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ORIGINAL ARTICLE

Alterations of *gyrA*, *gyrB*, and *parC* and Activity of Efflux Pump in Fluoroquinolone-resistant *Acinetobacter baumannii*

Sunok Park, Kyeong Min Lee, Yong Sun Yoo, Jung Sik Yoo, Jae Il Yoo, Hwa Su Kim, Yeong Seon Lee, Gyung Tae Chung*

Division of Antimicrobial Resistance, Korea National Institute of Health, Osong, Korea.

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Abstract

Objectives: This study investigated the fluoroquinolone-resistant mechanism of 56 clinical cases of *A baumannii* infection from 23 non-tertiary hospitals, collected between 2004 and 2006.

Methods: Susceptibility testing was performed by broth microdilution and Epsilometer test. Analyses of quinolone resistance-determining region (QRDR) were done by sequencing. The activity of the efflux pump was measured using inhibitors.

Results: The sequences from selected 56 isolates were divided into seven groups (I–VII) on the basis of mutations in *gyrA* (S83L), *parC* (S80L, S80W and S84K) and *gyrB* (containing the novel mutations E679D, D644Y and A677V). The 27 isolates with triple mutations in *gyrA*, *gyrB* and *parC* (groups IV–VII) showed higher levels of resistance to ciprofloxacin (minimal inhibitory concentration [MIC] of 16–256 μg/mL) than the 26 isolates with double mutations in *gyrA* and *parC* (groups II and III, MIC of 8–64 μg/mL; $p < 0.05$). Alterations in the efflux pump were observed in four isolates with the *parC* S80L mutation (group II) or E84K mutation (group VII), but no effect was observed in an isolate with the *parC* S80 W mutation (group III).

Conclusion: These results suggest that triple mutations in clinical isolates of *A baumannii* contribute to the development of high levels of resistance to fluoroquinolones and that mutations in *parC* S80L or E84K (groups II and VII) may contribute to alterations in efflux pump activity in *A baumannii*.

1. Introduction

Over the past three decades, *Acinetobacter baumannii* has emerged as an important nosocomial pathogen worldwide [1]. Certain strains of *A baumannii* are now resistant to many common antimicrobial agents, including fluoroquinolone, and multidrug resistance is often responsible for the failure of antibiotic therapy

*Corresponding author.
E-mail: gtchung@nih.go.kr

[2,3]. Resistance to fluoroquinolone is mediated primarily through spontaneous mutations of genes in the quinolone resistance-determining region (QRDR), namely, DNA gyrase and topoisomerase IV. Alterations in the drug target due to modifications in the genes for DNA gyrase subunit A (*gyr*A) or topoisomerase IV subunit C (*par*C) have been associated with high levels of resistance to fluoroquinolones [4–6]. A secondary cause of resistance to fluoroquinolone is alterations in the efflux pump that lead to active efflux of the drug. Efflux pumps typically have broad substrate specificity that contributes to resistance against multiple unrelated classes of drug, including aminoglycosides, tetracycline, fluoroquinolones, trimethoprim and chloramphenicol [7,8].

DNA gyrase is the target of a number of quinolones, including ciprofloxacin, levofloxacin, gemifloxacin and gatifloxacin [9,10]. Quinolones produce bacteriostatic activity by binding rapidly to the enzyme–DNA complex, probably before DNA cleavage occurs, thereby blocking DNA replication and transcription. The quinolones can bind to the complexes even with mutations in the genes for DNA gyrase or topoisomerase IV that result in an inability to cleave DNA [11,12]. The protein product of *gyr*B consists of two domains: a 43-kDa N-terminal domain containing a site for ATP binding and hydrolysis that is thought to act as a DNA clamp; and a 47-kDa C-terminal domain that is thought to play a role in strand passage and interactions with *gyr*A and DNA. GyrA and GyrB form a functional A2B2 tetramer [11,12].

Many fluoroquinolone-resistant clinical isolates of *A baumannii* have emerged rapidly in South Korea [13,14]. There are, however, few studies on the resistance to fluoroquinolone and the prevalence of mutations in the genes for DNA gyrase and topoisomerase IV in clinical isolates of *A baumannii* from the South Korean population. The role of the *A baumannii* efflux pump on fluoroquinolone resistance has not been fully investigated. Therefore, the aim of this study was to investigate the mechanisms of resistance to fluoroquinolone in clinical isolates of *A baumannii* from non-tertiary hospitals in South Korea. Specifically, we assessed: (i) the presence of mutations in the *gyr*A, *gyr*B and *par*C genes and the effect of different mutations on resistance to fluoroquinolone; and (ii) the presence of alterations in the efflux pump mechanism and their effects on resistance to fluoroquinolone.

2. Method and Materials

2.1. Bacterial strains and growth conditions

Fifty-six nonrepetitive clinical isolates of fluoroquinolone-resistant *A baumannii* from non-tertiary hospitals, collected between 2004 and 2006, were selected for this study. The *A baumannii* isolates were compared with the reference *Escherichia coli* ATCC 25922 strain (American Type Culture Collection, Manassas, VA, USA) for minimal inhibitory concentration (MIC) analysis and with the *A baumannii* ATCC 19606 strain for QRDR analysis. The clinical isolates were propagated at 37°C in nutrient broth or agar. The clinical isolates and reference bacteria were propagated aerobically at 37°C in Luria-Bertani (LB) broth until they reached mid-log growth. The clinical isolates were identified using the Vitek II automatic system (bioMerieux, Carcy-I'Etole, France).

2.2. Antimicrobial susceptibility testing

The MICs for ciprofloxacin, gemifloxacin, levofloxacin, norfloxacin and gatifloxacin were determined by Epsilometer test (Etest, AB BIODISK, Piscataway, NJ, USA) and the broth microdilution method, following the manufacturer's instructions, which were based on the guidelines of the Clinical and Laboratory Standards Institute [15]. The MIC for each drug was determined by a serial dilution of test compounds (Sigma-Aldrich, St Louis, MO, USA) in Mueller-Hinton broth (Becton Dickinson & Co., Sparks, MD, USA) with the bacteria at a density of 5×10^5 colony-forming units/mL. Plates were incubated at 37°C for 20 hours and growth was then assayed by measuring the optical density at 595 nm.

2.3. Quinolone resistance-determining region analysis

The QRDR analysis was conducted using the control sequence for *gyrA* (DQ270238), *gyrB* (CU468230) and *parC* (X95816). The primers were used to amplify the DNA and for sequencing (Table 1). Genomic DNA was extracted from the prepared isolates using the Qiagen genomic DNA purification kit (Qiagen, Hilden, Germany). Universal polymerase chain reaction (PCR) was used to confirm that the DNA samples contained the fluoroquinolone resistance genes (Table 1). A 1:100 dilution of DNA in Tris-EDTA (TE) buffer was used in the PCR. DNA amplification was carried out in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA, USA) instrument with initial denaturation at 95°C for 2 minutes followed by 30 cycles of amplification (denaturation at 95°C for 30 seconds, then annealing for 30 seconds at primer set-specific temperatures, and extension at 72°C for 1 minute), ending with a final extension at 72°C for 5 minutes. The PCR products were stained with ethidium bromide and photographed with ultraviolet illumination.

2.4. Treatment of efflux pump inhibitors

The growth inhibition assays were conducted as described previously [16], using efflux pump inhibitors

Table 1. Primer sequences

Target gene	Primer name	Sequences (5′ to 3′)	Usage	Temp (°C)	Product size (bp)	ATCC No.
gyrA	gyrA_F	AAATCTGCCCGTGTCGTTGGT	amp*	58	344	DQ270238
	gyrA_R	GCCATACCTACGGCGATACC	amp			
gyrB	gyrB_SDF_F	ATGAGTTCAGAGTCTCAATC	amp/seq†	60	2980	CU468230
	gyrB_SDF_R	TTAAGCATCAATATCCGCAATT	amp			
	gyrB_SDF_601	GCACGCCGTTTACGTGAGCT	seq			
	gyrB_SDF_1201	GCACGTGAAATGACACGCCG	seq			
	gyrB_SDF_1801	CAAGTTTCACACAAAGAGCTT	seq			
parC	parC_F	ATGAGCGAGCTAGGCTTAAA	amp	58	300	X95816
	parC_R	TTAAGTTGTCCTTGCCATTCA	amp			

amp = **[amplification OR amplified] prime; †seq = sequencing primer.*

(EPIs) including carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 1-(1-naphthylmethyl)-piperazine (NMP), phenyl-arginine-β-naphthylamide (PAβN), reserpine and verapamil (Sigma-Aldrich, St. Louis, MO, USA). The antimicrobial susceptibility test was conducted with levofloxacin, ciprofloxacin, gemifloxacin and norfloxacin in the presence or absence of each EPI at the following concentrations: 25 μg/mL PAβN, 10 μg/mL CCCP, 50 μg/mL NMP, 50 μg/mL reserpine and 100 μg/mL verapamil. Each EPI was tested at least three times.

2.5. Computer analysis of sequence data

Nucleotide sequence data were analyzed using the Clustal W software program (European Bioinformatics Institute, Cambridge, UK) [17]. The amino acid sequences were analyzed at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST). The GenBank and protein databases were screened for sequence similarity. The primer sets were designed using the sequences of the genes for DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC*) from the 56 clinical isolates of *A baumannii*, using the PrimerSelect program from the DNA Lasergene analysis software package (DNASTAR, Inc., Madison, WI, USA).

2.6. Statistical analysis

Statistical testing to determine differences in antimicrobial resistance was conducted using SPSS for MS Windows (version 11.5; SPSS, Inc., Chicago, IL, USA). Continuous variables were compared using Student's *t* test, and categorical variables were compared using Fisher's exact test or chi-squared analysis. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Novel *gyrB* mutant sites and amino acid substitutions within QRDRs

The 56 clinical isolates were identified from the QRDR analysis. The isolates were divided into seven groups according to the amino acid substitutions in the protein products of *gyrA*, *gyr*B and *par*C (Table 2). A mutation encoding Ser83 → Leu was detected in the QRDR of *gyrA* in all of the *A baumannii* isolates studied. Three different mutations were observed in the QRDR of *gyrB* encoding Glu479 → Asp (3.6%), Asp644 → Tyr (12.5%) and Ala677 → Val (37.5%) in 30 (53.6%) *A baumannii* isolates. Fifty-three of the 56 *A baumannii* isolates (94.6%) showed a single mutation in *parC* encoding Ser80 → Leu (30; 53.6%), Ser80 → Trp (2; 3.6%) and Glu84 → Lys (21; 37.5%). Overall, 27 (48.2%) of the 56 fluoroquinolone -resistant *A baumannii* isolates showed mutations in the QRDR of *gyrA*, *gyrB* and *parC*.

Table 2. Mutations in the GyrA, GyrB and ParC subunits in *A baumannii* isolates, and the MIC for different fluoroquinolone in groups of mutant isolates

Mutation groups	No. of isolates	Agent	No. of isolates with MIC (μg/mL)									GyrA	GyrB			ParC	
			1	2	4	8	16	32	64	128	>256	S83 (tca)	E479 (gaa)	D644 (gat)	A677 (gcg)	S80 (tcg)	E84 (gaa)
I	3	Ciprofloxacin		1	2							L(tta)		Y(tat)			
		Gatifloxacin	3														
		Gemifloxacin	3														
		Levofloxacin	1	2													
II	25	Ciprofloxacin				2	1	20	2			L(tta)				L(ttg)	
		Gatifloxacin	1	2	16	6											
		Gemifloxacin		2	3	14	5	1									
		Levofloxacin		2	17	4	2										
III	1	Ciprofloxacin							1								
		Gatifloxacin				1						L(tta)				W(tgg)	
		Gemifloxacin					1										
		Levofloxacin				1											
IV	1	Ciprofloxacin								1		L(tta)	D(gat)			L(ttg)	
		Gatifloxacin					1										
		Gemifloxacin					1										
		Levofloxacin					1										
V	1	Ciprofloxacin									1	L(tta)	D(gat)			W(tgg)	
		Gatifloxacin						1									
		Gemifloxacin						1									
		Levofloxacin						1									
VI	4	Ciprofloxacin					1		2	1		L(tta)		Y(tat)		L(ttg)	
		Gatifloxacin				2	1	1									
		Gemifloxacin				1		3									
		Levofloxacin				2	1	1									
VII	21	Ciprofloxacin						11	9			L(tta)			V(gtg)		K(aaa)
		Gatifloxacin			18	2	1										
		Gemifloxacin			4	9	8										
		Levofloxacin			5	16											

A = alanine, D = aspartic acid, E = glutamic acid, K = lysine, L = leucine, MIC = minimal inhibitory concentration, S = serine, V = valine, W = tryptophan, Y = tyrosine.

3.2. MIC for fluoroquinolone in the QRDR mutants

Group I mutants had mutations in *gyrA* and *gyrB* and showed resistance to four fluoroquinolones, but their MIC was ≤4 μg/mL. Group II and III mutants had double mutations in *gyrA* and *parC* and showed resistance to ciprofloxacin (MIC 8−64 μg/mL), gatifloxacin (MIC 1−8 μg/mL), gemifloxacin (MIC 2−32 μg/mL) and levofloxacin (MIC 2−16 μg/mL). Groups IV, V, VI and VII mutants had triple mutations *in gyrA*, *gyrB* and *parC*. They were resistant to ciprofloxacin (MIC 16−256 μg/mL), gatifloxacin (MIC 4−32 μg/mL), gemifloxacin (MIC 4−32 μg/mL) and levofloxacin (MIC 4−32 μg/mL), $p < 0.05$). Overall, isolates with triple mutations of *gyrA*, *gyrB* and *parC* had a higher level of resistance to fluoroquinolone than isolates with double mutations of *gyrA* and *parC* ($p < 0.05$).

3.3. Efflux pump analysis

To evaluate the contribution of the efflux pump to the fluoroquinolone-resistant phenotype of five isolates from different QRDR mutation groups, we compared the MIC for each fluoroquinolone in the presence or absence of CCCP, PAβN, NMP, reserpine and verapamil (Table 3). For four isolates (5733, 5A38, 6A69 and 6A80), the four EPIs PAβN, NMP, reserpine and verapamil significantly reduced the MIC (by two- to eightfold) for levofloxacin. These four isolates belonged to groups II (GyrA S83L; ParC S80L) and VII (GyrA S83L; GyrB A677V; ParC E84K). They showed a high level of resistance to levofloxacin (MIC >32 μg/mL). The remaining isolate, which was not affected by the EPIs, belonged to group III (GyrA S83L; ParC S80W) and showed less resistance to levofloxacin (8 μg/mL) than the other four isolates.

4. Discussion

The purpose of this study was to investigate mechanisms of resistance to fluoroquinolone in clinical isolates of *A baumannii*. The results of the present study

Table 3. The MIC for fluoroquinolone in the absence or presence of EPIs*

			MIC (μg/mL)				QRDR mutation		
Group	Isolate	+ EPI	NOR	CIP	GEM	LEV	GyrA	GyrB	ParC
II	5733	alone	>256	>32	25.3	>32	S83L		S80L
		+ CCCP	>256	>32	12.7	4.0			
		+PAβN	>256	>32	8.0	4.0			
		+ NMP	>256	>32	19.3	6.0			
		+ reserpine	>256	>32	24.0	4.0			
		+ verapamil	>256	>32	15.3	12.0			
II	5A38	alone	>256	>32	54.3	>32	S83L		S80L
		+ CCCP	>256	>32	11.0	>32			
		+ PAβN	>256	>32	14.7	4.0			
		+ NMP	>256	>32	49.3	6.0			
		+ reserpine	>256	>32	8.0	6.0			
		+ verapamil	>256	>32	5.5	4.0			
III	6018	alone	>256	>32	16.0	8.0	S83L		S80W
		+CCCP	>256	>32	13.3	8.0			
		+PAβN	>256	>32	15.3	8.0			
		+NMP	>256	>32	13.3	8.0			
		+Reserpine	>256	>32	36.7	8.0			
		+Verapamil	>256	>32	38.7	>32			
VII	6A69	alone	>256	>32	13.0	>32	S83L	A677V	E84K
		+ CCCP	>256	>32	7.3	>32			
		+ PAβN	>256	>32	6.0	4.0			
		+ NMP	>256	>32	10.3	6.0			
		+ reserpine	>256	>32	11.3	6.0			
		+ verapamil	>256	>32	10.0	12.0			
VII	6A80	alone	>256	>32	7.0	>32	S83L	A677V	E84 K
		+ CCCP	>256	>32	6.0	>32			
		+ PAβN	>256	>32	3.7	4.0			
		+ NMP	>256	>32	7.7	6.0			
		+ reserpine	>256	>32	7.3	4.0			
		+ verapamil	>256	>32	3.3	6.0			

**Average MIC values were determined by Etest at least three times (standard deviation ± 0.2−12).*

CCCP = carbonyl cyanide 3-chlorophenylhydrazone, CIP = ciprofloxacin, EPI = efflux pump inhibitor, GEM = gemifloxacin, LEV = levofloxacin, MIC = minimal inhibitory concentration, NMP = 1-(1-naphthylmethyl)-piperazine, NOR = norfloxacin, PAβN = phenylarginine-β-naphthylamide.

correspond well with those of earlier studies, which have shown that the most common mechanism of resistance to fluoroquinolone in *A baumannii* involves alterations in the genes that encode subunits of the quinolone targets DNA GryA (S83L, G81V or C and A84P) and ParC (S80L or W, E84K and G78C) [4−6]. Mutations in the GyrA and GyrB subunits of DNA gyrase and the ParC and ParE subunits of topoisomerase IV play a major role in conferring a high level of resistance to fluoroquinolone in other Gram-negative bacteria, such as *E coli* and *Pseudomonas aeruginosa* [18,19]. The outer membrane of *A baumannii* has been associated with intrinsic antimicrobial resistance resulting from the over-expression of the *adeABC* and *adeFGH* efflux pump genes [20,21]. These findings indicate that active efflux is involved in both intrinsic and acquired resistance to fluoroquinolones. The outer membrane of *P aeruginosa* shows little permeability to small hydrophobic molecules, which may account for the intrinsic resistance of *P aeruginosa* to quinolones. In fact, the outer membrane of *P aeruginosa* is 10- to 100-fold less permeable to antimicrobial agents than the outer membrane of *E coli* [22]. Additional efflux pumps associated with resistance to fluoroquinolone have also been characterized in *E coli* and other Gram-negative bacteria [22,23].

In the present study, clinical isolates of *A baumannii* with identical mutations in the QRDR of *gyrA*, *gyrB* and *parC* genes showed very different levels of resistance to fluoroquinolones.

It is controversial whether the efflux pump has a contributory effect on the resistance of *A baumannii* to fluoroquinolone [2,24]. Although we cannot exclude the presence of QRDR mutations outside of the region that we sequenced, our results suggest that the differences in resistance that were observed were due to differences in the active efflux of fluoroquinolone.

The effects of CCCP and PAßN−that primarily inhibit RND-family efflux pumps−and reserpine and verapamil−that primarily inhibit non-RND-family efflux pumps−on antimicrobial susceptibility have been examined previously [16,25]. Although these inhibitors have preferences for certain classes of pumps, their activity cannot be assumed to be exclusive to one class. The present study showed that the EPIs also reduced the MIC of fluoroquinolone, but only in isolates with clonally related *parC* mutations (S80L in group II and E84K in group VII). It did not have this effect in the S80W *parC* mutation (group III).

In conclusion, although a single mutation in DNA gyrase is sufficient for resistance to fluoroquinolone, the accumulation of triple mutations in the QRDR regions of the *gyrA*, *gyrB* and *ParC* genes is expected to significantly contribute to high-level fluoroquinolone resistance. We have demonstrated a role for the efflux pump in conferring resistance to fluoroquinolone in clinical isolates that carry S80L or E84K mutations in *parC*. Further studies are needed to elucidate the role of over-expression of the efflux pump on fluoroquinolone resistance.

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