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Phenotypic changes in pseudomonas aeruginosa induced by sub-inhibitory exposure to chlorohexidine

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Abstract

Background and objective: Many bacterial isolates show an increased antimicrobial resistance due to biofilm production. Repeated exposure to sub lethal concentrations of antimicrobial agents certainly contributes to the resistance as compared to planktonic bacteria. The aim of this study was to test whether the bacterial phenotypes of P. aeruginosa can be changed during exposures to the concentrations at sub lethal doses of chlorhexidine.

Methods: Sheep blood agar plates were used for evaluation of haemolysin assay for isolates of P. aeroginusa**.** A 96-flat bottom well microtiter plates were used for determination of MIC of antibiotic and biofilm formation.

Results: All tested isolates were able to lyse RBCs after exposure to sub-MIC of chlorhexidine. Effectiveness of sub-lethal doses of chlorhexidine on biofilm formations varied depending on the contact time. In general, long contact time exhibited increasing biofilm than short time. No significant difference in biofilm was detected among contact times: day I, day II and day III ($P = 0.132$, $P = 0.139$ and $P = 0.125$, respectively). The most effective sub-MIC of CHX was against azithromycin, since the resistance increased significantly ($P = 0.008$).

Conclusion: Surviving P. aeruginosa to low concentration of chlorhexidine can exhibit stronger biofilm and increased resistance to antibiotics.

Keywords: Biofilm, Chlorhexidine, Antibiotics, P. aeruginosa.

Introduction

Antiseptics and disinfectants have been used in clinical and domestic applications for over half a century. Currently, the use of these products are in question, since persistent exposure to such agents can have harmful effects on human health and can select for less susceptible strains towards biocides and antibiotics.[1] Chlorhexidine (CHX) as a cationic antimicrobial agent of quaternary ammonium compounds (QACs) has different behavior, which differs from other cationic biocides in that they interact only superficially with the lipid bilayer altering fluidity through cation displacement and head group bridging.[2] Many bacterial isolates of Pseudomonas aeruginosa show an increased antimicrobial resistance due to producing biofilm, which is defined as a surface-attached (sessile) community of microorganisms growing embedded in a self-produced matrix of extracellular polymeric substances (EPS).[3] The basic modes of action of bacterial resistance to antimicrobials are generally well documented, although data continue to accumulate about the nature and importance of efflux systems.[4] Biofilm is commonly associated with major clinical consequence of different infectious disease correlates with the problems of therapeutic killing of attached cells.[5] The mechanisms by which Pseudomonas aeruginosa resist killing by antimicrobial and biocides are still poorly defined, even though repeated exposure to sub lethal concentrations of antimicrobial agents certainly contributes to the resistance as compared to planktonic.[6] Decontamination and disinfection is an important and often challenging task, due

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to increasing number of nosocomial infections, which was the motive to set up a sanitation program that indicates the appropriate chemical agents were chosen for application in the most effective way. Minimal inhibitory concentration (MIC) was submitted to study and compare the behavior of selected microorganisms.[7] The term sub-MIC will be used to refers to concentrations of antimicrobial that do not affect the growth of the organism being tested.[8,9] A wide variety of active biocides have been used for hundreds of years for antisepsis, disinfection, but less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. In fact, the widespread use of antiseptic and disinfectant has prompted some assumption on the development of microbial resistance, in particular cross-resistance to antibiotics.[10] On the other hand, various studies have shown that sub-MIC of antimicrobial may induce biofilm formation. This process may have clinical relevance, since bacteria are continuously exposed to sub-MIC during low concentrations of disinfectants.[9] This inappropriate procedure has become difficult to control bacterial biofim through cleaning and disinfection.[11] The aim of this study was to test whether the bacterial phenotypes such as biofilm formation, haemolysis and antibiotic resistance of P. aeruginosa isolated from patients can be changed during exposures to the concentrations at sub lethal doses of chlorhexidine, a commonly used hospital disinfectant.

Methods

Bacterial isolates

Eight clinical isolates of Pseudomonas aeruginosa were collected from patients suffering from otitis media at Rizgary Teaching Hospital in Erbil, Kurdistan region, Iraq.

Antimicrobial agent and biocicides

The susceptibility of P. aeruginosa isolates to the following antimicrobial agents and biocides were tested: ceftriaxone at a concentration of 1000 mg/ml, ampiclox at 500 mg/ml, azithromycin at 250 mg/ml, and ciprofloxacin at 500 mg/ml. All antibiotics were used as raw materials (Mepha/Switzerland). Biocide 4% (w/v) chlorhexidine (CHX) (Al-Rhma pharmaceutical co.) as laboratory standard solution. All solutions were filtered sterilized using a 0.2 µm cellulose syringe filter (Jet-biofilm, China).

Inoculation preparation

The stock cultures were maintained in trypticase soy agar (TSA, Difco) at 4 °C. Weekly transferences were developed with the purpose to maintain the viability of microorganisms.[12]

Determination of sub-minimum inhibitory concentrations (sub-MICs) of CHX

The sub-MIC values of CHX against P. aeruginosa were tested.[1,5] Standard MICs were determined by macrodilution using a set of 10 ml capacity tubes. Briefly, 50 µL of adjusted bacterial suspension containing 1×10^8 cfu/ ml adjusted with MacFarlad 0.5 (biomerieux) in TSB broth were added to 5ml of serial twofold dilutions of the CHX. The tubes were incubated for 24 h at 37°C and observed for turbidity. The MIC was defined as the lowest concentration of CHX needed to inhibit visible bacterial growth compared to the control culture, while sub-MIC are those concentrations below the MIC, that inhibit normal cellular functions without causing death.[13] The bacterial growth were grown in tube treated with sub -MIC of CHX were kept at contact-time (control, day I, day II, day III and week I), subsequently. The effect of CHX at different contact-time was evaluated against bacterial isolates. Tests were performed in triplicate with negative and positive controls.

Influence of sub-MICs of CHX on haemolysin production

The method used for evaluation of haemolysin assay for eight selected

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producing-hemolysin bacterial isolates of P. aeroginusa. One loopful of bacterial growth was transferred from each sub-MIC tube of CHX at different contact-time onto sheep blood agar plate, then observed for their ability to lyse blood cells following incubation for 24 hrs at 37°C.[14] Bacteria that could completely lyse the blood cells forming a clearing zone around the colonies were called β-haemolytic, while partially break down the blood cells causing brown or green discoloration of the agar around the colony were called α-haemolytic and bacteria that cannot lyse the blood cells were called γ-haemolytic.

Influence of sub-MICs of CHX on biofilm formation

Biofilm formation on polystyrene surface was conducted by growing bacterial isolates on 96-flat bottom well microtiter plates (Costar/USA).[1] 200 µl of nutrient broth were added to all 96 wells. Bacterial cells from different contact-time of sub-MIC of CHX tube were prepared. The turbidity of each bacterial suspension was adjusted with McFarland tube 0.5. All plates were inoculated with the bacterial suspension (5µl per well) and were incubated at 37ºC for 24 hrs. After incubation, the plates were washed by PBS pH 7.2 for three times, then were exposed to air-dry, 200 µl of 0.1% crystal violet was added to each well, the plates were incubated at room temperature for 30 minutes, then washed off using distilled water and kept for air dry. The bound bacteria were quantified by addition of ethanol 70% and measurement of the dissolved crystal violet at absorbance of 630 nm using 96-flat wells microtiter plate of the ELISA reader ELX800 (Biotek / USA). Each result represents the mean of at least three separate experiments.

Influence of sub-MICs of CHX on antibiotic resistance

Standard broth microdilution 96- flat well plate and MIC-value was used for the test.[6] Two fold serial dilution (1 up to 512 µg/ml) of four types of selected antibiotics were prepared. The concentrations of antibiotics allocated on the wells (200 µl per well) starting from well number one up to well number ten, while well 11 and 12 were kept as a control negative and positive respectively. All wells except control negative were inoculated with 5 µL of a bacterial suspension that was transferred from tubes at different contact time. The plate was incubated for 24 hrs at 37ºC. The MIC was taken as the lowest concentration of the antibiotic that inhibits the bacterial growth. This procedure took place for a week contact time (day I, day II, day III and week I). Each result represents the mean of at least three separate experiments.

Statistical analysis of data

The paired-sample t-test was performed for statistical data analysis to determine whether there was a significant difference between MIC-value and biofilm formation at different time contacts. A $P \leq 0.05$ was considered statistically significant.

Results

Hemolysin production

All tested isolates of P. aeruginosa kept their ability to β-hemolysis after exposure to sub-MIC doses of CHX for different contact times from one day up to seven days.

Biofilm formation

Effectiveness of sub-lethal doses of CHX on biofilm formations varied depending on the contact time. In general, long contact time exhibited increasing biofilm than short time. Paired samples statistics showed no significant difference in biofilm among contact time: day I, day II and day III ($P = 0.132$, $P = 0.139$ and $P = 0.125$, respectively). The results changed significantly ($P < 0.001$) following one week, since this time showed stronger biofilm than three days (Figure 1).

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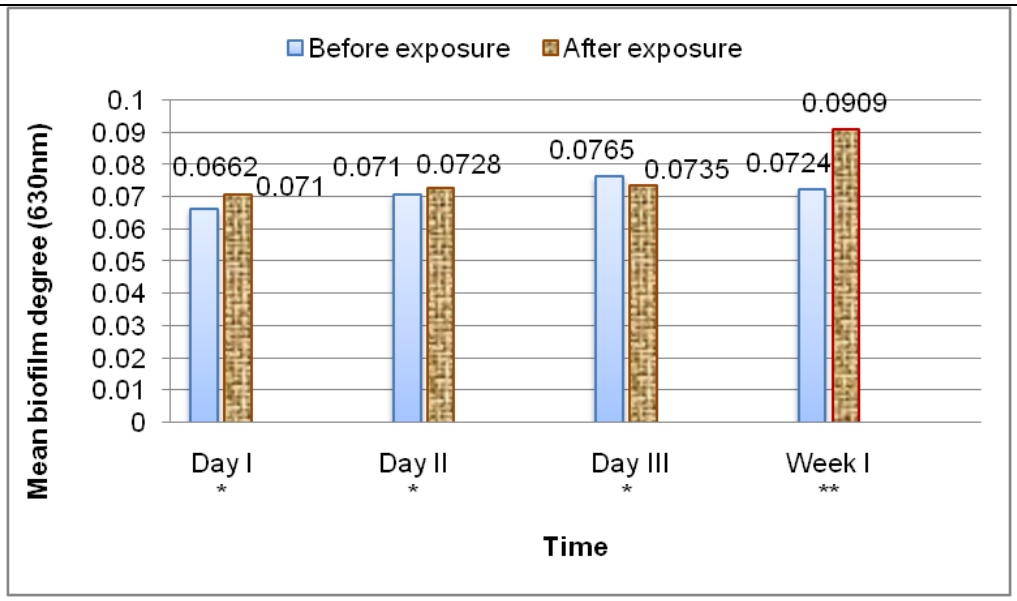

The mean difference is non-significant at (*) P > 0.1 and significant at (**) P < 0.001

Figure 1: The degree of biofilm formation by P. aeruginosa following exposure to sub-MIC of CHX at different contact time.

Antibiotic resistance

The efficacy of CHX showed no significant difference on bacterial isolates against ceftriaxone at contact time of day I and day II (P = 0.174 and P = 0.567, respectively).

Bacterial isolates could increase their resistance to the same types of antibiotics at day III and week I, as raised the MIC values (P = 0.02 and P = 0.014, respectively) as shown in Table 1.

Table 1: The MIC determination of ceftriaxone against eight bacterial isolates of P. aeruginosa at different contact time of CHX.

P. aeruginosa Isolates	Day I		Day II		Day III		Week I	
	Before	After	Before	After	Before	After	Before	After
P.1	8	128	512	256	64	512	256	256
P.2	32	128	256	256	64	256	256	512
P.3	32	32	32	64	32	64	32	128
P.4	64	64	64	128	64	128	64	256
P.5	128	128	128	128	128	256	128	256
P.6	128	128	128	256	128	128	128	512
P.7	64	64	64	256	128	512	512	512
P.8	128	128	64	128	128	256	128	256
Mean ±SD	**73± 49.02**	**100± 39.88**	**156± 159.94**	**184± 79.77**	**92± 39.88**	**264± 169.11**	**188± 153.40**	**336± 152.03**
p value	**0.174**		**0.567**		**0.02**		**0.014**	

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The susceptibility of isolates to ampiclox is presented in Table 2. The results showed various effects as the bacterial isolates started with no significantly resistance at day I and day II (P = 0.059 and P = 0.0640, respectively), then at day III could adapt the conditions (P = 0.424). At week I, The isolates showed significant resistance through raising the MIC-value (P = 0.011) in comparison to control. In contrast to above-mentioned results, the CHX could not change the sensitivity of bacterial isolates against ciprofloxacin at all contact times (Table 3).

Table 2: The MIC determination of ampiclox against eight bacterial isolates of P. aeruginosa at different contact time of CHX.

P. aerugiousa **Isolates**	**Day I**		**Day II**		**Day III**		**Week I**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
P.1	16	256	1024	1024	1024	512	512	512
P.2	32	256	64	512	128	512	128	1024
P.3	64	64	64	64	64	128	64	256
P.4	64	128	64	256	256	512	256	512
P.5	64	64	128	256	128	256	256	512
P.6	128	256	128	256	256	256	256	512
P.7	128	128	128	256	128	256	256	512
P.8	64	64	128	128	64	256	128	512
Mean ±SD	**70± 40.05**	**152± 90.10**	**216± 328.01**	**344± 303.93**	**256± 319.08**	**336± 152.03**	**232± 136.5**	**544± 213.6**
p value	**0.059**		**0.046**		**0.424**		**0.011**	

Table 3: The MIC determination of ciprofloxacin against eight bacterial isolates of P. aeruginosa at different contact times of CHX.

P. aerugiousa **Isolates**	**Day I**		**Day II**		**Day III**		**Week I**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
P.1	2	2	8	8	2	4	128	128
P.2	2	4	4	8	4	8	4	16
P.3	2	2	2	2	2	2	2	4
P.4	2	2	2	2	2	2	2	8
P.5	2	2	2	2	2	2	2	2
P.6	2	2	2	2	2	2	2	4
P.7	2	2	2	2	2	2	2	2
P.8	2	2	2	2	2	2	2	2
Mean ±SD	**2±0.00**	**2.25±0.7**	**3±2.1**	**3.5±2.7**	**2.25±0.7**	**3±2.1**	**18±44.4**	**20.7±43.5**
p value	**0.351**		**0.351**		**0.197**		**0.111**	

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Table 4: The MIC determination of azithromycin against eight bacterial isolates of P. aeruginosa at different contact times of CHX.

P. aerugiousa **Isolates**	**Day I**		**Day II**		**Day III**		**Week I**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
P.1	8	32	16	16	8	16	32	128
P.2	64	64	64	64	64	64	64	128
P.3	256	256	512	512	512	512	512	1024
P.4	256	256	256	512	256	512	512	1024
P.5	128	128	128	256	128	256	128	256
P.6	256	256	256	256	256	512	256	512
P.7	128	128	128	256	128	256	256	1024
P.8	256	256	128	256	256	512	256	512
Mean ±SD	**169± 100**	**172± 95.1**	**186± 155.8**	**266± 179.1**	**201± 156.9**	**330± 211.3**	**252± 182.9**	**576± 398.9**
p value	**0.351**		**0.049**		**0.017**		**0.008**	

The most effectiveness of CHX at sub-MIC concentrations on tested bacteria was against azithromycin. The MIC-value increased significantly starting from day II, day III and week I (P = 0.049, P = 0.017 and P = 0.008, respectively) as shown in Table 4.

Discussion

This study was designed to assess the effectiveness of CHX at their sub lethal concentrations on some phenotypic changes of P. aeruginosa. Three virulence factors were included; hemolysin production, biofilm formation and bacterial resistance to antibiotic. Hemolysin is recognized as potential virulence factor produced by most pathogenic bacteria, which can put a human's health at risk. Hemolysin phenotype was observed with the method of sheep blood agar. The results demonstrated no changes of hemolysin production by the eight isolates after they were exposed to CHX. In fact, hemolysin production is regulated by gene expression to get iron for nutrition. Research has revealed that gene expression of hemolysin is repressed in the presence of iron.[16] This ensures that hemolysin is produced only when needed. Thus, the mechanism of gen inhibition might be caused unchanged hemolysin phenotype following exposures to CHX in contact time. In recent works, considerable progress has been made in understanding the response of bacterial isolates to antiseptics. For example, the nature and composition of outer layer of P. aeruginosa may act as a permeability barrier, in which there may be reduced uptake as described by Russell.[17] It appears that the efficacy of CHX is associated with changes in the cell envelope. This observation is reinforced by finding presented by Prince et al[18] who reported that resistant strains to CHX did not show altered biochemical properties of changed virulence. Further study reviewed by McDonnell and Russell[19] could support these finding, who revealed that only the high concentration CHX can inhibit only the membrane-bound, which suggested that the enzymes is not a primary target for CHX action. The second aim of the study was to evaluate the efficacy of CHX on

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biofilm formation at sub lethal concentrations in different contact times. The goal of this line was to demonstrate the bacterial tolerance, which permit microorganisms to survive in the presence of an active biocide. Many reports of this style were reviewed in hospital environments, which include inadequate cleaning, incorrect products use and on effective infection control practice. In fact, microorganisms can adapt to variety chemical conditions and therefore not surprising that resistance to extensively used disinfectants and antiseptics has been reported.[19] The most significant mechanism is clearly intrinsic, in particular the ability to adaptation that develops protective biofilm of Pseudomonas. In these cases "resistance" may be incorrectly used and "tolerance" may be more correct as reviewed by McDonnell and Russell.[19] This study showed no significant difference in biofilm formation after three days of exposure to sub-MIC doses, but could change significantly after one week. This change might have a relation to adaptation or bacterial tolerance as described above. Several instances in hospitals are known of contamination of antiseptic and disinfectant solutions by bacteria can support our observation. For example, studies demonstrated that prolonged survival of bacterial isolates in sub-lethal dose (2% CHX) was attributed to the embedding these microorganisms in a thick matrix that adhered to the walls of a container.[20,21] One certain conclusion about what mentioned above, the interaction of bacteria with surface is usually reversible and eventually irreversible. Irreversible adhesion is initiated by binding of bacteria to the surface through exopolysaccharide glycocalyx matrix. New cells then arise by cell division are bound within glycocalyx polymers.[19] The last field of the experiment included the efficacy of CHX at low doses on bacterial resistance against antibiotics, through, determination of MIC-value following exposures. Step-wise exposure of P. aeruginosa isolates to gradually increasing contact time of CHX resulted in having the organism exhibiting increased MIC-value to antibiotics. Ciprofloxacin was the only antibiotic that P. aeruginosa isolates showed no less sensitive to CHX than the remained types of antibiotics, which exhibited raised MIC-value particularly after day III and week I. In fact, antibiotic resistance by bacterial isolates may be the result of phenotypic adaptation and survivals in environment where growth conditions are limiting or the cells are under stress.[22] However, sub lethal exposure to CHX can provoke antibiotic resistance through expression of efflux pump, which plays an important role in multidrug resistance in P. aeruginosa in hospitals where CHX are used frequently.[22] The present study showed bacterial biofilm that induced by low concentrations of CHX, can also adopt a part of resistance to antibiotics. The objective of the work presented in the last field was to evaluate the significance of penetration limitation as a mechanism of biofilm resistance to different groups of antibiotics such as β-lactams, azithromycin and ciprofloxacin. P. aeruginosa isolates showed no changes in sensitivity to ciprofloxacin before and after exposures to CHX. In contrast to the situations with ciprofloxacin, the sensitivity decreased against β-lactams and azithromycin through raised MIC-value. Poor penetration contributed to biofilm resistance to β-lactams but not to ciprofloxacin as described by Andrel et al[23] who hypothesized that the failure of β-lactams penetrate biofilms was due to its deactivation in the surface layers of biofilm faster than could diffuse in. Azithromycin is known to inhibit biofilm growth of P. aeruginosa, but the exact mechanism that mediates the azithromycin anti-Pseudomonas activity remains uncertain. Recent study proposed a two-step process in which azithromycin first permeabilizes the outer membrane and then causes cell death by inhibiting protein synthesis and/or ribosome assembly.[24] The present work showed that P. aeruginosa isolates revealed much greatest resistance to

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azithromycin through increased MIC-value. In fact, the above-mentioned mechanism of low concentration of CHX act on membrane-bound could change the permeability. This process might cause a barrier to prevent azithromycin penetration to reach its primary internal target. However, in some situations antibiotic resistance can be achieved without any genetic alteration; this is called phenotypic resistance, which is associated to specific processes such as growth in biofilm.[25]

Conclusion

Surviving *P. aeruginosa* exposed to CHX at low concentrations exhibited various phenotypic changes in pathogenicity. The isolates can build up stronger biofilm and increased resistance to antibiotics by MIC value. Therefore, incorrect use or dilution of disinfectants could lead to microbial population that is more resistance to antibacterial agents.

Conflicts of interest

The authors report no conflicts of interest.

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