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ORIGINAL ARTICLE

Ciprofloxacin Resistance due to *gyr A* Mutation in *Pseudomonas aeruginosa* Isolates at Zagazig University Hospitals

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ABSTRACT

Background: *Pseudomonas aeruginosa has developed resistance to multiple antibiotics. Unfortunately, ciprofloxacin-resistant P.aeruginosa has emerged with multiple resistance mechanisms.* ***Objectives:*** *This study aims to determine the frequency of gyrA (Topoisomerase II) mutation and its contribution to ciprofloxacin resistance in P. aeruginosa isolates from patients at Zagazig University Hospitals.* ***Methodology****: Cultivation of pus specimens, identification of isolates by Gram stain, oxidase test and API20NE. Antibiotic susceptibility testing and ciprofloxacin minimal inhibitory concentration (MIC) were done for all isolates. PCR –RFLP was used to detect mutation in gyrA gene.* ***Results****: Among 192 examined specimens, 30 (15.6%) P. aerguinosa were isolated mainly from Surgery and Burn Unit. Fourteen isolates (46.7%) were resistant to ciprofloxacin. Ten (71.4%, P<0.001) isolates had shown mutation in gyr A gene by PCR-RFLP.* ***Conclusion:*** *Mutation in gyrA gene is a major mechanism of ciprofloxacin resistance in P. aeruginosa.*

INTRODUCTION

Pseudomonas aeruginosa is a Gram negative bacterium that can be isolated from hospital environment as well as from other environments[1].

Immune compromised patients, burn patients and patients with metabolic disorders are considered at risk for infection with that opportunistic pathogen[2]. It is also a common cause of surgical wound infections, blood stream infections, ventilator associated pneumonia and urinary tract infections in catheterized patients as a result of biofilm formation[3].

Treatment of *P. aeruginosa* infections is difficult due to its natural resistance to antibiotics. The excessive use of broad spectrum antibiotics has led to emergence of highly resistant strains associated with increased morbidity, mortality and cost of patient care[4].

Fluoroquinolones have been widely used in the treatment of *Pseudomonas* infections because of good antibacterial activity, tissue diffusion, and oral bioavailability. Ciprofloxacin is a second-generation agent of quinolones derivatives. It inhibits the activity of bacterial DNA gyrase, type II DNA topoisomerase, thus inhibiting DNA transcription and replication[5].

Fluoroquinolones inhibit this enzyme by stabilizing the DNA-DNA gyrase complex. So, replication fork could not move any more with irreversible DNA-strand breaks generation causing damage to DNA resulting in cell death due to inhibition of DNA synthesis [6].

The aim of our study was to determine the frequency of *gyrA* mutation encoding the DNA gyrase and its contribution as a mechanism of ciprofloxacin resistance in *P. aeruginosa* isolated from Zagazig University Hospitals, Egypt.

METHODOLOGY

Samples and bacteriological methods

A cross-sectional study was carried out over one year in Microbiology and Immunology Department, Faculty of Medicine, Zagazig University. The study was approved by the Institutional Review Board (IRB), Faculty of Medicine, Zagazig University, Egypt. Informed consent from each patient was obtained.

One hundred and ninety two pus samples were collected from wounds, ulcers and bed sores from patients admitted at Surgery and Burn Unit, Surgical ICU, Orthopedic unit, Medicine ICU and Oncology Unit at Zagazig University Hospitals. All samples were examined by Gram stain, cultivated on MacConkey agar plates (Oxoid, UK) and incubated at 37°C for 24 hours aerobically. Gram negative, lactose non fermenting colonies were further identified using oxidase test, triple sugar iron (Oxoid, UK) and API 20 NE (Bio-Merieux, Marcy L Etoile. France).

Antibiotic sensitivity tests:

Isolates presumptively identified as being *P. aeruginosa* were tested for antibiotic sensitivity by disc diffusion method according to Clinical Laboratory Standards Institute guidelines[7]. Ciprofloxacin disc 5ug

(Oxoid, UK) was included to detect ciprofloxacin-resistance. Minimal inhibitory concentration (MIC), using ciprofloxacin E-test strip with concentrations ranged from 0.002 – 32 µg/ml (Bio-Merieux, Marcy L Etoile, France), was also determined.

PCR-REIP:

Using QIAamp® DNA Mini kit (Qiagen, Germany), DNA extraction from isolated colonies was done. PCR amplification was performed in 35 cycles. Each cycle consisted of "denaturation for 1 minute at 94°C, annealing for 1 min at 65°C for *gyrase A* and extension for 1 minute at 72°C". Primers (iNtRON Biotechnology, Korea) used were: "Gyrase A forward GACGGCCTGAAGCCGGTGCAC and Gyrase A reverse GCCCACGGCGATACCGCTGGA". *Sac II* enzyme (Thermoscientific, USA) was used for restriction of target amplified PCR product[8]. Amplification results and fragments lengths were analyzed using electrophoresis with a suitable DNA marker.

Statistical analysis:

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. A P-Value<0.05 was considered to be statistically significant at 95% confidence interval. Chi-square test was used to compare proportions.

RESULTS

Out of 192 collected pus specimens, 30 *P. aeruginosa* were isolated with an isolation rate of 15.6%. Table 1 demonstrates the different isolation rates among different hospital wards where the highest rate was obtained from Surgery and burn unit (73.3%), followed by Surgical ICU (13.3%), Orthopedic unit (6.7%) and then both Medicine ICU and Oncology Units with 3.3% for each. This was statistically significant (P < 0.05).

Table 1: Distribution of isolated *P. aerguinosa* in different hospital wards

Hospital ward	No. of samples	No. and % of isolates		X^2	P value
		No.	%		
Surgery and burn unit	84	22	73.3	12.87	0.01*
Surgical ICU	49	4	13.3		
Orthopedic unit	37	2	6.7		
Medicine ICU	13	1	3.3		
Oncology unit	9	1	3.3		
Total	192	30	100.0		

*** Significant difference**

On testing for ciprofloxacin resistance, 46.7% of *P. aeruginosa* isolates (14/30) were found to be resistant by disc diffusion method (table 2). Regarding the MIC results, isolates that had a MIC of 2 µgm/ml or more, were considered resistant (n=14).

Table 2: Frequency of ciprofloxacin-resistant *P. aeruginosa* by disc diffusion

Resistant No. (%)	Susceptible No. (%)	Total No. (%)
14 (46.7)	16 (53.3)	30 (100)

The frequency of *gyrA* mutation was assessed among both ciprofloxacin-resistant as well as susceptible isolates by PCR-RFLP analysis. Mutant gene was found to be significantly (P < 0.001) more prevalent in resistant isolates (71.4%) compared to susceptible ones (0.0%) (table 3, fig 1 & 2).

Table 3: Frequency of mutant type II topoisomerase gene (*gyrA*) by PCR-RFLP analysis among *P.aerguinosa* isolates

Isolates (n=30)	Mutant gene		Non –mutant gene		X^2	P value
	No.	(%)	No.	(%)		
Quinolone resistant isolates (n=14)	10	(71.4%)	4	(28.6 %)	13.65	<0.001*
Quinolone sensitive isolates (n=16)	0	(0.0%)	16	(100.0%)		

***A high statistical significant difference**

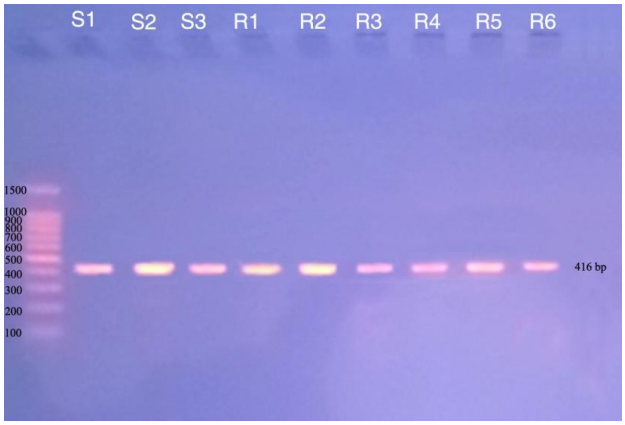

Fig 1: *gyrA* gene amplification before RFLP.
Lane 1: molecular weight marker, Lane 2-4: *gyr A* gene amplicon of 3 sensitive *P. aerguinosa* before RFLP, Lane 5-10: *gyr A* gene amplicon of 6 ciprofloxacin resistant *P. aerguinosa* before RFLP.

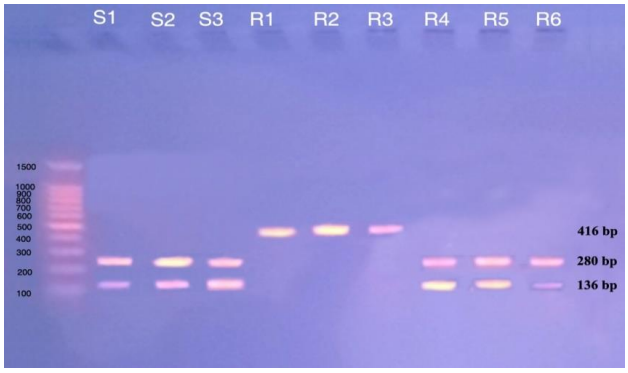

Fig 2: *gyrA* gene digestion fragments after RFLP
Lane 1: molecular weight marker, Lane 2-4: *gyr A* gene amplicon of 3 sensitive *P. aerguinosa* with non mutant gene showing 2 bands; 280bp and 136bp, Lane 5-7: *gyr A* gene amplicon of 3 ciprofloxacin resistant *P. aerguinosa* with mutant gene showing one band; 416bp.Lane 8-10: *gyr A* gene amplicon of 3 ciprofloxacin resistant *P. aerguinosa* with non mutant gene showing 2bands; 280bp and 136bp

DISCUSSION

For the treatment of *P. aeruginosa* infections, fluoroquinolones such as ciprofloxacin are a cornerstone class of antibacterial agents that could be used. However, the widespread application of these agents has resulted in increasing levels of bacterial resistance. The principle mechanism of fluoroquinolones resistance in *P. aeruginosa* involves mutations in the genes of DNA gyrase and topoisomerase IV[9].

In our study, the rate of *P. aerguinosa* isolation (15.6%), comes in agreement with the rates reported previously in Egypt by Gad and his colleagues[10] who detected 81 *P. aerguinosa* isolates out of 445 hospital samples with a percentage (18.2%) and Hassuna and his colleagues [11]who detected 20% isolation rate (50 *P. aerguinosa* out of 250 pus samples).

Among different hospital wards, the highest prevalence of *P.aerguinosa* was detected in samples collected from Surgery and Burn Unit (73.3%) followed by Surgical ICU Unit (13.3%), Orthopedic Unit (6.7%), then equal prevalence in Medicine ICU and Oncology Unit (3.3%) with a statistically significant difference (p=0.01). These results were in keeping with previous two Egyptian studies where Mansour and his colleagues [12] declared that the highest isolation rate of *P.aerguinosa* was from burn exudates (66.7%) and Gad and his colleagues[10] who detected 72% of their *P.aerguinosa* isolates from burn exudates. This appears logical as burn patients are more prone to infection due to skin deficiency, long hospital stays and multiple invasive operations.

Sensitivity to ciprofloxacin was tested by the Kirby–Bauer disk diffusion method and E test according to Clinical Laboratory Standards Institute guidelines[7], we found 46.7 % (n=14) of *P. aeruginosa* isolates were resistant to ciprofloxacin versus 53.3% (n=16) were sensitive. This comes in accordance with Rieuwpassa and his colleagues [8]who found 11 sensitive isolates out of 24 with a percentage of 45.8 % versus 54.1% resistant to ciprofloxacin (n=13) and Avsar and Yegin [5] who found 21 samples (38.9%) sensitive and 33 samples (61.1%) resistant to ciprofloxacin. In contrast, other studies have found lower level of ciprofloxacin resistance and recommended it for routine use among *P.aeruginosa* isolates. Anil and Shahid[13]had found that 72.41% of their *P. aeruginosa* isolates were susceptible to ciprofloxacin with 27.6% found to be resistant. Also Koirala and his colleagues[14] found that 70.3% of examined *P. aeruginosa* were sensitive to ciprofloxacin. Furthermore, Corona-Nakamura and his colleagues [15] showed that *P. aeruginosa* was absolutely susceptible to ciprofloxacin (sensitivity 100%).This controversy could be attributed to the continuous development of multidrug resistant strains of *P. aeruginosa* in different parts of the world due to uncontrolled use of ciprofloxacin in treatment of *P. aeruginosa* infections.

In order to determine the frequency of *gyrA* gene mutation among the isolated *P. aeruginosa* and its contribution to ciprofloxacin resistance, a PCR-RFLP analysis was performed. We detected the mutant gene in 10 resistant isolates out of 14 resistant isolates (71.4%) while no mutant gene at all was detected in the sensitive isolates with a highly significant statistical difference (p<0.001) which indicated a strong relationship between resistance of *P. aeruginosa* clinical isolates to ciprofloxacin and mutation in the gene encoding DNA gyrase enzyme.

These findings come in keeping with Avsar and Yegin[5] who found *gyrA* mutation in 51.5% of ciprofloxacin resistant isolates, and found it absent in ciprofloxacin sensitive one. This is also consistent with Rieuwpassa and his colleagues[8]who found *gyrA* mutation in 54.5% of ciprofloxacin resistant isolates.

In our study, 4 out of 14 (28.6%) ciprofloxacin resistant isolates were non mutant *P. aeruginosa* which suggests another mechanism of ciprofloxacin resistance such as alteration in DNA gyrase by mutations in *gyrB* genes and topoisomerase IV, decreased drug accumulation by the innate impermeability of the membrane or over expression of efflux pump system[9].

CONCLUSION

Mutation in type II topoisomerase gene (*gyr A*) is frequent among ciprofloxacin-resistant *P. aerguinosa* isolates in Zagazig University Hospitals suggesting it a key mechanism for ciprofloxacin resistance.

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Conflict of interest: None
Limitations: None

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