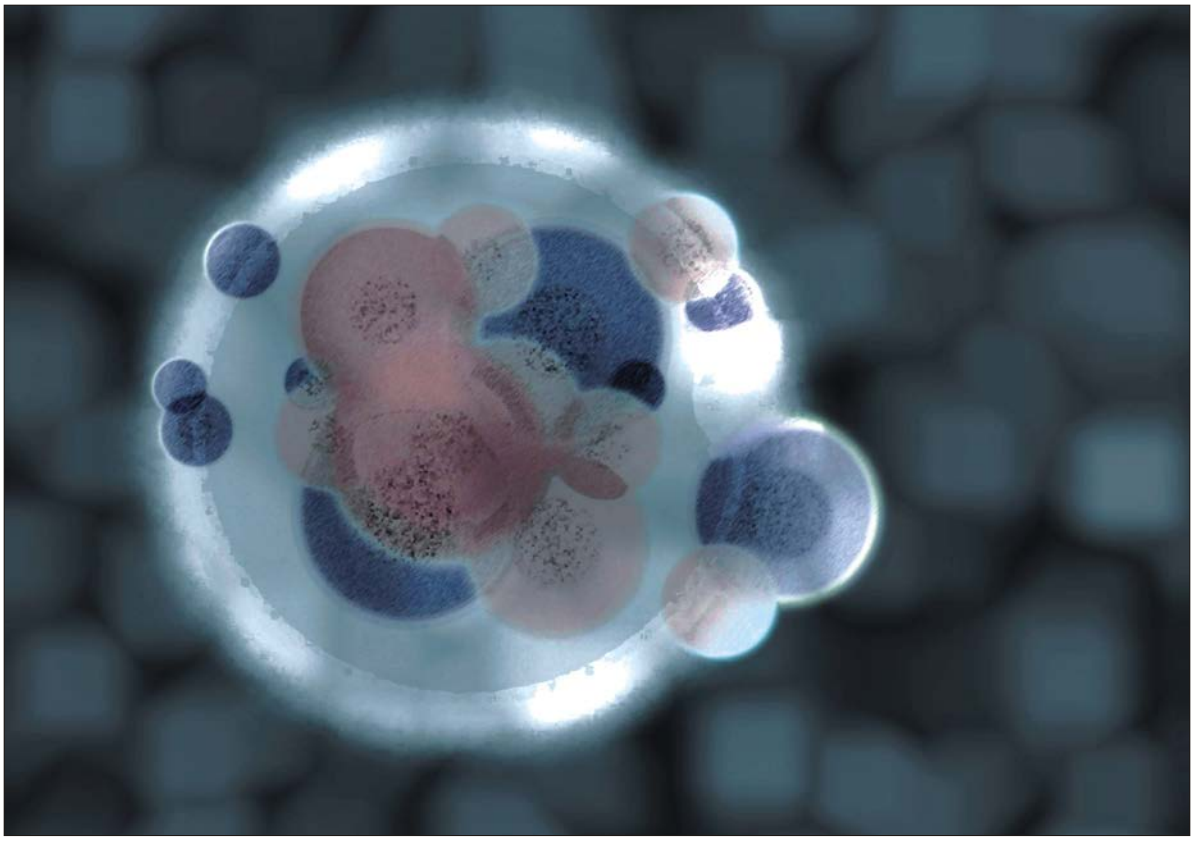

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Research Article
Investigation of Plasmid-Associated Fluoroquinolone Resistance in Nosocomial *Pseudomonas aeruginosa* Isolated from Infected Burn Wounds

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Abstract

Background and Objective: In recent years, there has been an increasing interest in discovering the role of plasmids in emerging fluoroquinolones resistance implicated in serious difficulties of nosocomial infections' therapy. Therefore, this study aimed to explore the association of *Pseudomonas aeruginosa* plasmid DNA in emerging fluoroquinolone resistance. **Materials and Methods:** Thirty *P. aeruginosa* isolates were diagnosed in 45 specimens collected from patients with infected burns who attended the local hospitals in Baghdad. Antibiotic susceptibility of the studied isolates to different fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin and ofloxacin) has been investigated and showed variable responses. Plasmid DNA profiles of *P. aeruginosa* cells were also investigated utilizing QIAprep Spin Miniprep Kit and distinguished by agarose gel electrophoresis. In order to investigate the association between the fluoroquinolone resistant isolates and their content of plasmid DNA, two strategies were adopted: (1) SDS-based plasmid curing technique and (2) Bacterial transformation by plasmid DNA. **Results:** The results illustrate that the plasmid elimination from *P. aeruginosa* progeny cells has slightly increased fluoroquinolones response in comparison to the parental cells and Ciprofloxacin was the more susceptible antibiotic to *P. aeroginosa*. Further evidence was obtained from transformation of TOP10 *Escherichia coli* by *P. aeruginosa* extracted plasmids. Transformed *E. coli* cells exhibited resistance to some of the fluoroquinolones (Ciprofloxacin). **Conclusion:** The findings suggested that *P. aeruginosa* plasmid content could be a preliminary determinant for fluoroquinolone-based therapeutic regimens of nosocomial infections caused by *P. aeruginosa*.

Key words: *P. aeruginosa*, plasmid elimination, ciprofloxacin, transformation, fluoroquinolone, nosocomial infections, antibiotic susceptibility

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas aeruginosa is a leading nosocomial pathogen causing an emergence of multidrug resistance[1] and thus higher rates of nosocomial mortalities, especially in hospitalized burn individuals[2]. Beside their use in treating a variety of bacterial infections, such as respiratory, urinary tract and joint and bone infections, typhoid fever, septicaemia, bacterial gastroenteritis and gynecological infections[3,4], fluoroquinolones are widely administrated to treat burn-related infections caused by *P. aeruginosa* alongside with other drugs of choice[5]. Nonetheless, emergence of fluoroquinolones-resistant strains of *P. aeruginosa* represents a serious growing medical problem that challenges their clinical use[6,7]. Mutations in drug efflux pumps regulated genes as well as DNA gyrase and topoisomerase IV encoding genes, gyrA and parC, respectively, are the main molecular mechanisms leading to fluoroquinolones resistance in these pathogenic bacteria[8]. It is well established that these mechanisms are chromosomally regulated[9]. However, recent years witnessed growing evidence about the fluoroquinolone regulated genes carried by extra chromosomal plasmid DNA in nosocomial Enterobacteria members[10], in particular *Klebsiella pneumonia* and *Eschirechia coli*[11,12]. However, a modicum is reported regarding the relation between the plasmid content of nosocomial *P. aeruginosa* regarding emergence of their antimicrobial resistance to fluoroquinolones[13,14]. In addition, the available evidence was even ambiguous in terms of determination the direct link between nosocomial *P. aeruginosa* plasmids and their response to the fluoroquinolones[15]. Since exploring such association, which may stand behind decreasing the fluoroquinolone cellular response of *P. aeruginosa*, represents unmet clinical need particularly in Iraqi hospitals, the aim of this study was, therefore, to investigate the plasmid implication in emergence of fluoroquinolones resistance in nosocomial isolates of *P. aeruginosa*.

MATERIALS AND METHODS

Sample collection and bacterial isolation: This study has been conducted within one year of time starting from September, 2017. In order to explore the nosocomial infections with *P. aeruginosa*, two hospitals located in Baghdad (AL-Yarmook Teaching Hospital and Imamein Kadhimein Medical city) were targeted in this research. Forty five clinical samples had been autonomously collected from individuals with infected burn wounds. The samples were stored in the hospitals' laboratories, where they obtained from. The collection was performed during the period of 5\12\2016-18\2\2017. Accordingly, no ethical approval was needed since the routine microbiological investigation was taken place in the hospitals' laboratories.

Bacterial diagnosis: The selective condition of 42 °C overnight incubation on 0.3% Cetrimide agar was applied for the preliminary detection. A single isolated colony of each isolate was subjected for confirmative routine diagnosis of *P. aeruginosa* by conducting; (1) Oxidase (10 mg mL^{-1} tetramethyl para phenylenediamine dihydrochloride) and Catalase (3% hydrogen peroxide) tests and (2) Biochemical tests based on the principles of microbiological techniques[16].

Antimicrobial susceptibility: The examined isolates were tested for investigating their antimicrobial response using disc diffusion method and Minimal Inhibitory Concentrations (MICs). Inocula taken from overnight grown colonies of examined isolates were suspended in PBS buffer and adjusted in accordance to 0.5 Mc Farland standard to obtain approximate rate of 1.5×10 CFU mL^{-1}. About 0.1 mL of bacterial suspension of each isolate was seeded on either Muller Hinton broth or agar plates by spreading method. The antibiotic discs of ciprofloxacin, levofloxacin, Nalidixic acid, norfloxacin and ofloxacin were applied and the plates were incubated for overnight. The standard strain "*P. aeruginosa* ATCC 27853™" was utilized for quality control of antibacterial susceptibility test purpose. The findings were read based on the CLSI and EUCAST 2018 Guidelines.

Plasmid DNA extraction: Plasmid DNA was extracted utilizing QIAprep Spin Miniprep Kit (Catalogue No. 27104-QIAGEN) according to the manufacture protocol. In brief, 2 mL of overnight grown bacteria in LB broth was spun at 5000 rpm for 10 min. The bacterial cell pellet was re-suspended with 2 mL of TE buffer by vigorous shaking. About 0.4 mL of the provided Lysis Buffer was added to the re-suspension and mixed carefully. About 0.6 mL of cold Neutralization Buffer was added to the mixture and then applied to the centre of the spin column after converting the colour from blue to yellow by inverting several times. The yellowish mixture was spun down at 13,000 rpm for 1 min for removing retained lysate. After washing with 400 µL of Wash Buffer at 13,000 rpm for 1 min, the spin column was transferred into a 1.5 mL microfuge tube for plasmid DNA elution by 40 µL TE buffer at 13,000 rpm centrifugation for 2 min.

Plasmid curing: Chemical-based sodium dodecyl sulphate (SDS) method was adopted to eliminate the plasmid DNA from their bacterial hosts. About 10 mL Luria Bertani Broth (LB) was inoculated with a single colony of each selected *P. aeruginosa* isolate then incubated for overnight at 37°C in a shaking incubator. About 0.1 mL of the culture was grown in 1 mL of 10% SDS in LB medium[17]. The plasmid curing efficiency was confirmed by agarose gel electrophoresis. The antibiotic susceptibility test of cured bacterial cells was carried out.

Bacterial transformation with plasmid DNA: In order to gain further evidence about the association of the genetic content of plasmid DNA in augmentation of cellular resistance to fluoroquinolones. One Shot TOP10 Competent *E. coli* bacterial cells (Catalogue No. C4040-03-Life technologies) were transformed with the plasmid content based on the manufacture protocol. Briefly, 2 µL of 50 ng µL plasmid DNA was added into the competent *E. coli* cells contained vial. The mixture was incubated on ice for 30 min. Afterward, the cells were exposed to heat-shock for 30 sec at 42°C without shaking. The vial was re-incubated on ice for 2 min. Subsequently, 500 µL of pre-warmed SOC broth was added to the vial under sterilised conditions and then incubated in a shaking incubator at 37°C for 1 h at 200 rpm. Finally, 100 µL from transformation mixture was inoculated on a pre-warmed selective medium plate (Luria-Bertani medium contained 100 µg mL^{-1} ciprofloxacin).

Statistical analysis: Chi-squire tests were conducted for statistical analyses of the study results, where p-values of less than 0.05 have been considered as significant.

RESULTS

Based on the screening analysis, 67% of *P. aeroginosa* isolates (n = 30) were detected in the collected clinical specimens. The antimicrobial sensitivity test of the detected isolates showed variable responses to the examined fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin and ofloxacin). However, the common susceptibility trend demonstrates that most of the isolates possess moderate to high resistance to the studied antibiotics and Ciprofloxacin was the more susceptible antibiotic to *P. aeroginosa* (Table 1).

Based on their resistant to the selected antibiotic marker (Ciprofloxacin), the most resistant *P. aeroginosa* isolates (n = 6) were subjected for preliminary molecular analysis. The plasmid DNA profiles of the selected isolates were investigated. The results demonstrate that the examined bacterial cells contain distinctive plasmid profiles ranged between 6-2.5 kbp (Fig. 1).

In order to explore the implication of the plasmid DNA in augmentation of fluoroquinolone resistance, the investigated *P. aeruginosa* bacterial cells were subjected for eliminating their plasmid content using SDS-based chemical treatments. The agarose gel electrophoresis analysis revealed that the plasmid curing was significantly efficient ($p<0.05$) with elimination rate ranged between 80-90% (Fig. 2). It was also illustrated from this figure that SDS-based chemical method was efficient to eliminate the bacterial plasmid content. The cured daughter *P. aeruginosa* cells exhibited

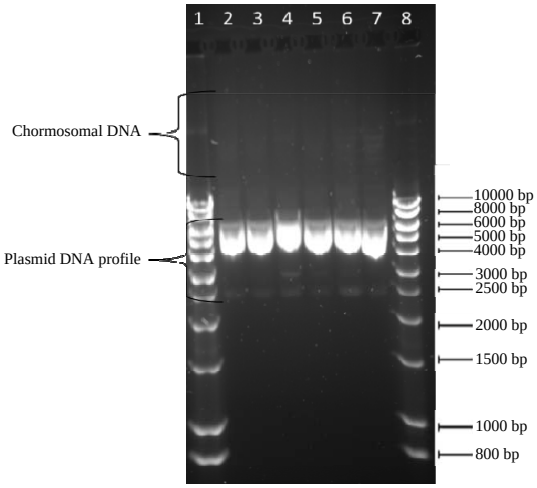

Fig. 1: Gel electrophoretic figure depicting plasmid profile extracted from the most ciprofloxacin resistant *P. aeroginosa* isolates
Electrophoretic lanes represent, 1 and 8: HyperLadder DNA markerand, 2-7: Plasmid DNA profiles of the examined *P. aeroginosa* isolates, which ranged between 6-2.5 kbp

Table 1: Susceptibility testing results of Fluoroquinolone antibiotics (Ciprofloxacin, Levofloxacin, Norfloxacin and Ofloxacin) alongside the zone diameter breakpoint guide

		Zone diameter breakpoint (mm)			Susceptibility testing results		
Fluoroquinolones	Disk content (µg)	S ≥	I	R ≤	Susceptible strains	Intermediate strains	Resistant strains
Ciprofloxacin	5	26	25-17	16	6 (20%)	18 (40%)	6 (20%)
Levofloxacin	5	22	21-13	12	0 (0%)	17 (56.7%)	13 (43.3%)
Norfloxacin	10	17	13-16	12	3 (10%)	22 (73.3%)	5 (16.7%)
Ofloxacin	5	16	13-15	12	1 (3%)	19 (63.3%)	10 (33.3%)

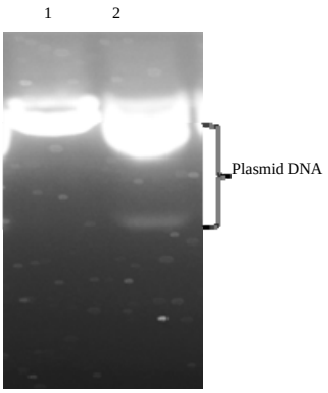

Fig. 2: Representative gel electrophoretic figure depicting 1: Plasmid removal from the daughter cells of the most ciprofloxacin resistant *P. aeroginosa* clinical isolate and 2: Plasmid DNA of the parental *P. aeroginosa* cells

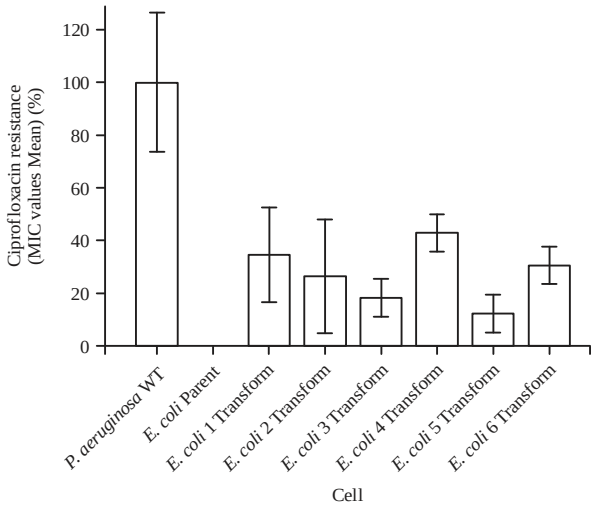

Fig. 3: Variable resistance to ciprofloxacin among the transformed *E. coli* bacteria cells
Bars represent mean values of MIC from biological triplicates and error bars represent 95% confidence intervals

higher response to ciprofloxacin among the tested fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin and ofloxacin).

In order to achieve further evidence to support the hypothesis that the plasmid DNA content of nosocomial *P. aeruginosa* is implicated in raising the fluoroquinolones resistance, bacterial transformation of *E. coli* by the plasmid DNA extracted from the most ciprofloxacin resistant isolates (n = 6) has been carried out. Three successful transformed clones of each plasmid extract were investigated for their ciprofloxacin response. The findings proved the reduction of ciprofloxacin response of successful transformed *E. coli* cells. On the other hand, the ciprofloxacin MIC values of transformed *E. coli* cells were lower than that of plasmid harbouring *P. Aeruginosa* isolates (Fig. 3). The figure illustrated the variable resistance to ciprofloxacin among the transformed *E. coli* bacteria significantly higher than that of parental *E. coli* and lower than that of *P. aeruginosa*.

DISCUSSION

Thirty (67%) *P. aeroginosa* isolates were detected in the collected specimens. The result indicated the high prevalence of nosocomial *P. aeroginosa* in the screened clinical specimens. The finding is in agreement with previous reports that confirm the high prevalence of *P. aeruginosa* casing hospital acquired infections during the last three decades[1,18-20]. In fact, the examined isolates manifested moderate to high resistance to the tested fluoroquinolone, which could be an indicator for higher expression levels of quinolones resistance regulated genes[8,14,21]. Exploring the plasmid DNA content of the most resistant isolates uncovered distinguishing profiles ranged between 6-2.5 kpb. The outcome coincides with previous studies[22,23].

The evidence achieved from exploring the implication of the plasmid content in augmentation of fluoroquinolone resistance by plasmid curing clearly explain that the elimination of the plasmid profile modulate the cured resistant isolates to be fluoroquinolone sensitive forms. Therefore, the plasmid DNA content might be directly implicated in increasing antibiotic resistance to fluoroquinolones in the clinical isolates of burn wounds[14,23].

Towards exploring further evidence to support the potential role of *P. aeruginosa* plasmids in emergence fluoroquinolones resistance, bacterial transformation strategy was adopted. Transformed *E. coli* cells by *P. aeruginosa* plasmids revealed significant increase in their resistance to ciprofloxacin ($p<0.05$) compared to the non-transformed parental *E. coli*, although the ciprofloxacin MIC values of transformed cells were lower than that of plasmid harbouring *P. aeruginosa* cells (Fig. 3). In view of the fact that the quinolone resistance factors are chromosomally regulated[24-26], the difference in ciprofloxacin response between *P. aeruginosa* and transformed bacteria shown in Fig. 3 could be explained by additive impact by both plasmid and chromosomal gene expression of either topoisomerase IV and gyrase enzymes or efflux pump factors in wild type *P. aeruginosa* bacteria as being up-regulated in fluoroquinolone resistant bacteria[27]. Such an additive ciprofloxacin resistant was previously observed in *E. coli*[28,29]. As a result, transformed *E. coli* clones'

responded slightly higher to ciprofloxacin than that of wild type *P. aeruginosa*, where the chromosomal associated resistance is lacked. This study may help in future for further molecular investigation of plasmid-mediated fluoroquinolone resistance in *P. aeruginosa*[15,30].

CONCLUSION

Taken together both of achieved evidence from plasmid curing and *E. coli* transformation studies, the outcome was clearly uncovered the significant impact of plasmid DNA in elevating ciprofloxacin resistance in nosocomial *P. aeruginosa* isolates. Thus, the plasmid profile may foresee fluoroquinolone response in patients with hospitalized acquired pseudomonal infections in the Iraqi medical centres. Nevertheless, this study requires further molecular analysis on a larger sample size.

SIGNIFICANCE STATEMENT

This study discovers the association between plasmid DNA content and fluoroquinolone resistance of nosocomial *P. aeruginosa* that can be beneficial for stratifying patients with hospitalised acquired pseudomonal infections for ciprofloxacin-based regimen. This study will help the researcher to uncover the critical areas of molecular investigation of plasmid-mediated fluoroquinolone resistance in *P. aeruginosa* that many researchers were not able to explore. Thus a new theory on plasmid-based fluoroquinolone personalized treatment of nosocomial *P. aeruginosa* infections may be arrived at.

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