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ORIGINAL ARTICLE

Amino acid substitutions of quinolone resistance determining regions in GyrA and ParC associated with quinolone resistance in *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU

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Background and purpose: Amino acid substitutions in GyrA and ParC are associated with resistance to quinolones in *Acinetobacter baumannii* (*A baumannii*), but this association is rarely elucidated in *Acinetobacter* genomic species (AGS) 13TU. This study aims to compare the association of amino acid substitutions in GyrA and ParC with quinolone resistance in *A baumannii* and AGS 13TU in Taiwan.
Methods: Eleven representative strains of *A baumannii* and 13 strains of AGS 13TU were selected from 402 bacteremic isolates. The sequences of quinolone resistance determining regions of *gyrA* and *parC* were determined. Minimal inhibitory concentrations (MICs) of nalidixic acid, ciprofloxacin, levofloxacin and moxifloxacin were determined by agar dilution method.
Results: Ser83Leu substitution in GyrA in *A baumannii* (one strain) was associated with resistance to all tested quinolones. This substitution plus a Ser80Leu or Ser80Tyr in ParC in *A baumannii* (four strains) and AGS 13TU (two strains) were associated with higher MICs of all quinolones. All but one quinolone MICs of *A baumannii* (one strain) and AGS 13TU (two strains) carrying a single substitution Ser56Asn in ParC remained in the susceptibility breakpoint. The

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Ser83Leu substitution in GyrA, even with additional Ser56Asn substitution in ParC, was associated with resistance to only nalidixic acid, but not other newer quinolones in AGS 13TU (two strains).
Conclusion: A baumannii and AGS 13TU possessed similar quinolone resistance associated with amino acid substitutions in GyrA and ParC. Further study with more strains is needed to determine whether a single Ser83Leu substitution in GyrA was associated with a high level of quinolone MIC only in *A baumannii*, but not in AGS 13TU.
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Introduction

Acinetobacter species, especially *A baumannii*, *Acinetobacter* genomic species (AGS) 3 and 13TU, have emerged as important pathogens of nosocomial infection with high mortality and morbidity in critically ill patients.[1,2] They also cause outbreaks in intensive care units and are difficult to eradicate because of their ability to survive in harsh environments for a prolonged time.[3] With their ability to accumulate different mechanisms of resistance and increasing numbers of more vulnerable hosts, the prevalence of multidrug resistant *Acinetobacter* spp. has been rising in the past decades and the choice of treatment has become limited.[4,5] Although they are phenotypically undifferentiated, they have distinct resistance mechanisms for antimicrobial agents. As for resistance to aminoglycosides, *A baumannii* carries *armA* and *aph(3′)-Ia*, whereas AGS 13TU possesses *aac(3)-Ia* and *aph(3′)-VI*.[6] For resistance to carbapenems, $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-11}}$, which belong to class B metallo-beta-lactamase genes, are more commonly found in AGS 13TU, and the class D carbapenemase genes are observed more often in *A baumannii*.[7] Quinolones have been used for the treatment of *Acinetobacter* spp. because of their good activity, even compared with broad-spectrum cephalosporins and aminoglycosides, until a high rate of resistance to quinolones was detected recently.[8–10] Three mechanisms of resistance to quinolones have been recognized: (1) mutations in target enzymes; (2) changes in drug entry and efflux; and (3) plasmid-mediated Qnr protein, which prevents DNA from quinolone binding and compromises the efficacy of quinolones.[11–13] The most commonly identified mechanism in Gram-negative bacilli is mutations in target enzymes including DNA gyrase, encoded by *gyrA* and *gyrB*, and topoisomerase IV, encoded by *parC* and *parE*. In *A baumannii*, rapid resistance to ciprofloxacin and nalidixic acid is associated with the chromosomal mutations in the quinolone resistance determining regions (QRDRs) of *gyrA* and/or *parC*.[4,5,8,9,14] Single amino acid substitution in GyrA (Ser83Leu) is associated with high level resistance to ciprofloxacin and nalidixic acid.[4,5,9] An additional amino acid substitution in ParC, mostly Ser80Leu, is associated with higher resistance in *A baumannii*.[8,9,14] To date, the majority of the data regarding quinolone resistance focused on *A baumannii*.[4,5,8,9,14] In contrast, the mechanism of quinolone resistance in AGS 13TU, which is genetically closely related to and phenotypically undifferentiated from *A baumannii*,[3] is not elucidated. Recent studies showed that *A baumannii* and AGS 13TU possess remarkably distinct phenotypic and genotypic traits against antimicrobial agents.[15,16] Lee et al reported *A baumannii* bacteremia was associated with a higher 14-day mortality rate, a higher 30-day mortality rate and a higher in-hospital mortality rate than bacteremia due to AGS 13TU or 3.[17] Chuang et al revealed higher rates of antimicrobial resistance and poorer outcome for patients infected with *A baumannii* than for those infected with AGS 13TU or 3.[18] The prevalence of resistance to ciprofloxacin was different in these studies, but the mechanism was not analyzed. The major aim of the present study was to compare the association of amino acid substitutions in GyrA and ParC with quinolone resistance between the clinical isolates of *A baumannii* and AGS 13TU in Taiwan.

Materials and methods

Bacterial strains and antimicrobial susceptibility testing

During 2006 and 2008, 402 bacteremic isolates of *Acinetobacter* spp. were collected. Twenty-four representative strains of *Acinetobacter* spp. with different susceptibility to quinolones and pulsotypes were selected for the study. All strains had been identified to the genomic species level by either a multiplex PCR method[19] or sequencing analysis of 16S-23S rRNA internal transcribed spacer.[20] Susceptibility against nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin were established by the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI).[21] The breakpoints proposed by CLSI were used for nalidixic acid (susceptible $\leq$ 16 μg/mL; resistant $\geq$ 32 μg/mL),[22] ciprofloxacin (susceptible $\leq$ 1 μg/mL; resistant $\geq$ 4 μg/mL) and levofloxacin (susceptible $\leq$ 2 μg/mL; resistant $\geq$ 8 μg/mL).[21] The breakpoint of moxifloxacin (susceptible $\leq$ 1 μg/mL; resistant $\geq$ 4 μg/mL) was proposed by the manufacturer.

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

The QRDRs of *gyrA* and *parC* were amplified with the following primer pairs: 5′-atgagcgtatcggaaatccg-3′ and 5′-ggtatttaccacgaatgtgtaa-3′ for *gyrA*, and 5′-atgaccagccttgcgcatc-3′ and 5′-gttatcttgccattcgctagt-3′ for *parC* with a proof-reading polymerase (Takara Bio Inc, Otsu, Shiga, Japan), yielding amplicons of 733 and 450 base pairs, respectively. Polymerase chain reaction (PCR) program was as follows: 94°C for 1 minute, 35 cycles at 98°C for 10 seconds, and 60°C for 1 minute with a final extension at

72°C for 10 minutes. The amplified DNA product was resolved by electrophoresis in agarose 2% w/v gels, stained with ethidium bromide, and purified according to the manufacturer's instruction (Geneaid Biotech Ltd, Taipei, Taiwan). The purified PCR product was cloned into a pCRII-TOPO® vector and transformed into *Escherichia coli* (Invitrogen Corp., Carlsbad, CA, USA). The sample was then processed for DNA sequencing (Mission Biotech, Taipei, Taiwan).

Results

A total of 24 representative strains comprising *A baumannii* (11 strains) and AGS 13TU (13 strains) were included in the study. The amino acid substitutions in the QRDRs of the GyrA and ParC of these strains and their association with quinolone MICs were presented.

For *A baumannii*, five strains (strain number 16, 129, 202, 312, 305) had mutation on QRDRs of *gyrA*, leading to an amino acid Ser83Leu substitution (Table 1). A strain with only the single Ser83Leu substitution in GyrA displayed resistance to all tested quinolones. Among these five strains, four strains (strain number 129, 202, 312, 305) had simultaneous Ser80Leu or Ser80Tyr substitutions in ParC. These four strains had higher level of quinolone MICs, compared to those with only Ser83Leu in *gyrA*. Strain number 514 had a single Ser56Asn substitution in ParC, but the MICs of all quinolones tested were similar to those without the amino acid substitution.

For AGS 13TU, four strains (number 242, 254, 405, 454) had mutations on QRDRs of the *gyrA* gene, resulting in an amino acid Ser83Leu substitution (Table 2). Six strains (number 23, 242, 254, 405, 454, 502) had either ParC 56 (Ser56Asn) or ParC 80 (Ser80Leu) amino acid substitution. The two strains (405 and 454) with simultaneous substitutions in GyrA 83 and ParC 80 displayed the highest level of quinolone MICs. For the two strains (number 242 and 254) that carried the simultaneous substitutions in GyrA 83 and ParC 56, the MICs of nalidixic acid were still high, but MICs of ciprofloxacin, levofloxacin and moxifloxacin were within susceptibility or just intermediate breakpoint. For the two strains (number 502 and 23) with just a single substitution Ser56Asn in ParC, strain number 502 did not have significant MIC change, compared with the strains without any amino acid substitution in the QRDRs of both GyrA and ParC. For strain number 23, the MIC of nalidixic acid was slightly higher than that found in strains without any amino acid substitution, but the MICs of ciprofloxacin, levofloxacin and moxifloxacin were similar to those of strains 242 and 254, which simultaneously had amino acid substitutions in GyrA 83 and ParC 56.

Discussion

Among *Acinetobacter* spp., *A baumannii* and AGS 13TU are the most clinically relevant pathogens of nosocomial infections.[23] They have different clinical features, outcomes and resistance rates to several antibiotics,[17] including fluoroquinolones. In Sheng's study,[24] as compared with carbapenem-resistant *A baumannii*, carbapenem-resistant AGS 13TU and 3 isolates have higher antimicrobial susceptible rates to ciprofloxacin. It is well-known that, for resistance to quinolones, amino acid substitutions in GyrA and ParC play important roles in Gram-negative bacilli.[25] The contribution of amino acid substitutions in GyrA and ParC to quinolone resistance may be unequal among different Gram-negative bacilli. In *Pseudomonas aeruginosa*, the fluoroquinolone resistance is mainly due to *gyrA* mutations, with *parC* mutations being less significant.[26] Double mutations in *gyr*A and *par*C were needed for high level resistance to quinolones in *A baumannii*, but three or four mutations in both *gyrA* and *parC* genes are required for high-level resistance to ciprofloxacin in *E coli*.[8,27,28] Therefore, our study compared the association of amino acid substitutions in GyrA and ParC with quinolone resistance in the two most clinically important *Acinetobacter* spp., *A baumannii* and AGS 13TU.

Our study revealed that the association of amino acid substitutions of GyrA and ParC with quinolone resistance in *A baumannii* and AGS 13TU was largely similar, with only a mild discrepancy. Single amino acid Ser83Leu substitution in GyrA was associated with high level MICs of all quinolones in *A baumannii*, but only associated with a high level of MIC of nalidixic acid in AGS 13TU. Further substitution in ParC

Table 1 Minimal inhibitory concentrations (MICs) of quinolones and amino acid substitution in quinolone resistance determining regions of GyrA and ParC in Acinetobacter baumannii

Strains	MIC(μg/mL)				Amino acid substitution		
	Nalidixic acid	Ciprofloxacin	Levofloxacin	Moxifloxacin	GyrA Ser83	ParC Ser56	ParC Ser80
514	8	0.25	0.25	≤0.125	Ser	Asn	Ser
5	8	0.25	0.25	≤0.125	Ser	Ser	Ser
294	8	0.5	0.25	0.25	Ser	Ser	Ser
24	8	1	0.5	0.5	Ser	Ser	Ser
15	16	0.5	1	0.25	Ser	Ser	Ser
25	16	1	0.5	0.5	Ser	Ser	Ser
16	>64	16	8	8	Leu	Ser	Ser
129	>64	64	16	16	Leu	Ser	Tyr
202	>64	>64	16	32	Leu	Ser	Leu
312	>64	>64	16	32	Leu	Ser	Leu
305	>64	>64	16	32	Leu	Ser	Leu

Table 2 Minimal inhibitory concentrations (MICs) of quinolones and amino acid substitution in quinolone resistance determining regions of GyrA and ParC in Acinetobacter genomic species 13TU

Strains	MIC(μg/mL)				Amino acid substitution		
	Nalidixic acid	Ciprofloxacin	Levofloxacin	Moxifloxacin	GyrA Ser83	ParC Ser56	ParC Ser80
20	4	0.25	≤0.125	≤0.125	Ser	Ser	Ser
500	4	0.125	0.125	<0.125	Ser	Ser	Ser
502	4	0.125	0.125	<0.125	Ser	Asn	Ser
12	8	0.25	0.25	≤0.125	Ser	Ser	Ser
27	8	0.25	0.25	≤0.125	Ser	Ser	Ser
316	8	2	1	1	Ser	Ser	Ser
356	16	0.5	0.5	0.5	Ser	Ser	Ser
13	16	1	0.5	0.125	Ser	Ser	Ser
23	16	4	1	1	Ser	Asn	Ser
242	>64	2	1	2	Leu	Asn	Ser
254	>64	2	1	1	Leu	Asn	Ser
405	>64	>64	>32	32	Leu	Ser	Leu
454	>64	>64	>32	16	Leu	Ser	Leu

80, which combined the occurrence of the substitution in GyrA 83, achieved a higher level of MICs of all the quinolones in both *Acinetobacter* species (Tables 1 and 2). These mechanisms resembled those in other Gram-negative bacilli.[8,9,14] This study also demonstrated that newer fluoroquinolones were less influenced by amino acid substitutions in GyrA and ParC in *Acinetobacter*.[14, 29] This may be partially explained by the intrinsic structure of different quinolones.[12,30–32] Unexpectedly, with s similar Ser83Leu substitution in GyrA, *A baumannii* became more resistant than AGS 13TU to newer fluoroquinolones. If this result can be demonstrated in more strains, it may be one of the reasons to explain the higher rate of resistance to quinolones in *A baumannii* than AGS 13TU.

To the best of our knowledge, a novel substitution in ParC 56 was found in *Acinetobacter* in the current study. Substitutions of ParC at Ser80 and Glu84 have been previously detected and contribute to MIC change in *A. baumannii*. Chiu et al[33] observed a novel *parC* gene mutation leading to amino acid substitution, Lys59Gln. In the absence of concurrent amino acid substitution in GyrA, the Lys59Gln in ParC was associated with ciprofloxacin resistance. The result disclosed that in *A baumannii* substitution of ParC might be as important as substitution of GyrA. However, a high level of expression of AdeB, an efflux pump protein, was found in the resistant strain. Thus, the author suggested that ParC Lys59Gln mutation and the efflux pump may function synergistically to induce resistance to ciprofloxacin. In our study, substitution in ParC, without substitution in GyrA, were noted both in *A baumannii* and AGS 13TU. Although the substitution was not associated with significant MIC change in strain 514 of *A baumannii* and strain 502 of AGS 13TU, the MICs of nalidixic acid and ciprofloxacin in strain 23 of AGS 13TU increased (MIC of 16 μg/mL for nalidixic acid and 4 μg/mL for ciprofloxacin). It implied that in AGS 13TU, ParC may be more than a secondary target, although the synergistic role should also be considered.

In conclusion, *A baumannii* and AGS 13TU possess similar substitutions in QRDRs in GyrA and ParC that are associated with quinolone resistance. Single substitution Ser83Leu at GyrA could confer high level resistance to quinolones in *A baumannii*, but may not confer high level resistance to newer fluoroquinolones in AGS 13TU. An additional substitution at ParC 80 was associated with a higher level of resistance to quinolones in both *Acinetobacter* spp. The role of substitution at ParC 56 needs to be further elucidated.

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