it is costly, time-consuming, and laborious when analyzing numerous clinical strains. As an alternative, PCR-restriction fragment length polymorphism (RFLP) has been used to detect mutations associated with FQ resistance in A. baumannii [6, 7, 13]. However, this approach was insufficient for identifying significant mutations linked to high-level FQ resistance because it determined only the presence or absence of gyrA mutations at codon 83 or parC mutations at codon 80.

We have previously reported a mismatched PCR-RFLP assay for detecting gyrA and parC mutations associated with FQ resistance in Enterobacteriaceae [14]. We aimed to develop a mismatched PCR-RFLP assay to detect mutations in gyrA (codons 83 and 87) and in parC (codons 80 and 84) associated with FQ resistance in A. baumannii.

**METHODS**

**Bacterial strains**

We used 58 A. baumannii strains and 37 non-baumannii Acinetobacter strains, including some of the strains used in previous studies [15, 16]. These strains were collected between 2009 to 2018 and stocked in our laboratory. The 37 non-baumannii Acinetobacter strains included 10 A. nosocomialis, eight A. pittii, two A. calcoaceticus, two A. soli, two A. ursingii, two A. colistiniresistens, two A. johnsonii, two A. gerneri, one A. radiore sistens, one A. bereziniae, one A. towneri, one A. grimon ti, one A. junii, one A. tandoii, and one A. haemolyticus strain. These strains were identified by DNA polymerase β-subunit gene sequence analysis [17]. All strains were used to confirm the practicality of the mismatched PCR-RFLP assay. A. baumannii strain ATCC 19606 was used for comparison with the A. baumannii strains. According to the ethical guidelines for epidemiological studies released by the Ministry of Health, Labour, and Welfare in Japan [18], ethical approval and written or verbal informed consent are not required for this type of study.

**Antimicrobial susceptibility testing**

Susceptibility was tested using an agar dilution assay according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. The minimum inhibitory concentrations (MICs) of levofloxacin and ciprofloxacin were determined. To examine the effect of efflux pumps in FQ resistance, MICs were also measured in the presence of the efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP), which was incorporated into the Mueller–Hinton agar at a concentration of 12.5 μM [20].

**Amplification and DNA sequencing of the gyrA and parC QRDRs**

The sequence of A. baumannii strain ATCC 19606 was used as the reference susceptible strain. The gyrA and parC QRDRs from 58 A. baumannii strains were amplified using PCR and sequenced using Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA), as previously described [21]. The results were compared with those of the mismatched PCR-RFLP.

**Development of the mismatched PCR-RFLP assay**

Based on the conserved sequences of A. baumannii gyrA and parC, we designed two sets of mismatched PCR-RFLP primers to detect mutations in gyrA (codons 83 and 87) and parC (codons 80 and 84) by introducing an artificial restriction enzyme cleavage site into the PCR products, as described previously [22]. The reverse primers for gyrA and parC are located immediately downstream of the nucleotide sequences, corresponding to GyrA87 and ParC84, with mismatched nucleotides to create recognition site (XmnI), respectively (Fig. 1). The primer sequences and PCR conditions were expected to yield 143 and 120 bp DNA fragments for gyrA and parC, respectively. We performed PCR amplification of gyrA or parC from each strain using the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions and as described in Table 1. The gyrA and parC PCR products were digested with HinfI or XmnI at 37°C for one hour, and the digested products were analyzed by electrophoresis using 3.0% MetaPhor Agarose gels (Takara, Shiga, Japan).
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**RESULTS**

Susceptibility testing and DNA sequencing of the *gyrA* and *parC* QRDRs

We analyzed 58 *A. baumannii* strains (47 FQ-resistant and 11 FQ-susceptible). Point mutations were present only in *gyrA* (codon 83) and *parC* (codons 80 and 84). The strains with no mutations (N=11) resulting in amino acid changes at Ser-83 and Glu-87 in *GyrA* and at Ser-80 and Glu-84 in *ParC* were susceptible to levofloxacin and ciprofloxacin. The FQ-resistant strains (N=47) carried two mutations, in *gyrA* (codon 83) and *parC* (codon 80 or 84), and all but one strain had ciprofloxacin MICs ≥32 μg/mL (Table 2). Of these 47 FQ-resistant strains, 45 possessed *gyrA* codon 83 TCA (Ser) → TTG (Leu) and *parC* codon 80 TCG (Ser) → TGG (Leu) mutations, and the remaining two possessed *gyrA* codon 83 TCA (Ser) → TTA (Leu) and *parC* codon 84 GAA (Glu) → AAA (Lys) mutations. CCCP did not appreciably affect the levofloxacin and ciprofloxacin MICs for the *A. baumannii* strains. The FQ-resistant strains were still classified as resistant based on the CLSI breakpoints for levofloxacin and ciprofloxacin, even in the presence of this efflux pump inhibitor. The mutations identified in *gyrA* and *parC* and the MICs of levofloxacin and ciprofloxacin are shown in Table 2.

Mismatched PCR-RFLP

We performed PCR-RFLP on 58 *A. baumannii* strains and *A. Table 1. Primer sequences and restriction enzymes for mismatched PCR-RFLP

| Target | Primer | Oligonucleotide sequence (5´ to 3´)*  | PCR conditions | Product size (bp) | Restriction enzyme (Recognition site)† | QRDR amino acid (codon)‡ |
|--------|--------|--------------------------------------|----------------|------------------|----------------------------------------|--------------------------|
| *gyrA* | Forward | GAGCTAGGCTTAAAAAGCAGTGG            | 95°C for five minutes and 35 cycles of 95°C for one minute, 48°C for one minute, and 72°C for 30 seconds | 120              | *Hinf* I (GANTC)                       | Ser80 in ParC (TGG)      |
|        | Reverse | TCTTGAGCCATACGAA                      |                |                  | *Xmn* I (GANNNTTC)                     | Glu84 in ParC (GAA)      |
| *parC* | Forward | GAGCTAGGCTTAAAAAGCAGTGG            | 95°C for five minutes and 35 cycles of 95°C for one minute, 48°C for one minute, and 72°C for 30 seconds | 143              | *Hinf* I (GANTC)                       | Ser83 in GyrA (TCA)      |
|        | Reverse | GAGCTAGGCTTAAAAAGCAGTGG            | 95°C for five minutes and 35 cycles of 95°C for one minute, 48°C for one minute, and 72°C for 30 seconds | 143              | *Xmn* I (GANNNTTC)                     | Glu87 in GyrA (GAA)      |

*Boldface represents mismatched nucleotides that introduce artificial restriction sites. Underlined nucleotides indicate restriction sites; †Underlined nucleotides correspond to QRDRs in the *gyrA* or *parC* genes; ‡Underlined nucleotides indicate restriction sites.Abbreviations: QRDR, quinolone resistance-determining region; RFLP, restriction fragment length polymorphism.

![Fig. 1. Strategy used for mismatched PCR-RFLP of *gyrA* and *parC* QRDRs in *A. baumannii*. The reverse primers for *gyrA* and *parC* are located immediately downstream of the nucleotide sequences corresponding to GyrA87 and ParC84, respectively. The reverse primer for *gyrA* was designed with one mismatched nucleotide to create an XmnI recognition site (GAAANNTTC) in the *gyrA* region containing the codon for Glu-87 (GAA). The reverse primer for *parC* was designed with two mismatched nucleotides to create an XmnI recognition site in the *parC* region containing the codon for Glu-84 (GAA). Boldface represents codons 83 and 87 of *gyrA* and codons 80 and 84 of *parC*. Underlined DNA sequences indicate restriction sites present in the QRDRs of FQ-susceptible strains.](https://doi.org/10.3343/alm.2020.40.1.27)
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*gyrA* and *parC* mutations were successfully obtained for all strains. The PCR products of *gyrA* contained both one natural *Hinf*I recognition site and one artificially created *Xmn*I recognition site. Similarly, the PCR products of *parC* contained both one natural *Hinf*I recognition site and one artificially created *Xmn*I recognition site (Fig. 1). Consequently, *Hinf*I and *Xmn*I digested the amplified 143 bp fragment to generate two fragments of 103 and 40 bp and of 122 and 21 bp, respectively. The 21 bp fragment, which was produced by *Xmn*I digestion, was not visible in Fig. 2; however, it was easy to recognize that the amplified 143 bp fragment was digested by *Xmn*I. Similarly, *Hinf*I and *Xmn*I digested the amplified 120 bp fragment to generate two fragments of 85 and 35 bp and of 104 and 16 bp, respectively. The 16 bp fragment, produced by *Xmn*I digestion,

### Table 2. Levofloxacin and ciprofloxacin MIC ranges, amino acid (codon) changes in GyrA and ParC QRDRs, and mismatched PCR-RFLP results for *A. baumannii* strains

| Strain (N) | MIC range (μg/mL) | Amino acid (codon) change at position* | Mismatched PCR-RFLP† |
|------------|-------------------|----------------------------------------|----------------------|
|            | Levofoxacin | Ciprofoxacin | GyrA | ParC | gyrA | parC |
| ATCC 19606 | 0.5 | 0.5 | TCA (Ser) | GAA (Glu) | TCG (Ser) | GAA (Glu) | + | + | + | + |
| (10) | 0.125–0.25 | 0.125 | – | – | – | – | + | + | + | + |
| (1) | 1 | 0.5 | – | – | TCG (Ser) | – | + | + | + | + |
| (2) | 8 | 32 | TTA (Leu) | – | – | AAA (Lys) | – | + | + | – |
| (45) | 8–128 | 16–256 | TTA (Leu) | – | TGG (Leu) | – | – | + | – | + |

*Compared with ATCC 19606. Underlining indicates point mutations; –, no change.
†+ indicates PCR products that were digested by the restriction enzyme; – indicates PCR products that were not digested by the restriction enzyme.

Abbreviations: QRDR, quinolone resistance-determining region; RFLP, restriction fragment length polymorphism; MIC, minimum inhibitory concentration.

### Fig. 2. PCR-RFLP patterns obtained following digestion with *Hinf*I or *Xmn*I for *gyrA* and *parC*, respectively. Lanes 1 to 3 and 4 to 6 show PCR-RFLP results for *gyrA* and *parC*, respectively. Lane: M, 20 bp DNA ladder marker. (A) PCR-RFLP results for *A. baumannii* ATCC19606. Lanes: 1, undigested (143 bp); 2, *Hinf*I-digestion (103 bp and 40 bp); 3, *Xmn*I-digestion (122 bp and 21 bp); 4, undigested (120 bp); 5, *Hinf*I-digestion (85 bp and 35 bp); and 6, *Xmn*I-digestion (104 bp and 16 bp). (B) PCR-RFLP results for the representative FQ-resistant *A. baumannii* strain possessing mutations in *gyrA* (83) and *parC* (80). Lanes: 1, undigested (143 bp); 2, *Hinf*I-digestion (143 bp); 3, *Xmn*I-digestion (122 bp and 21 bp); 4, undigested (120 bp); 5, *Hinf*I-digestion (120 bp); and 6, *Xmn*I-digestion (104 bp and 16 bp).

Abbreviations: FQ, fluoroquinolone; RFLP, restriction fragment length polymorphism.

*baumannii* strain ATCC 19606. Amplification products with the expected sizes of 143 bp for *gyrA* and 120 bp for *parC* were successfully obtained for all strains. The PCR products of *gyrA* from FQ-susceptible strains contained both one natural *Hinf*I recognition site and one artificially created *Xmn*I recognition site. Similarly, the PCR products of *parC* from FQ-susceptible strains contained both one natural *Hinf*I recognition site and one artificially created *Xmn*I recognition site (Fig. 1). Consequently, *Hinf*I and *Xmn*I digested the amplified 143 bp fragment to generate two fragments of 103 and 40 bp and of 122 and 21 bp, respectively. The 21 bp fragment, which was produced by *Xmn*I digestion, was not visible in Fig. 2; however, it was easy to recognize that the amplified 143 bp fragment was digested by *Xmn*I. Similarly, *Hinf*I and *Xmn*I digested the amplified 120 bp fragment to generate two fragments of 85 and 35 bp and of 104 and 16 bp, respectively. The 16 bp fragment, produced by *Xmn*I digestion,
was not visible in Fig. 2; however, it was easy to recognize that the amplified 120 bp fragment was digested by XmnI. *HinfI* and *XmnI* failed to digest the PCR products at the site containing the mutations that resulted in amino acid changes at Ser-83 in GyrA or at Ser-80 or Glu-84 in ParC.

Furthermore, we applied this assay to 37 non-A. *baumannii* strains. While *gyrA* amplicons were obtained for 28 strains, namely 10 *A. nosocomialis*, eight *A. pittii*, two *A. calcoaceticus*, two *A. ursingii*, two *A. gerneri*, two *A. johnsonii* strains, one *A. grimon- tii*, and one *A. tandoi*, no *parC* amplicons were obtained in any of the 37 strains tested.

**DISCUSSION**

We developed a mismatched PCR-RFLP assay to detect mutations in *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84), which are associated with FQ resistance in *A. baumannii*. This assay specifically detected significant mutations associated with reduced susceptibility to FQs in *A. baumannii* and accurately classified all the FQ-resistant and FQ-susceptible strains according to the MIC results.

The regions containing the mutation site resulting in amino acid change within the Ser-83 codon in GyrA or Ser 80 codon in ParC have a naturally occurring *HinfI* restriction site; thus, these regions are amplified by PCR, and the mutations at these positions are detected when the PCR products are not digested with *HinfI*, as analyzed by electrophoresis on agarose gels [6, 7]. However, the mutation sites within the Glu-87 codon in GyrA and the Glu-84 codon in ParC are not involved at any restriction cleavage site. To detect mutations in *gyrA* (codon 87) and *parC* (codon 84), we introduced base substitutions near the mutation sites to create *XmnI* cleavage sites using the primer-specified restriction site modification method [22]. Our results demonstrated that while *HinfI* and *XmnI* digested the *gyrA* and *parC* amplicons from FQ-susceptible strains, they did not digest those from FQ-resistant strains with mutations in *gyrA* and *parC*. The mutations detected by this assay were concordant with the DNA sequencing results shown in Table 2. The high specificity of the restriction enzyme *XmnI* to each set of the three nucleotides for codon 87 (Glu) of GyrA and codon 84 (Glu) of ParC in FQ-susceptible strains allows our assay to accurately detect mutations at codon 87 in *gyrA* and at codon 84 in *parC*. Therefore, our assay can identify significant mutations in *gyrA* and *parC* QRDRs linked to high-level FQ resistance in *A. baumannii* without the need for DNA sequencing; this assay may thus serve as an alternative to other PCR-RFLP assays that are limited by their ability to detect mutations at only codon 83 of *gyrA* or codon 80 of *parC* [6, 7].

Our assay accurately identified FQ-susceptible strains and FQ-resistant strains. When our assay did not detect mutations in *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84), the MICs showed susceptibility to FQs. On the other hand, when our assay detected mutations in both *gyrA* and *parC*, the MICs showed FQ resistance. In addition, our assay enables simultaneous analysis of many strains and provides results within four hrs. Thus, it could aid in the rapid identification of FQ-resistant *A. baumannii* strains in the clinical setting.

Previous studies have reported several rapid assays for detecting *gyrA* and *parC* mutations associated with FQ resistance in *A. baumannii* and determining FQ resistance, including PCR followed by electrospray ionization mass spectrometry and pyrosequencing assay [21, 23]. These assays successfully detected FQ resistance mutations in *gyrA* (codon 83) and *parC* (codons 80 and 84) and identified FQ-resistant strains. However, these assays require expensive equipment; our assay does not.

Another important and advantageous finding was that the mismatched PCR-RFLP primers for *parC* amplified *parC* from *A. baumannii* strains, whereas these primers did not amplify *parC* from any non-*baumannii* Acinetobacter strains. This assay may therefore differentiate *A. baumannii* from other Acinetobacter species in bacterial colonies, and determine FQ resistance for *A. baumannii* without the need for precise species identification within the genus Acinetobacter. This could also be an advantage of our assay. However, further studies are needed to confirm this finding.

The limitation of our assay is that it is unable to detect mutations at other locations of *gyrA* and *parC* or in other genes. Nevertheless, our data suggest this assay specifically amplifies *gyrA* and *parC* from *A. baumannii* and allows for simple, specific, rapid, and inexpensive detection of significant FQ resistance mutations. Thus, this assay may be useful for rapid assessment of FQ resistance in *A. baumannii* and for epidemiological studies of resistant strains in the clinical setting; moreover, it might be used to differentiate *A. baumannii* from other Acinetobacter species.

**Author Contributions**

The contributions of the authors are as follows: Research conception and design: Nakano R, Yano H. Data acquisition: Kakuta N, Nakano R, Nakano A, Suzuki Y, Tanouchi A, Masui T, Horiuchi S, Endo S, Kakuta R, Ono Y, Yano H. Data analysis and interpretation: Kakuta N, Nakano R, Nakano A, Suzuki Y, Tanou-
chi A, Masui T, Horiuchi S, Endo S, Kakuta R, Ono Y, Yano H. Manuscript writing (original draft): Kakuta N, Nakano R. Manuscript writing (review and editing): Nakano R, Yano H. All authors have accepted their responsibility for the entire content of this manuscript and approved submission.

Conflict of Interest

None declared.

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REFERENCES

1. Hooper DC. Clinical applications of quinolones. Biochim Biophys Acta 1998;1400:45-61.
2. Kim ES and Hooper DC. Clinical importance and epidemiology of quinolones. Biochim Biophys Acta 1998;1400:45-61.
3. Dalhoff A. Resistance surveillance studies: a multifaceted problem—the fluoroquinolone example. Infection 2012;40:239-62.
4. Gaynes R, Edwards JR, National Nosocomial Infections Surveillance System. Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis 2005;41:848-54.
5. Wispelghoff H, Bischoff T, Tal lent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 2004;39:309-17.
6. Vila J, Ruiz J, Goñi P, Marcos A, Jimenez de Anta T. Mutation in the gyrA gene of quinolone-resistant clinical isolates of Acinetobacter baumannii. Antimicrob Agents Chemother 1995;39:1201-3.
7. Vila J, Ruiz J, Goñi P, Jimenez de Anta T. Quinolone-resistance mutations in the topoisomerase IV parC gene of Acinetobacter baumannii. J Antimicrob Chemother 1997;39:757-62.
8. Wispelghoff H, Decker M, Haefs C, Kutz O, Plun G, Seifert H. Mutations in gyrA and parC associated with resistance to fluoroquinolones in epidemiologically defined clinical strains of Acinetobacter baumannii. J Antimicrob Chemother 2003;51:177-80.
9. Coyne S, Couvralin P, Périchon B. Efflux-mediated antibiotic resistance in Acinetobacter spp. Antimicrob Agents Chemother 2011;55:947-53.
10. Correa S, Poeta P, Hébraud M, Capelo JL, Igrejas G. Mechanisms of quinolone action and resistance: where do we stand? J Med Microbiol 2017;66:551-9.
11. Valentine SC, Contreras D, Tan S, Real LJ, Chu S, Xu HH. Phenotypic and molecular characterization of Acinetobacter baumannii clinical isolates from nosocomial outbreaks in Los Angeles County, California. J Clin Microbiol 2008;46:2499-507.
12. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003;51:1109-17.
13. Hamouda A and Amyes SG. Novel gyrA and parC point mutations in two strains of Acinetobacter baumannii resistant to ciprofloxacin. J Antimicrob Chemother 2004;54:695-6.
14. Nakano R, Okamoto R, Nakano A, Nagano N, Abe M, Tansho-Nakagawa S, et al. Rapid assay for detecting gyrA and parC mutations associated with fluoroquinolone resistance in Enterobacteriaceae. J Microbiol Methods 2013;94:213-6.
15. Endo S, Yano H, Hirakata Y, Arai K, Kanamori H, Ogawa M, et al. Molecular epidemiology of carbapenem-non-susceptible Acinetobacter baumannii in Japan. J Antimicrob Chemother 2012;67:1623-26.
16. Mu X, Nakano R, Nakano A, Ubagai T, Kikuchi-Ueda T, Tansho-Nakagawa S, et al. Loop-mediated isothermal amplification: Rapid and sensitive detection of the antibiotic resistance gene ISAba1-blaOXA-51-like in Acinetobacter baumannii. J Microbiol Methods 2013;94:213-6.
17. La Scola B, Gundi VA, Khamis A, Raoult D. Sequencing of the genome of Acinetobacter baumannii and molecular characterization of its clinical isolates. J Microbiol Methods 2016;121:36-40.
18. Ministry of Health, Labour and Welfare. Ethical Guidelines for Medical and Health Research Involving Human Subjects. https://www.lifescience.me/2003/1109-17.
19. CLSI. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100-S22. 22nd ed. Wayne, PA: Clinical and Laboratory Standards Institute, 2012.
20. Pourmaras S, Markogiannakis A, Ikonomidou K, Kondylis L, Bithoulou K, Maniatis AN, et al. Outbreak of multiple clones of imipenem-resistant Acinetobacter baumannii isolates expressing OXA-58 carbapenemase in an intensive care unit. J Antimicrob Chemother 2006;57:575-61.
21. Hujer KM, Hujer AM, Endimiani A, Thomson JM, Adams MD, Goglin K, et al. Rapid determination of quinolone resistance in Acinetobacter spp. J Clin Microbiol 2009;47:1436-42.
22. Hallassos A, Chomel JC, Tesson L, Baudis M, Kruh J, Kaplan JC, et al. Modification of enzymatically amplified DNA for the detection of point mutations. Nucleic Acids Res 1989;17:3606.
23. Deccache Y, Irenge LM, Savoy E, Ariciuc M, Macovei A, Trifonova A, et al. Development of a pyrosequencing assay for rapid assessment of quinolone resistance in Acinetobacter baumannii isolates. J Microbiol Methods 2011;86:115-8.