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ORIGINAL ARTICLE

Cross Resistance against some Microbicides Exposure in Clinically MDR *Pseudomonas aeruginosa* isolates from Iraqi Patients

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Key words:
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ABSTRACT

Background: *Microbicide resistance poses a severe danger to the protection of life, health, and the prudent use of resources in order to prevent the spread of nosocomial infections.* ***Objectives:*** *This study aims to investigate the linkages between tolerance towards microbicides and induction of antibiotics resistance, in addition to evaluate the expression response of efflux pump qacEΔ1 gene in clinically MDR Pseudomonas aeruginosa isolates.* ***Methodology:*** *The susceptibility of 38 identified clinical P. aeruginosa isolates to certain microbicides (benzalkonium chloride and chlorhexidine digluconate) was examined in addition to determine the minimum inhibitory concentration (MIC) of these agents. Furthermore, the presence and expression level of qacEΔ1 gene was measured using PCR and RT-PCR.* ***Results:*** *The results indicated that the MIC of benzalkonium chloride ranged from 128-8µg/ml while for chlorhexidine digluconate ranged from 512-128 µg/ml. The presence of efflux pump encoded gene qacEΔ1 was identified in (89.47%) of examined isolates. Quantifying the expression of qacEΔ1 gene showed significantly increased in fold change upon treated with Chlorhexidine digluconate and Benzalkonium chloride about (3.953 ±0.99) and (3.783 ±0.71) respectively.* ***Conclusion:*** *Due to widespread use of microbicides in hospitals, concerns about the emergence and transmission of microbicide resistance genes have raised which induce the resistance antibiotic in pathogenic P. aeruginosa. In addition, a crucial correlation between the investigated microbicide and the efflux pump-encoded gene qacEΔ1.*

INTRODUCTION

Microbicides are widely used in hospital environments for sterilization and disinfection in order to avoid the spread of nosocomial infections[1]. However, because of the decreased efficiency of microbicides, the formation of resistance to them poses a significant risk to the prudent use of resources as well as the safety of life and health [2].

The key to understanding the effectiveness of quaternary ammonium compounds (QACs) like ethanol, chlorhexidine digluconate (CHG) and benzalkonium chloride (BKC) is comprehending their mode of action and the molecular initiating event. The deleterious impact of QACs on bacterial cell walls and membranes results from their interaction with negatively charged structural proteins and the head groups of acidic phospholipids in the membrane. The bacterial cell membrane and QACs interact ionically to disrupt the membrane and permit intracellular low-molecular-weight material, proteins, and nucleic acids to leak out, resulting in rapid cell lysis [3].

Moreover, the key hypothesized processes behind cross-resistance are an increase or decrease in outer membrane permeability (OMP), alterations of the lipopolysaccharide (LPS) profile and outer membrane electrostatic activity, and activation or overexpression of multidrug efflux pumps [4]. Due to the broad range of substrate specificity to antimicrobial drugs, multidrug resistance (MDR) efflux pumps could be significant mediators of cross-resistance [5,6].

Efflux pumps are involved in the regulation of the internal environment through the extrusion of toxic substances, quorum sensing molecules (autoinducers), biofilm formation molecules, and virulence factors of the bacteria [7]. The overproduction of specific efflux pumps is one way that bacterial pathogens exploit to fight microbicides. Among the small multidrug-resistance (SMR) family of efflux pumps are the QAC-specific ones. They are related to the processes that cause microbicide resistance, which in turn causes antibiotic resistance [1].

Pseudomonas aeruginosa (*P. aeruginosa*) such as uses efflux pumps, a distinct mechanism of antibiotic resistance, to eliminate antibiotics [8]. Also, it is able to adapt to changing environmental conditions because of its ubiquity, high metabolic flexibility, innate, acquired, and adaptive resistance mechanisms, and capacity to create a wide spectrum of virulence factors [9,10].

A common feature of microorganisms is the efflux pump system, which contributes significantly to bacterial resistance. It can actively excrete poisonous chemicals and antibiotics in addition to promptly eliminating metabolic waste [11].

The most commonly reported genes in the study of microbicide resistance genes are the *qac* genes, which are present on specific plasmids and have the ability to transfer resistance by conjugative transfer. How the *qac* genes can be transmitted by transformation or transduction is still a mystery [12].

Changes in membrane permeability, enzyme modification of the microbicide, and efflux pumps to lower the microbicide's intracellular concentration are some of the techniques that bacteria have been shown to employ to lessen the effects of microbicides [13].

Thus, the goal of the current study is to assess how the expression of the efflux pump *qac*EΔ1 gene responds to exposure to various microbicides in clinically isolates of MDR *P. aeruginosa* that exhibit cross resistance.

METHODOLOGY

Sample collection

Out of 100 clinical samples (burns, ear infections, wound infections, and UTIs), 38 clinical isolates of P. aeruginosa were obtained during the period from June 2023 until November 2023 for both sexes of different ages from Baghdad Medical City (Specialized burns hospital, Ghazi Al-Hariri Hospital and The National Center of Teaching Laboratories) were incorporated in the study.

Bacterial identification

To identify the bacterial isolates, the colonies' appearance on nutritional agar, MacConkey agar, and cetrimide agar were utilized. Analysis was done on the color, texture, and sharpness of the colony. In addition to biochemical tests using the oxidase and catalase tests. The VITEK 2 compact system's conformation revealed that 38 isolates were P. aeruginosa.

Antibiotics susceptibility testing

Growth-based methods were employed in the automated microbiology compact system VITEK 2 using colorimetric reagent cards that were automatically interpreted after being incubated, it was utilized to detect the presence of bacteria, test for antibiotic susceptibility, and identify resistance mechanisms. Using information and breakpoints from the Clinical Laboratory Standards Institute, lists of antibiotic categories that are advised for antibiotics susceptibility testing were developed [14].

Biofilm quantitation

According to Phuengmaung et al.[15] the micro-titer plate (MTP) is a quantitative method used with a micro-titer plate reader to evaluate biofilm production. The strains were separated into three categories based on their capacity to form biofilm: weak, moderate, and robust biofilm makers. in contrast to the control group, which consisted mainly of broth media-filled wells.

Microbicides susceptibility test

The sensitivity of *P. aeruginosa* isolates to the microbicide compounds was assessed using the agar well diffusion method. In order to prepare the microbicides utilized in this investigation, which include benzalkonium chloride (>95%) and chlorhexidine digluconate (20%) from Sigma-Aldrich (USA), they were dissolved in sterile distilled water according to the CLSI technique to create a stock solution [13].

Minimum inhibitory concentration (MIC) of Microbicides

MIC is the lowest antimicrobial agent concentration that may inhibit apparent bacterial growth but not kill it. Microbicides were dissolved in distilled water to a final concentration of 1024 µg/ml and then poured into 10 ml containers to create a stock solution. Muller-Hinton broth (MHB) was used in 96-well microtiter plates to perform two-fold serial dilutions from the stock solution to gate concentrations ranging from 512µg/ml to 2µg/ml[13].

Molecular identification

From a pure culture of *P. aeruginosa*, genomic DNA was extracted (FavorPrep Total DNA Mini Kits, Korea). This kit extracted DNA using the bacterial technique. Using a specific primer (16S rRNA as housekeeping gene), *P. aeruginosa* isolates were molecularly identified in this study. It was made in a lyophilized form, based on information supplied by the source, Macrogen.

Table 1, lists the genes used in this study along with the amplicon size, annealing temperature, and genetic sequence. Using electrophoresis and 2% agarose gel dyed with Red Safe, the PCR products were seen.

Table 1. Primers utilized in this study with annealing temperature.

TARGET GENE	PRIMER SEQUENCE 5`to 3`	PRODUCT SIZE (BP)	ANNEALING TEMPRATURE (˚C)	REFRANCE
16*S* *r*RNA	**F**-CAGCTCGTGTCGTGAGATGT	150	57	**16**
	R-CGTAAGGGCCATGATGACTT			
***qac*EΔ1**	**F**-TTACTAAGCTTGCCCCTTCCG	197	57	Newly designed
	R-CCCCATACCTACAAAGCCCC			

Real time- quantitative PCR (RT-qPCR)

Before and after applying the microbicides, RNA was extracted, and sub-MIC dosages of the agents were used to encourage bacterial growth. TRIzol™ Reagent was utilized to extract total RNA. The expression levels of *qac*EΔ1 genes were measured using RT-qPCR, with 16S rRNA acting as the reference gene. The expression was examined using the Easy Script® 1-Step RT-qPCR system in compliance with the manufacturer's instructions.

The following ingredients were present in each PCR tube's reaction mixture: To get 20 µl, add 10 µl of master mix, 3 µl of cDNA, 1 µl of each primer, and 5 µl of nuclease-free water. The SaCycler-96 Real Time PCR machine (Sacace, Italy) was used to perform the qRT-PCR. The initial denaturation step for the prepared reactions was 60 seconds at 95°C. This was followed by a 15-second denaturation stage at 95°C, a 30-second annealing step at 60°C, and a 40-minute melt curve step at 60-95°C in the protocol.

The Livak formula was utilized to calculate the mean Ct value statistical approach, which was then applied to three duplicate reactions to determine the levels of gene expression.

Statistical analysis

Using SAS (2018), the Statistical Analysis System, the impacts of the experimental variables were examined. Using the analysis of variance (ANOVA) test with the least significant difference (LSD), a significant comparison of the means was obtained. The chi-square test was used in this study to assess the statistical significance of an outcome difference between 5% and 1%.

RESULTS

According to the current study's findings, 38% of local isolates were identified as*P. aeruginosa.* The highest rates (47.37% and 23.68%) were assigned to burn and wound infections, while the lowest frequency (10.53% and 18.42%) was assigned to ear and UTI infection as shown in Figure 1.

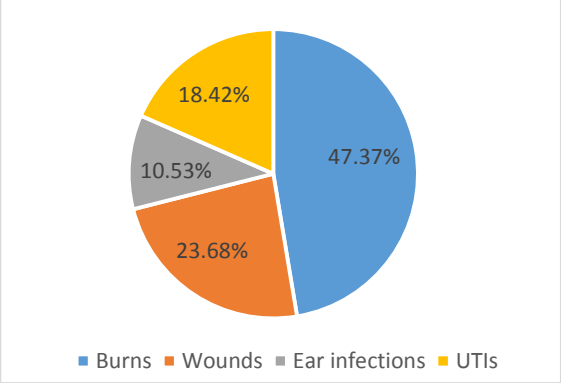

Fig. 1: A pie chart showing the percentage of *P. aeruginosa* isolates

Antibiotic susceptibility test by VITEK 2 compact system was divided the examined isolates to MDR (73.86%), Extensively drug-resistant (XDR) (13.15%), and Pan drug-resistant (PDR) (10.52%) while (2.63%) were sensitive as illustrated in Figure 2.

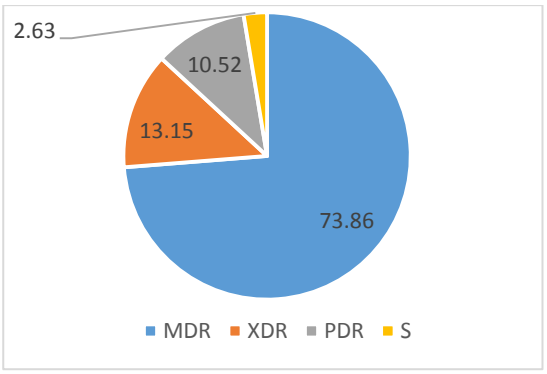

Fig. 2: A pie chart showing the percentage of Antibiotics susceptibility

The MIC of examined Microbicides agents were displayed in Figure 3, wherein the concentration of (BKC) ranged from 128- 8µg/ml, while the concentration of (CHX) ranged from 256-128µg/ml.These bacteria may have had a high level of resistance and caused infections in the patients in our study, since the high MICs needed were only enough to stop the isolates from growing, not to kill them.

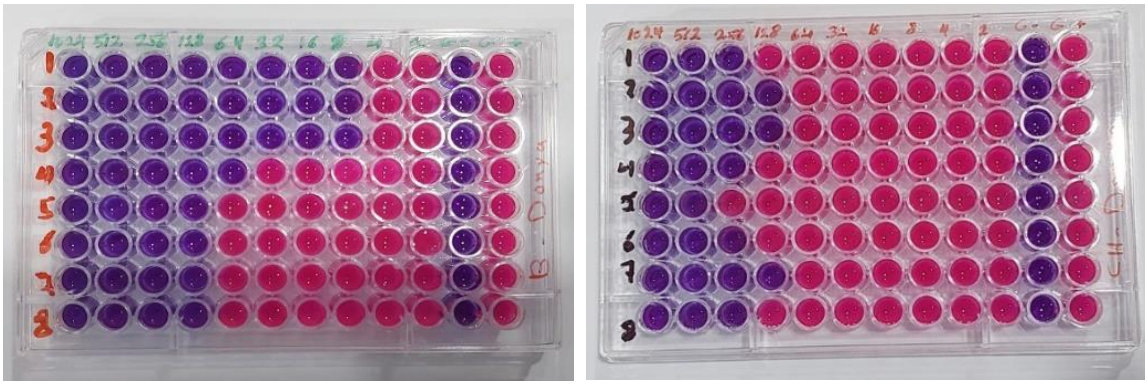

Fig. 3: The results of Broth micro- dilution method to determine (MIC).
Left-BKC treatment; right-CHG treatment; (C+) Positive control (only bacteria, broth & microbicide agent); (C-) Negative control (only broth & microbicide agent).

The study's findings regarding biofilm development demonstrated that *P. aeruginosa* isolates (38) had the ability to create biofilm and adhere to surfaces. The results were classified as strong (79.41%), moderate (14.17%), and weak biofilm formation (5.88%).

Figure (4) illustrates that 34 (89.47%) of the isolates contained the efflux pump gene detected molecularly using a particular primer for the *qac*EΔ1 gene. This was done by evaluating the bands on gel electrophoresis and comparing their molecular weight to the 100 bp DNA ladder. Four isolates (10.52%) lacked the bands, but all positive isolates had bands that fell within the expected gene size (197 bp).

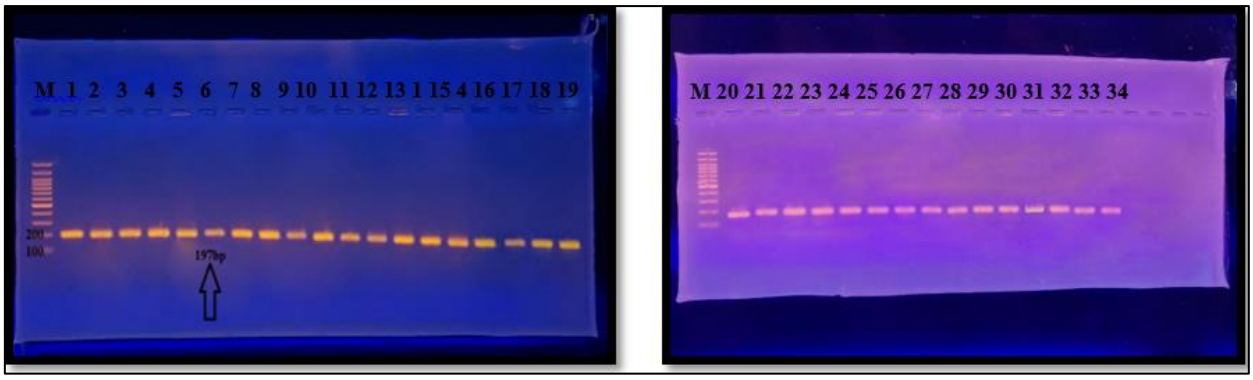

Fig. 4: Electrophoresis the amplification products of *qac*EΔ1 gene. Lane M: 100bp DNA ladder; Lanes 1-34: PCR product of samples with expected size 197 bp (2% agarose at 70 volts for 60 min).

With Quantification of efflux pump gene expression in real time using PCR, the fold change of *qac*EΔ1 gene expression in Chlorhexidine digluconate- treated isolates was about (3.953 ±0.99 a). The fold change for Benzalkonium chloride- treated was about (3.783 ±0.71 a) in comparison to the level of *qac*EΔ1 expression before treatment which about (1.154 ±0.22 b) as shown in Table 2.

Table 2: *qac*EΔ1 gene expression fold change in response to various treatments

Group	Fold change ± SE of *qac*EΔ1
Before	1.154 ± 0.22 b
After treatment 1 (CHX)	3.953 ± 0.99 a
After treatment 2 (BKC)	3.783 ± 0.71 a
LSD	2.109 *
P-value	0.0194
Significant differences were between the means that had different letters in the same column. * (P≤0.05).	

Since the *16S r*RNA gene's expression is consistent in the cells under investigation under all conditions, it was chosen as the housekeeping gene for this experiment. The results show that there was a statistically significant difference in the Ct values between the treated groups. The concentrations of benzalkonium chloride, which varied from 128 µg/ml to 8 µg/ml, had Ct values higher than those of the isolates that were not treated, while the isolates treated with Chlorhexidine digluconate concentrations ranged from 512 µg/ml, 256 µg/ml, and 128 µg/ml.

DISCUSSION

This research sheds light on the microbicides (QAC genes) resistance in significant ways on *P. aeruginosa* as one of the primary factors contributing to hospital-acquired illnesses associated with antibiotic resistance. Since the SARS-CoV-2 outbreak, microbicide products such as disinfectants and antiseptics have been used more frequently to manage and stop the spread of diseases. In order to reduce the risk of nosocomial infections, hospitals also frequently utilize them to sanitize equipment and surfaces, these goals were documented by 2 reports[17,18].

The findings of our investigation are in line with those of other investigations that looked at the distribution of *P. aeruginosa* isolates connected to different medical conditions [19, 20].

Furthermore, the results showed that the highest rates of phenotypic frequencies were found in MDR (73.86%) and XDR (13.15%), which differed from studies in China (MDR 18.5%, XDR 3.5%)[21] and Pakistan study's (MDR 36.3%, XDR 18.1%)[22].

According to our study findings on biofilm formation, most *P. aeruginosa* isolates could form biofilm and were classified as strong, medium, or weak adherents. Different rates of biofilm generation with *P. aeruginosa* isolates have been observed in a number of investigations. In the line of our findings, a study reported that 80% of isolates had biofilm morphologies[23]. In contrast to the mean biofilm production of 80.35%, moderate biofilm formation was seen in 29.65% of the isolates. Moreover, Biofilm production was seen in at least 87% of isolates examined, according to Egypt[24] investigation that found 10 isolates (13%) Egyptian were unable to create any apparent biofilm, whereas 15 (20%) were strong, 26 (35%) were medium, and 24 (32) were weak biofilm producers.

Our results are consistent with the observation that 58.6% of MDR isolates produce substantial biofilm. According to a study by Bakht and Rahimi[25] their results showed a substantial association between MDR isolates and biofilm growth. Furthermore, in the current investigation, it was discovered that the microbicides with the highest MBC values also had the greatest MIC values against the isolates, BKC and CHX. According to a study conducted in Turkey by Uzunbayir-Akel et al.[26], the CHX had the lowest MIC against *P. aeruginosa*, which contradicted these findings.

In an Egyptian investigation, the *qac*EΔ1 gene was detected in 21.4% of susceptible bacteria and 57.8% of multidrug-carrying isolates which were MDR / XDR, indicating a link between this gene and resistant isolates[27]. While, another study reported that the percentage of *qac*EΔ1-positive *P. aeruginosa* isolates in Iran ranged from (73.7% to 92.5%), which is lower than the current study's (89.47%) [28]. Additionally, they demonstrate how the strong link between the existence of the *qac*EΔ1 microbicide resistance gene and better phenotypic resistance to BKC and CHX and the gene's high prevalence corroborate these conclusions.

In our study, the results demonstrated that the high frequency of the microbicide resistance gene *qac*EΔ1 and the strong correlation between it and higher phenotypic resistance to BKC and CHX in *P. aeruginosa* that is infectious. Furthermore, previous studies in Iran [29] and in Egypt [30] have documented that the notable rises in minimum inhibitory concentration (MIC) against BKC and CHX in *P. aeruginosa* isolates carrying the *qac*EΔ1 gene which agreed with our study. Moreover, Overexpression of the multidrug efflux pump demonstrated a direct correlation between specific efflux pumps and cross-resistance to diverse classes of antibiotics and microbicides; these results agreed with our findings that *P. aeruginosa* isolates have facilitated the spread of MDR infections throughout the environment. Hospital microbicide treatments are frequently used, resulting in a wide range of antibiotic and microbicide classes that have been linked to cross-resistances [31]. On the other hand, the one report looked into the idea that hospitalized infections could become more resistant because of a decreased tolerance to microbicide chemicals and the existence of QAC genes, like *qac*E and *qac*ΔE1[32].

Finally, distinct microbicides strategies and antibiotic types are employed differently in hospitals due to regional differences in natural settings and economic status [33]. This has led to a diversity of microbicides resistance mechanisms among strains from various areas. Consequently, it is critical that hospital infection control strategies are grounded in regional epidemiological research[34, 35] and our findings regarding the microbicide resistance gene support this viewpoint.

CONCLUSION

The study's findings demonstrate that the extensive use of microbicides in hospitals has sparked worries about the development and spread of genes resistant to the microbes, which is linked to the growth of antibiotic resistance in pathogenic *P. aeruginosa*. Phenotypic results and the existence of QAC and antibiotic

resistance genes suggest that MDR may be selectively pressed and transmitted through horizontal gene transfer or efflux pump-mediated resistance mechanisms if QACs are present at sub-MIC levels. Furthermore, a crucial correlation between the investigated microbicide and the efflux pump expressed gene *qac*EΔ1. Therefore, it is necessary to continuously monitor the environment and apply infection management measures that specifically target the suppression of genes encoded for resistance to microbicides.

Declarations: The manuscript has been read and approved by all named authors. The manuscript is not published elsewhere.

Ethics Approval: The ethics committee of the Genetic Engineering and Biotechnology Institute for Post Graduate Studies approved the study proposal. (No.: Ref: EC/2148, dated: 8/5/2023).

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