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ORIGINAL ARTICLE

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

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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

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[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