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Topoisomerases II Mutations in Ciprofloxacin-resistant Clinical Isolates of *Pseudomonas aeruginosa* in Makassar, Indonesia

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Abstract **Background**: Ciprofloxacin constitute a clinically successful and widely used class of broad-spectrum antibiotics; however, the emergence and spread of resistance increasingly limits the use of this fluoroquinolone in the treatment and management of microbial disease. Ciprofloxacin is the most frequently used member of the fluoroquinolones during initial eradication therapy of *Pseudomonas aeruginosa*. The emergence of ciprofloxacin-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) has been reported. In this study, we examined mutations in the quinolone resistance-determining regions of the topoisomerase II (GyrA) genes of 11 clinical isolates of *P. aeruginosa* that resistance to ciprofloxacin. **Methods**: In this study we examined the relationship between gene mutations of topoisomerase II (gyrA) with ciprofloxacin resistance of *P. aeruginosa*. The examination performed by disc diffusion test followed by RFLP-PCR. **Results**: The results of disc diffusion test showed that 13 samples were sensitive to ciprofloxacin, 11 samples were resistant to ciprofloxacin. In this study we found mutation in GyrA in 6 (54.5%) of ciprofloxacin resistant samples. There was no mutation found in ciprofloxacin sensitive samples. **Conclusion**: Mutation in topoisomerases genes are the main mechanism of fluoroquinolone resistance in *P. aeruginosa*.

Keywords: *Topoisomerase II (GyrA), Pseudomonas aeruginosa, mutation, PCR-RFLP*

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1. Introduction

Fluoroquinolon was originally developed as an excellent activity against aerobic Gram-negative bacteria, while its activity against Gram-positive organisms is limited. Ciprofloxacin, enofloxacin, lomefloxacin, levofloxacin, ofloxacin are agents that have activity both Gram-negative and of moderate to good activity against Gram-positive bacteria. In this group, ciprofloxacin is the most active agents against Gram-negative, especially *Pseudomonas aeruginosa* [1,2].

Ciprofloxacin is a second-generation agent, as one of the quinolones derivates. Ciprofloxacin inhibit the activity of bacterial DNA gyrase, it has bactericidal activity with a broad spectrum against Gram positive and Gram-negative. Ciprofloxacin effectively used for urinary tract infections, urethritis, typhoid and paratyphoid fever, respiratory infections, soft tissue infections and osteomyelitis. The drug can also be used as antimicrobial prophylaxis in patients with neutropenia [3].

Since the intensive use of ciprofloxacin as one of the most effective quinolones against *P. aeruginosa*, has led to the emergence of resistant strains [4]. The targets of quinolones are considered to be the type II topoisomerases, which are essential enzymes responsible for controlling the topological state of DNA during its replication and transcription. DNA gyrase is composed of two A and B subunits, which are encoded by the gyrA and gyrB genes [5]. Topoisomerases are play essential roles in every aspect of DNA metabolism [6,7]. The quinolone antibiotics inhibit DNA replication by forming complexes of the drug with DNA bound to the topoisomerase enzyme [6]. Quinolones interfere with the activity of DNA gyrase, an essential bacterial type II DNA topoisomerase [8]. Many studies have been demonstrated that mutations conferring fluoroquinolone resistance in *P. aeruginosa* may be due to alterations in DNA gyrase, because of the reduced sensitivity to fluoroquinolone inhibition of DNA supercoiling in fluoroquinolone-resistant isolates and

reduced inhibition of DNA synthesis by fluoroquinolone resistant isolates upon exposure to fluoroquinolones[9]. The gyrA mutations are the major mechanism of resistance to fluoroquinolones [10].

To characterize the prevalence of mutations in type II topoisomerase genes on ciprofloxacin resistance, we analyzed the gyrA gene of *Pseudomonas aeruginosa* isolates with resistance to ciprofloxacin.

2. Methods

2.1. Identification of P. aeruginosa

Specimens from the wound/ulcer, taken using a cotton stick. Specimens were inoculated into the Mac Conkey agar (MC) and incubated at a temperature of 35-37°C for 24 hours. The bacterial colonies on MC were confirmed by Gram staining test to find the Gram-negative rods then followed by biochemical tests to find non-fermenter bacteria. *P. aeruginosa* are non-fermenter, oxidase (+), (+/-), pyocyanin fluorescence (+), glucose (+), xylose (+), (+/-), mannitol lactose (-), maltose (-), 42°C (+), esculin (-), urea (+), D Nase (-), ONPG (-), indole (-), motility (+), flagella (1), H_2S (-), N_2 gas ¬ (+ / -), pigment (brown, green and fluorescence).

2.2. Disc Diffusion Test

The results obtained by measuring the resistance zone formed on the agar. Inhibition zone width compared with NCCLS standard tables in 1997, to determine the bacterial isolates of *P. aeruginosa* resistant, intermediate and sensitive.

2.3. Preparation of DNA

DNA was extracted from freshly collected culture of *P. aeruginosa* according to the diatom-guanidinium isothiocyanate (GuSCN) method. For the extraction of DNA from culture, a freshly colony of *P. aeruginosa* sample was mixed with 900 uL of lysis buffer (50 mM Tris-HCl, 5.25 M GuSCN, 20 mM EDTA, 0.1% Triton X-100) and centrifuged at 12,000 × *g* for 10 minutes. To obtain the DNA, samples were lysed by incubation for 15 minutes at 18°C and 20 uL of diatom suspension was added. The diatom containing the bound DNA was sedimented by centrifugation at 12,000 × g for 15 seconds. The diatom pellet was washed with washing buffer (5.25 M GuSCN in 0.1 M Tris-HCl, pH 6.4), rinsed with 70% ethanol and acetone, and dried by incubation at 56°C for 10 minutes. The pellet was mixed with 60 uLof10 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer and the DNA was eluted by incubation at 56°C for 10 minutes. After sedimentation of the diatom by centrifugation, the supernatant was collected and stored at -20°C until PCR was performed [11].

2.4. PCR Amplification

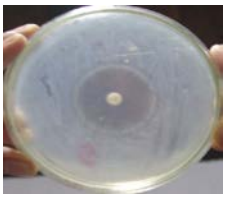
Nucleotide sequence from Gene Bank, *Pseudomonas aeruginosa* gi:459928, accession L29417 were use for PCR amplification [12]. Amplification on a PCR machine at 35 cycles, each cycle of denaturation for 1 minute to do with the temperature 94°C, annealing for 1 min at 65°C for gyrase A and extension for 1 minute at 72°C. Primers used are: Gyrase A forward GACGGCCTGAAGCCGGTGCAC and Gyrase A reverse GCCCACGGCGATACCGCTGGA. Sac II enzyme used for restriction of target amplified PCR product. Amplification results were analyzed using electrophoresis.

3. Results

In this study we examined the relationship between gene mutations of topoisomerase II (gyrA) with ciprofloxacin resistance of *P. aeruginosa*. The examination performed by disc diffusion test followed by RFLP-PCR. The results of disc diffusion test showed that 13 samples were sensitive to ciprofloxacin with an average diameter of inhibition zone 28.31 mm. We also found 11 samples were resistant to ciprofloxacin with average of inhibition zone 8.91mm (Figure 1).

P. aeruginosa isolate in TSIA medium

Disc diffusion test of Ciprofloxacin Resistant P. aeruginosa



Disc diffusion test of Ciprofloxacin Resistant P. aeruginosa

Figure 1. Disc diffusion test of *P. aeruginosa*

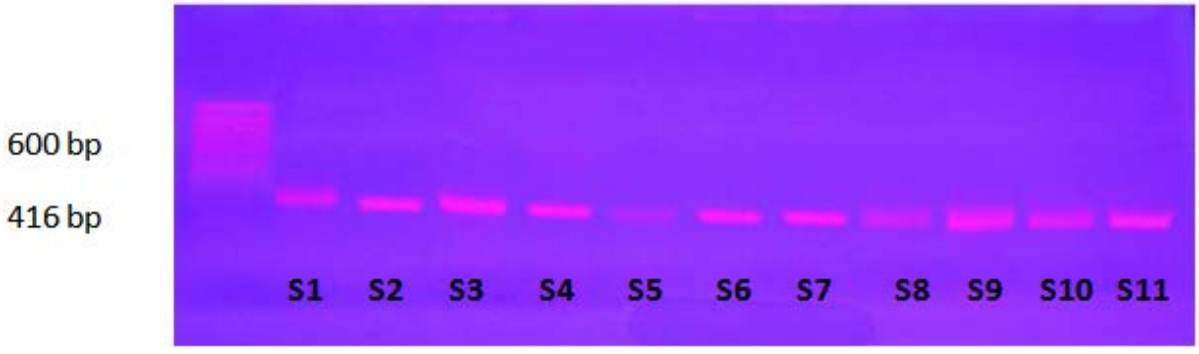


Figure 2. Electrophoresis results of Sensitive Samples (S1-S11) before the restriction

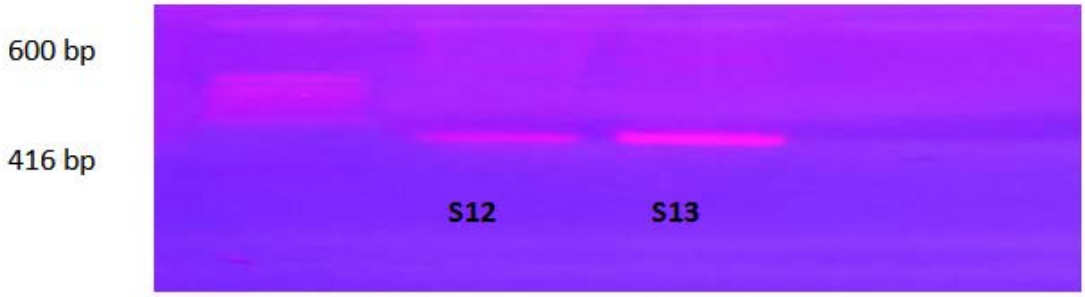


Figure 3. Electrophoresis results of Sensitive (S12 and S13) before the restriction

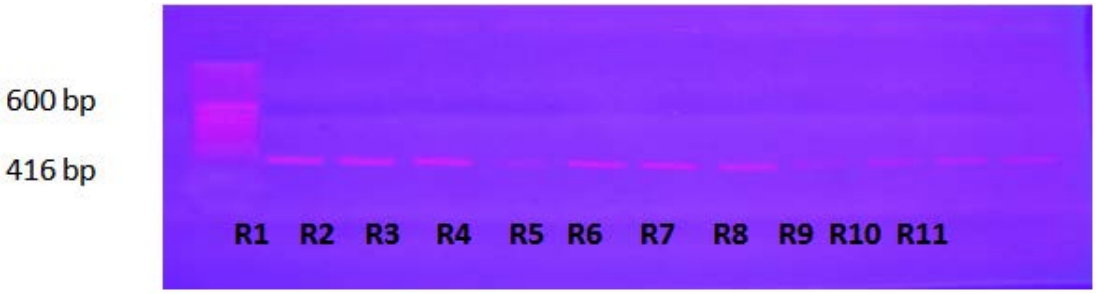


Figure 4. Electrophoresis results of resistant samples (R1-R11) before the restriction

Figure 2-Figure 7 shown illustration of electrophoresis result before and after restriction with the enzymes Sac II. Electrophoresis results before and after RFLP shown in Figure 2-Figure 7. Presence of mutation indicate by single fragment in electrophoresis after samples digested by enzymes Sac II. In this study we found mutation in GyrA in 6 (R2, R4, R5, R7, R8 and R9) of resistant samples (Figure 7).

RFLP-PCR

This study was done with PCR-RFLP using restriction enzymes Sac II to determine the mutations in the existing samples.

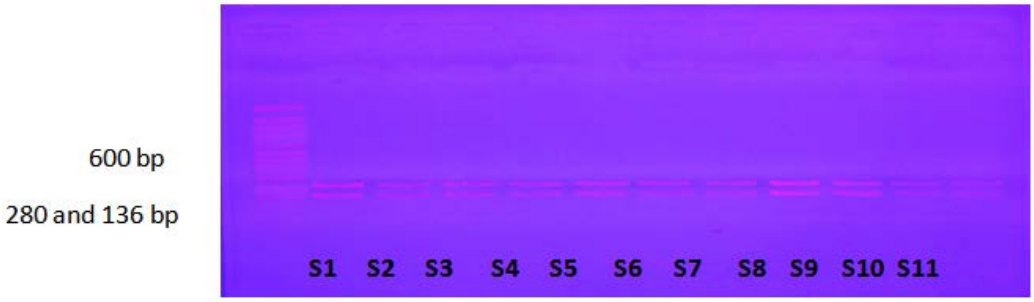


Figure 5. Electrophoresis Results of Sensitive Samples (S1-S11) after restriction enzyme Sac II

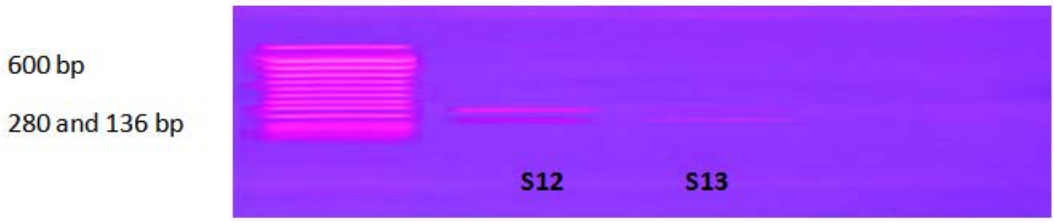


Figure 6. Electrophoresis Results of Sensitive Samples (S12 and S13) after restriction enzyme Sac II. Non Mutant strain indicate by double fragment/band

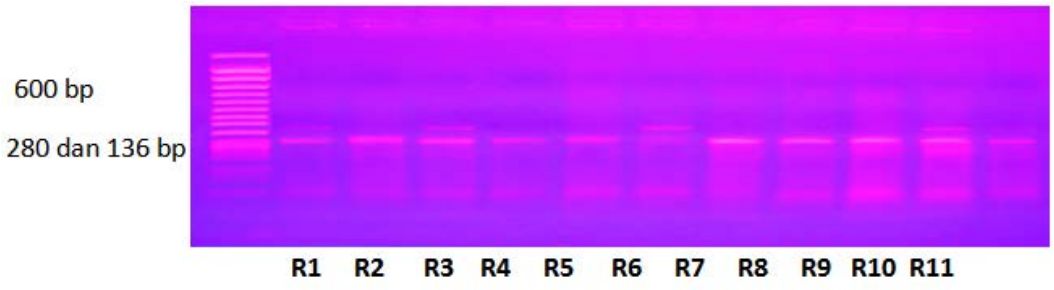


Figure 7. Electrophoresis results of resistant samples (R1-R11) after restriction enzyme Sac II Samples in Slot R2, R4, R5, R7, R8 and R9 were mutant strains, indicate by single fragment/band

4. Discussion

In the present study, we found mutations in type II topoisomerase genes (GyrA) in 6 (54.5%) of 11 ciprofloxacin resistant strains. There was no mutation found in other 5 resistant strains. Our results demonstrate that mutation in topoisomerase II (GyrA) related to presence of *P. aeruginosa* resistant strains. Ciprofloxacin is the most active new quinolone antimicrobial agents. It is bactericidal against a broad spectrum of gram-positive and gram-negative microorganisms, including *Pseudomonas aeruginosa*. Most of *P. aeruginosa* strains are sensitive to ciprofloxacin at 0.5ug/ml. Previous studies have been reports of decreased susceptibility to ciprofloxacin during therapy in clinical studies of *P. aeruginosa* infections [2]. Quinolone resistance mechanisms have been reported in a wide variety of organisms [13]. This resistance related to specific mutations that lead to amino acid alterations in the quinolone-resistance determining regions (QRDRs) [13]. This regions constituting topoisomerase II and topoisomerase IV, which are involve in DNA replication, recombination, transcription and in the partitioning of replicated chromosome. Topoisomerases are the important targets for antibacterial and anticancer drugs [13]. Topoisomerase inhibitors convert topoisomerases into topoisomerase-DNA complex or cleavable complex as a topoisomerase-drug-DNA ternary complex. These ternary complex formation is critical for the cytotoxicity [7]. Mutations in topoisomerases genes are the main mechanism of fluoroquinolone resistance in many bacteria [4,10]. The main mutations for gyrA on codons 83 and 87 found to be related to fluoroquinolone resistance [1,14].

In this study, 5 (45.4%) of 11 ciprofloxacin resistant samples found to be non mutant *P. aeruginosa*. This suggests that there is another mechanism of ciprofloxacin resistance in *P. aeruginosa*. Three mechanisms of resistance are known to cause quinolone resistance in *P. aeruginosa*: alteration in DNA gyrase by mutations in gyrA or gyrB genes, decreased drug accumulation by decreased permeability of the cell wall, and enhanced efflux [10].

5. Conclusion

Presence of mutations in topoisomerase II (gyrase A) by using PCR-RFLP technique suggest that mutation in topoisomerases genes are the main mechanism of fluoroquinolone resistance in *P. aeruginosa*.

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