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Molecular Study of Azithromycin-Resistant *P. aeruginosa*

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Abstract: The aim of the present study was to investigate the occurrence of azithromycin resistance among *P. aeruginosa* swabs isolates. Out of 60 pus, burn and wound swabs, 16 (26.6%) *P. aeruginosa* were isolated. The susceptibilities of the *P. aeruginosa* isolates to the macrolides azithromycin, chloramphenicol and aminoglycosides streptomycin, gentamicin and kanamycin were evaluated by disc diffusion experiments. The azithromycin was observed to be less resistance with high susceptibilities rate 13 (81.25) as compared to aminoglycosides and chloramphenicol. Partial 23S rDNA gene sequences of 12 isolates demonstrate new single base substitution in the resistant isolates in positions A1807G, C1808A, A1823G and A1819G which confer the azithromycin resistance with low frequency rate 0.9 for each one. As we recommend the azithromycin is the drug of choice in *P. aeruginosa* treatment, further study is needed to whole 23S rDNA gene in local isolates to identify mutations outside the partial selected region.

Key words: Azithromycin, 23Srrna, *P. aeruginosa*.

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Introduction

P. aeruginosa is a rod-shaped gram-negative obligatorily aerobic bacterium. *P. aeruginosa* is a worldwide opportunistic pathogen, which causes a wide spectrum of infections and leads to substantial morbidity in immuno-compromised patients. It is one of the three most abundant bacterial species causing nosocomial infections. Despite therapy, the mortality due to nosocomial pseudomonal pneumonia is approximately 70%. (1,6,25). As human *P. aeruginosa* infections are nosocomial in nature, hospital reservoirs of growth are many and include respiratory equipment, solutions, medicines, disinfectants, sinks, mops, food mixers and vegetables (18,26).

In particular, *P. aeruginosa* strains have developed resistance against antibiotics such as fluoroquinolones. As a consequence, this antibiotic has lost its effectiveness. Fluoroquinolones have been widely used for the treatment of *P. aeruginosa* infections in hospitals, and it is known that these bacteria are capable of acquiring resistance during antibiotic therapy (15, 17). Besides the

acquired fluoroquinolone resistance, *P. aeruginosa* has intrinsic resistance to several antibiotics. Once chronic infection is established, *P. aeruginosa* is extremely difficult, if not impossible, to eradicate using conventional antibiotic treatments (8, 22)**.** One antibiotic that has shown promise in treatment against chronic *P. aeruginosa* infections is the macrolide azithromycin (AZM) (16). The antibiotic-resistant *P. aeruginosa* is an important concern in the treatment of long-term infections. Azithromycin treatment has been used for patients chronically infected with *P. aeruginosa* , even though the use of azithromycin on *P. aeuginosa* infections has been found to have beneficial clinical effects (13). It remains unclear how azithromycin works on *P. aeruginosa* and if macrolide resistance can emerge. Treatment of chronic cases of *P. aeruginosa* like CF infections with AZM have shown promise in treatment against in CF. CF patients treated with AZM have shown improvement based on increased lung function and body weight (9, 24). The treatment is controversial, because the exact mechanism of killing of *P. aeruginosa* is unknown (20,24). Azithromycin is concederd in the treatment of *P. aeruginosa*, its mechanism of action as an inhibitor of bacterial protein synthesis, it is less clear how azithromycin ameliorates the disease associated with *P. aeruginosa*, which is considered to be resistant to the drug. Azithromycin inhibited *P. aeruginosa* protein synthesis by 80%, inhibiting bacterial growth. It acts by binding to the 50S ribosomal subunit of susceptible microorganisms and, thus, interfering with microbial protein synthesis (28). Explanation and further understanding of the AZM mechanism against *P. aeruginosa* infections are needed to optimize treatment efficacy. The using 23rRNA sequencing, showed the occurrence of azithromycin resistance among clinical *P. aeruginosa* isolates that associated with specific mutations (A2058G, A2059G, and C2611T in domain V of 23S rRNA and that introduction of A2058G and C2611T into strain PAO1 results in azithromycin resistance (20). Macrolides are a diverse class of antibiotics that inhibit bacterial protein synthesis by binding to the 23S rRNA of the 50S ribosomal subunit (**27**). A macrolide-binding pocket is formed mainly by 23S rRNA domain V nucleotides 2058 and 2059. Alteration of these two key contact sites could cause conformational rearrangements of the binding site of macrolides (19).

Materials and Methods

The study included 60 clinical specimens were taken from different Hospitals in Baghdad during a period of seven months from (October 2012 to May 2013). Clinical specimens used were pus, burn and wound swabs. Each swab taken carefully from the site of infection and placed in tubes containing readymade media to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on *Pseudomonas* isolation agar (Cetramide) (Hi-Media, Mumbai, India). All plates were incubated aerobically in incubator at 37ºC for 24 hrs. Identification was done by using colony morphology, Gram stain, motility, oxidase, citrate utilization, and pyocyanin production (12).

Antibiotic Disk Diffusion Method

Antibiotic susceptibility was done on Mueller and Hinton agar by single disc diffusion method, using Azithromycin 15µg, chloramphenicol 30µg, streptomycin 10µg, gentamicin 10µg and kanamycin 30µg (Hi-Media, Mumbai, India), tested the resistance of the isolates toward the antibiotics as described by Kirby & Bauer (3)**.** The end point, was compared with zones of inhibition determined by Clinical and Laboratory Standards Institute (**7**).

DNA Extraction

Genomic DNA extraction carried out based on Automated method using ExiPrep 16 Plus (Bioneer, Republic of Korea). 0.2 ml of the fresh bacterial culture were predicated by centrifugation 6000 RPM for 10 min. The pellet was resuspended with the lysis buffer (Provided by manufacturing company) and incubated at 37˚C for 30 min then loaded to extraction cartridge (Provided by manufacturing company). DNA was eluted by 50 µl elution buffer (Provided by manufacturing company). The DNA sample measured for their concentration and purity using Microvolume UV Spectrophotometer (ACTGene, USA).

DNA Sequencing and Inseleco

The primers 23srnaF: TTGAGCCCCGTTACATCTTC and 23srnaR: GGGGAACCCACCTAGG ATAA were designed, based on the 23S ribosomal RNA gene sequence in the GenBank (accession no.Y00432). PCR was performed in a 50 µl mixture containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl2, 50 mM KCl [pH 9]) (Merck, India), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Merck, India), 10 pM each of forward and reverse primers, and 100 ng of templet DNA. The program for PCR included an initial denaturation 94°C for 5min, 30 cycles of denaturation at 94°C for 60s, annealing at 58°C for 60s, extension at 72°C for 60 min. and a final extension at 72 °C for 7min. The PCR products were resolved on a 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and bands observed using a gel documentation system (ATTO, Japan). PCR products were sent for sequencing at Bioneer company, Korea. The generated sequences were compared and analyzed against the standard sequences in the GenBank by the MEGA4 software (26) to identify any probable mutation.

Results and Discussion

Sixteen (26.6%) isolates were recovered from 60 clinical swabs. Table (2) showed no inhibitory effect of azithromycin in 3(18.75%) isolates, interpreted according to the following criteria as reported by Clinical and Laboratory Standards Institute of the standard single-disk susceptibility test with a 15µg azithromycin disk, table (1). Whereas, the aminoglycosides streptomycin and kanamycin showed high resistance rate 16 (100%), followed by chloramphenicol and gentamycin 9 (56.25%), 7 (43.75%) respectively.

Table 1: Interpretation of Azithromycin Zone of Inhibition

Zone Diameter (mm)	Interpretation
≥ 18	Susceptible (S)
14-17	Intermediate (I)
≤ 13	Resistant (R)

P. aeruginosa emerged as an important pathogen and responsible for the nosocomial infections that is one of the important causes of morbidity and mortality among hospital patients. In our study the resistance pattern against Azithromycin was observed to be less as compared to aminoglycosides and chloramphenicol, table (2). These finding are in good agreement with the other similar studies (21). Furthermore, high rate of resistance to aminoglycosides and chloramphenicol recorded in this study appears to confirmed by a previous study (4). This could be attributed to the selective pressure of drugs usage, which should be controlled by successful application of infection control measures (11, 29). Regarding aminoglycosides, the sub-inhibitory concentrations induces both swimming and swarming of *P. aeruginosa*, increased level of expression of *mexXY* efflux pump genes and *aminoglycoside response* (*arr*) gene, which contributes to biofilm-specific aminoglycoside resistance (14). However *P. aeruginosa* is usually intrinsically resistant to chloramphenicol, in part due to the MexAB-OprM efflux system. Sub-inhibitory concentrations of macrolides cause substantial inhibition of the synthesis of virulence factors, including those implicated in QS regulation, killing of stationary-phase and/or biofilm-forming cells, and synergism with other antimicrobials and with serum complement (10, 29).

Table 2: Zone of inhibition of 12 *P. aeruginosa*

Samples	Zone of inhibition				
	AZM 15 µg	K 30 µg	Gn 10 µg	S 10 µg	C 30 µg
D1	S	R	S	R	R
D2	S	R	S	R	R
D3	S	R	S	R	R
D4	S	R	S	R	S
D5	S	R	S	R	R
D6	S	R	S	R	R
D7	S	R	S	R	R
D8	S	R	S	R	S
D9	S	R	R	R	S
D10	R	R	R	R	S
D11	S	R	R	R	S
D12	R	R	R	R	R
D13	R	R	R	R	S
D14	S	R	R	R	R
D15	S	R	S	R	R
D16	I	R	R	R	S
%Total of resistance	18.75	100%	43.75%	100%	56.25%

S:sensitive,S: S: sensitive, R: resist, I: intermediate. D1-D16: Number of samples. C: Chloramphenicol, AZM: Azithromycin, K: Kanamycin, Gn: Gentamycin, S: Streptomycin.

DNA Extraction, PCR Amplification for 23sRNA and Sequencing

The concentration and purity of total DNA extracted for each samples was measured by NanodropD-1000 (Thermo scientific, USA), it was in range (93-1238ng/µl) with purity of (1.6-2). The genomic DNA was amplified in PCR with specific primer to investigate partially amplified fragment of 23sRNA gene. The results showed that the band was approximate (900bp) compare with ladder (100bp) as shown in figure (1).

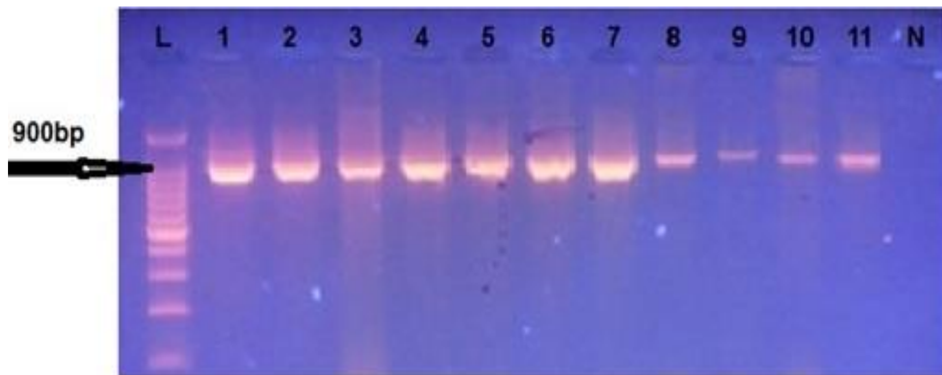

Figure 1: Agarose gel electrophoresis of 900bp PCR product of 23sRNA fragment amplified using F&R primers by electrophoresis on 2% agarose gel, 90V, 1hr. in TBE buffer and stained by Ethidium bromide under transilluminator UV. Lane L: 100 bp DNA ladder, lane 1,2,3,4,5,6,7,8,9,10 and 11: 900 bp PCR product of 23s DNA., lane N: negative control

Twelve isolates were selected for sequencing. As shown in figure (2), only 2 isolates showed base substitution and changing the amino acid sequence among 11 isolates, one of them was neglected due to poor or trace data. The target site for macrolides is the large (50S) subunit of the bacterial ribosome. Many cases of macrolide resistance in clinical strains can be linked to alteration of specific nucleotides in 23S rRNA within the large ribosomal subunit (**5**), Since the discovery of *erm* genes, another means of resistance involving alteration of rRNA structure has been identified. Under laboratory conditions, single base substitutions introduced into rRNA were shown to confer macrolide resistance. This form of resistance was first observed in the single rRNA (*rrn*) operon of yeast mitochondria, which was mutated at position A2058 in the large-subunit rRNA (23). We sequenced and analyzed partial 23S rDNA gene of 12 clinical *P. aeruginosa* isolates (figure2) to identify the mutations and their relation to Azithromycin reistance. Upon comparing the sequence of the partial 23S rRNA gene of the *P. aeruginosa* reference genome (GenBank, accession no. Y00432), to our isolates sequence gene and single nucleotide polymorphisms were called by Bio Edit, eligment use clustalW, we identified substitutions corresponding to positions A1807G, C1808A, A1819 G and A1823G. the base substitution A to G in position 1823 shift the amino acid Asparate (D) to Glycin (G) in S10, while S13 showed base pair substitution at position 1807, 1808 and 1819 which shift the amino acid Threonin (T) to Glutamate (E). The rate of mutation related to presence copy numbers of rRNA operons, so this may explain the low mutation rate 0.9. In our isolates especially the *P. aeroginosa* has 4 copy number, this in similarity with a study that showed a high potential for macrolide resistance to occur by mutations in the 23S rRNAs of the bacteria with low copy number than that with high copy number and the probability of resistance developing would of course depend on the types and

quantities of drug to which these organisms are exposed (5).

The emergence of antibiotic-resistant *P. aeruginosa* is an important concern in the treatment of such cases due to significant changes in microbial genetic ecology, as a result of superficial use of anti-microbials. Antibiotic resistance is a growing clinical problem which threat the public health especially if colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal (2). Hereafter, results from laboratory studies should be combined with evidence from clinical trials in order to summarize the information on the mode of action of AZM in *P. aeruginosa* infection, so as to generate data that would help clinicians to choose the correct practical treatment. The accessibility of antibiotics in shops and open markets as well as consumption of drugs without proper medical prescription, is probably an important factor worthy for consideration. Routine sensitivity screening of antibiotics before prescription is suggested.

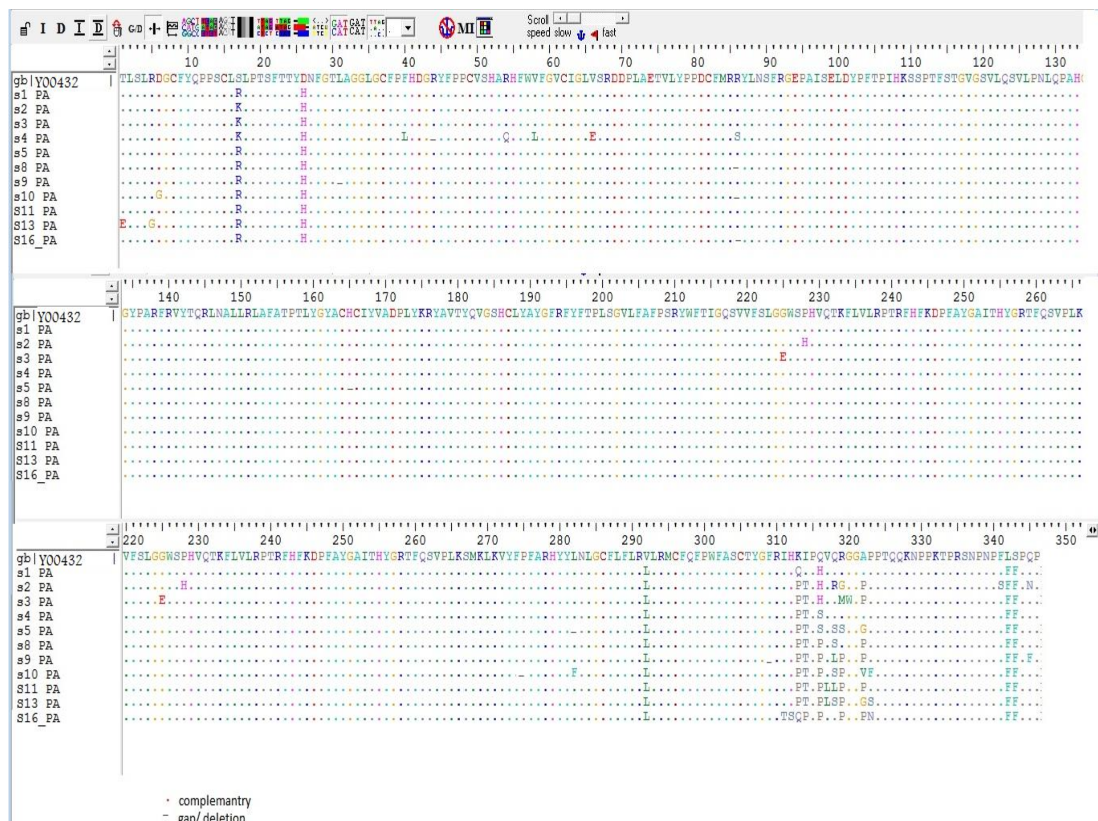

Figure 2: Aligment of partial 23SrDNA peptide sequences from 11 *P. aeroginosa* strains including the *P. aeroginosa* reference strain (AC NO.: Y00432)

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