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Short Communication

Mutations in the quinolone resistance determining region in isogenic mutant *Acinetobacter baumannii* strains

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The aim of the study is to explore the change or mutation of DNA gyrase (*gyr*A) and topoisomerase IV (*par*C) in Quinolone Resistance Determining Region (QRDR) in *Acinetobacter baumannii* isogenic mutant strains which were insensitivity or resistant to fluoroquinolones (FQs). A total of 11 strains were studied, one is isolated from clinical sample as a wide-type parent strain and the others isogenic mutant strains which were selected by ciprofloxacin *in vitro* environment. Three of the isogenic mutant strains have amino acid mutation in GyrA and ParC of QRDR, two of the three have the mutation Ser-97 to Thr and Asp-150 to His in GyrA, another one which is highly resistant to ciprofloxacin (MICs of ciprofloxacin, 64 µg/ml) has the mutations Ser-97 to Thr in GyrA and Ala-85 to Pro in ParC.

Key words: *Acinetobacter baumannii*, quinolone resistance determining region, mutation, isogenic strain.

INTRODUCTION

In the last few years, the importance of *Acinetobacter baumannii* in nosocomial infections has been steadily rising. Several outbreaks, many of them in intensive care units, caused by multiply resistant strains of *Acinetobacter* spp. have been documented (Peleg et al., 2008; Howard et al., 2012; Davies and Davies, 2010). Previously, fluoroquinolones (FQs) had good activity against *Acinetobacter* strains even compared with expanded-spectrum cephalosporins and aminoglycosides (Bergogne-Berezin and Joly-Guillou, 1985; Higgins et al., 2000; King and Phillips, 1986; Rolston et al., 1988). However, resistance to these antibiotics has rapidly emerged in clinical isolates (Acar et al., 1993; Seifert et al., 1993). The protein targets for FQs are type II topoisomerases (DNA gyrase and topoisomerase IV); both are tetrameric enzymes. DNA gyrase is composed with two A subunits and two B subunits encoded by the *gyr*A and *gyr*B genes, and topoisomerase IV is composed with two C and two D subunits encoded by the *par*C and *par*E genes (Hooper, 1998). There is a region in these genes that is known as the quinolone resistance determining region (QRDR), where mutations associated with the acquisition of quinolone resistance have been located. The mutations that play the most important role in the acquisition of resistance are located in the QRDR of the *gyr*A and *par*C genes (Hooper, 1998).

In GyrA, the most common changes are Ser-83 and Asp-87 (Hooper, 1998; Vila et al., 1995; Hamouda and Amyes, 2004; Lee et al., 2005) and in ParC Ser-80 and Glu-84 are the most common mutation (Hamouda and Amyes, 2004; Vila et al., 1997; Lee et al., 2005). This study is to explore the change or mutation of QRDR of the isogenic mutant *A. baumannii* strains which were insensitivity or resistant to FQs *in vitro* environment, and to comprehend the effect of antibiotics on bacterial resistance.

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Table 1. The MIC of *Acinetobacter baumannii* strains and the mutations of *gyr*A and *par*C.

Strain	MIC (μg/ml)	*gyr*A mutation	*par*C mutation
Parent strain	0.06	—	—
2C1	1	—	—
2C2	1	Ser-97→Thr	—
2C3	1	—	—
2C4	1	—	—
2C5	0.5	—	—
2C6	2	Asp-150→His	—
4C1	1	—	—
4C2	1	—	—
4C3	2	—	—
4C4	64	Ser-97→Thr	Ala-85→Pro

MATERIALS AND METHODS

Bacterial collection and antimicrobial susceptibility test

The wide-type parent strain was isolated from clinical sample, which was identified as *A. baumannii* by sequence analysis of the 16S rRNA gene. The primers used to amplify the 16s rRNA gene were 5′-TACCTTGTTACGACTT-3′ and 5′- AGAGTTTGATCITGGA-3′ (Vila et al., 1996). It is susceptible to all commonly used antibiotics, according to the Clinical and Laboratory Standards Institute (CLSI) 2012 standard; and the others 10 strains were the isogenic mutant strains of the wide-type parent strain selected by ciprofloxacin *in vitro* environment. Susceptibility testing was performed by an agar dilution method and in accordance with the guidelines established by the CLSI. Approximately 10^4 CFU of each isolate was inoculated onto MH agar plate containing serial dilutions of ciprofloxacin (China Drug and Biological Product Standardization Institution, Batch NO.130451-200302, contents 84.9%). *Pseudomonas aeruginosa* ATCC27853 was chosen as quality control reference strain. First, we tested the MIC of ciprofloxacin of the parent strain, and then approximately 10^4 CFU of the wide-type parent strain was inoculated onto the MH agar plate containing ciprofloxacin concentrations of 2×MIC and 4×MIC, cultivated at 37°C for 24 to 72 h. Then we selected the typical bacterial colony to inoculate onto the MH agar plate again, which was cultivated one generation and then inoculate onto the original MH agar plate containing 2×MIC or 4×MIC ciprofloxacin concentrations, respectively.

If there had the typical bacterial colony, we think that strain was the mutant strain. Altogether 10 mutant strains were selected and numbered as 2C1, 2C2, 2C3, 2C4, 2C5, 2C6, 4C1, 4C2, 4C3 and 4C4.

Amplification and sequencing of *gyr*A and *par*C fragments

The primers, 5'-AATCTGCCCGTGTCGTTGGT-3' and 5'-GCCATACCTACGGCGATACC-3' for *gyr*A, 5'-AAAAATCAGCGCGTACAGTG-3' and 5'-CGAGAGTTTGGCTTCGGTAT-3' for *par*C were used. The PCR was performed as follows. Half of a colony grown on MH agar was resuspended in 25 μl of sterile distilled water and boiled for 10 min. After a short centrifugation step at 15,000 × g, 25 μl of a reaction mixture containing 20mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, gelatin (0.1%, wt/vol), 400 μM deoxynucleoside triphosphates and 1 μM each primer was added together with 2.5 U of *Taq* polymerase [TAKARA Biotechnology (Dalian) Co., Ltd.]. Each reaction mixture was overlaid with mineral oil and amplified at the following temperature profiles: 35 cycles at 93°C for 30 s, 55°C for 30 s and 72°C for 1 min. The PCR products were purified with the DNA purification kit according to the manufacturers' instructions.

The sample was processed with a DNA sequencing kit and analysed in an automatic DNA sequencer, and compared with GenBank database genes by using the BioEdit software.

RESULTS

The ciprofloxacin MIC of isogenic mutant strains

The ciprofloxacin MIC of the parent strain was 0.06 μg/mL, the MIC of the isogenic mutant strains were higher than that (Table 1). Three isogenic mutant strains are insensitive to ciprofloxacin and one is highly resistant to ciprofloxacin which MIC is 64 μg/mL, the other two were intermediate according to CLSI 2012 standard (S is the MIC of ciprofloxacin ≤1 μg/ml, I is the MIC equal to 2 μg/ml and R is the MIC≥4 μg/ml).

QRDR mutations

The results of the PCR amplification are that all of the 11 strains obtain a 285 and a 276 bp segment of *gyr*A gene and *par*C gene, respectively (Figure 1). The *gyr*A gene compared with X82165 and the *par*C gene compared with X95819, which can be found in GenBank. The mutations in the *gyr*A and *par*C gene leading to amino acid changes are shown in Table 1. The sequence of *gyr*A gene has 1~2 nucleotide transversion in all the 11 strains including the wide-type parent strain, all the strains has a A to T transversion at nt486, this dose not induce the amino acid change. But two isogenic mutant strain 2C2 and 4C4 have a G to C transversion at nt291 and this leading to the change at Ser-97 to Thr in GyrA. Another isogenic mutant strain 2C6 has a G to C transversion at nt448 and this induce the change at Asp-150 to His in GyrA.

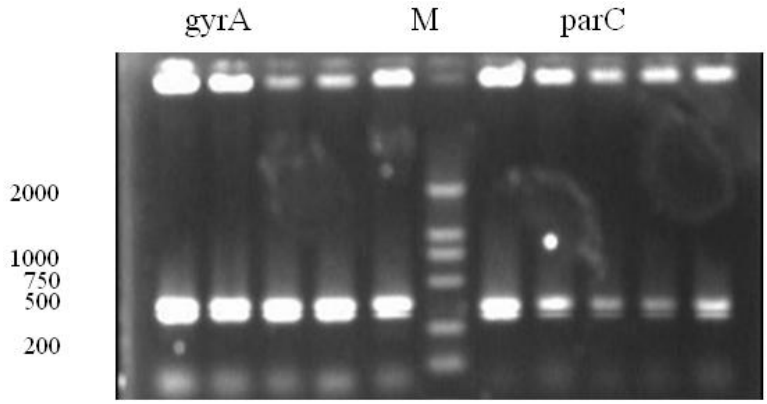

Figure 1. The PCR products of *gyr*A and *par*C M: Marker.

The *par*C gene sequence has 3~4 nucleotide transversions in all the 11 strains. But only the isogenic mutant strain 4C4, the change at Ala-85 to Pro.

DISCUSSION AND CONCLUSIONS

The mutations that play the most important role in the acquisition of resistance of FQs are located in the QRDR of the *gyr*A and *par*C genes (Hooper, 1998), and the mutation of GyrA usually occurs in the 67~106 amino acids. Mutations of the QRDR of the *gyr*B gene seem to be more frequent in quinolone-resistant strains obtained *in vitro* (Nakamura et al., 1989) than in clinical isolates (Ouabdesselam et al., 1995; Vila et al., 1994). Mutations affecting the *par*E gene are extremely unusual among clinical isolates of Gram-negative microorganisms (Everett et al., 1996; Ruiz et al., 1997), although mutations *in vitro* quinolone-resistant strains of *Escherichia coli* have been described by Breines et al. (1997). The mutations of quinolone-resistant strains of *A. baumannii* in GyrA are Gly-81, Ser-83, Ala-84 and Glu-87 those have been reported, and the mutation of Ser-83 to Leu is the most common (Hooper, 1998; Vila et al., 1995; Hamouda and Amyes, 2004). These amino acid mutations do not be found in our study. The mutations we found are Ser-97 to Thr and Asp-150 to His, these have not been reported before. In our study, we do not find any mutation of DNA sequence of the clinical parent isolate, the mutations we found are at the isogenic mutant strains which MIC of ciprofloxacin are raised. The change is Ser-97 to Thr at 2C2 and 4C4 isogenic strain, the MIC of ciprofloxacin was 1 and 64 µg/ml, respectively. This mutational site is in the range of QRDR of GyrA, so we think the MIC rising is caused by the mutation. The differences of our outcome with the others we think is because in our study the isogenic strains are those selected by ciprofloxacin *in vitro* environment, reported earlier is the clinical quinolone-resistant isolates. We also find the mutation of Asp-150 to His at isogenic strain 2C6 which MIC of ciprofloxacin was 2 µg/ml. This mutational site is out of the range of QRDR of gyrA, so we can not conclude that the raised MIC is caused by this mutation. This change may be spontaneous mutate of the chromosomes or the other reason we can not explain and may need additional empirical studies.

The mutation in ParC of the topoisomerase IV which have been mainly reported are Ser-80 and Glu-84 (Hamouda and Amyes, 2004; Vila et al., 1997; Lee et al., 2005); these also could not be found in our study; but at isogenic strain 4C4 the change of ParC we found is Ala-85 to Pro, this has not been reported yet. In previous study, the *par*C rarely mutates alone, at most station it mutates with the *gyr*A at the same time, especially at high resistance bacteria (Lee et al., 2005). The isogenic strain 4C4 is a resistance strain induced by ciprofloxacin *in vitro* in our study, the MIC is 64 µg/ml of ciprofloxacin, it has two mutations of GyrA and ParC at the same time; but we can not be sure the resistance or the MIC raise is caused by the two mutations of the strain, especially the mutation of Ala-85 to Pro in ParC, because this strain is a *in vitro* induced strain, not a clinical resistance isolate; another, the mutation of DNA is not the only mechanism for resistance; so the reason and significance of this mutation (Ala-85 to Pro in ParC) need additional studies.

DNA sequence analysis revealed several variations: a A to T transversion at nt486 in *gyr*A, a T to G transversion at nt321, a T to A transversion at nt327 and a T to C transversion at nt333 in *par*C. These are silent mutations and do not lead to amino acid changes. We select isogenic mutant strains of *A. baumannii in vitro* by ciprofloxacin using the clinical sensitive wide-type parent strain. This is similar to the unreasonable application of antibiotics, which may induce the bacteria resistance to the drugs. In our study, we find the MIC raised and the mutations of the isogenic mutant strains. This may explain the reason that the isolates of nosocomial infection are multi-drug resistance partly, and the clinician should reasonably prescribe antibacterial drugs to reducing the emergence of drug resistant bacteria.

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