

SCANNING ELECTRON MICROSCOPE OF DISINFECTANTS EXPOSED PSEUDOMONAS AERUGINOSA ISOLATED FROM DIFFERENT TYPES OF INFECTIONS

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Abstract— Disinfectants play an important role in health care-associated infection control by either minimizing or preventing microorganism dissemination. This article to study the morphological changes which may be related to the lose of antibiotic resistance after disinfectant exposure using SEM. Showed all isolates resistant to ampicillin, amoxicillin, cloxacillin, cephalixin, tetracycline, doxycycline, rifampin, chloramphenicol, trimethoprim cefotaxime and erythromycin, while one of burn isolates was susceptible for gentamicin, chloramphenicol and trimethoprim, and 15 of burn, 6 of wound, 5 of ear, and all urine isolates were susceptible to gentamicin using Kirby-Bauer method.

The MICs of four common in use disinfectants (Hexatane, Dettol, Savlon and Povidone – Iodine) were determined for all isolates. The results showed that the MICs of Hexatane ranged from (64–512) µg/ml, Dettol (2048–16384) µg/ml,

Savlon (4096:40960)–(32768:327680) µg/ml and for Povidone – Iodine MICs were (8192–32768) µg/ml. It has been found that burn and urine isolates were more resistant to disinfectants than wound and ear isolates. According to the effect of subMICs of disinfectants at different exposure patterns on antibiotic resistance, the results showed lose of resistance to tetracycline, doxycycline, rifampin, chloramphenicol, cefotaxime and trimethoprim in %72, %72, %68, %22, %28 and %36 of isolates, respectively. The results of SEM micrograph showed normal morphology and small sized bacteria with nub formation on some of them when exposed to dettol, and shape changes in cells with bulging in exposed to Povidone-iodine, while elongation and deformation were recorded in some cells in exposed to Savlon(chlorohexidine/ cetrimide) and Hexatane (chlorohexidine/ gluconate), respectively.

Key words— *Pseudomonas aeruginosa*, SEM, disinfectant.

I. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that is of public health significance, as it is the most common cause of hospital-acquired (nosocomial) infections(1, 2, 3). In the USA, nosocomial infections are estimated to involve approximately 2 million patients each year, leading to 90 000 deaths and a huge economic impact of 4.5 billion dollars (4). *P. aeruginosa* has also been reported to contaminate disinfectants in the hospital environment, thereby compromising their purpose of reducing or eliminating bacterial contamination (5).

P. aeruginosa is commonly encountered in the wider clinical environment, for example Intensive Care Units, hospital and clinic floors, surfaces, linen and utensils. It has a preference for moist environments (e.g. contaminated tap water, soap solutions, sink traps) and is often harbored by healthcare personnel via intestinal colonization and inadequate hand hygiene. The organism has been shown to cause wound sepsis (especially burn wounds) as well as a variety of nosocomial infections such as folliculitis, external otitis, infantile diarrhea and ventilator-associated pneumonia (6).

The scanning-beam electron microscope (SEM) allows examination of the surface morphology of large numbers of whole intact microorganisms at high magnification in 3-dimensional perspective (7). In vitro studies suggest that exposure to disinfectants results in reduced susceptibility to antibiotics and biocides by intrinsic or acquired mechanisms of resistance. In addition, microorganisms have adapted to disinfectant exposure by acquiring plasmids and transposons that confer biocide resistance, the same survival strategies to disseminate acquired mechanisms of resistance to disinfectant as they have for resistance to antibiotics (8).

In Iraq, there are many studies on the effect of disinfectants exposure to bacterial resistance to disinfectants and antibiotics. Al-Shakarji, who studied the effect of low-power diode laser light-with or without photochemical agents (povidone-iodine) on wound healing ,virulence factors, susceptibility to antibiotics ,Minimal Inhibitory Concentrations (MICs) of disinfectants for isolates and non-,firstly-,secondly-disinfectants exposed of *P. aeruginosa* (9) while Ràuf, who studied *P. aeruginosa* bacteriologically and genetically during some virulence factors and plasmids when exposed to Hibitane(chlorohexidine), savlon (chlorohexidine 0.3 % and cetrimide 3%), Povidone-Iodine and dettol(10).

The aim of this study is to evaluate the susceptibility of clinical isolates of *P. aeruginosa* to antibiotics and some disinfectants commonly used in health care settings in Iraq and to reduce the resistance of this bacterium by treatment with disinfectants and to determine changes morphological surface after disinfectants exposure, using scanning electron microscope.

II. MATERIALS AND METHODS

A. Bacterial isolates and Media

P. aeruginosa were isolated from clinical specimens using MacConky's, nutrient and blood agars. Isolates were identified by microscopic, and biochemical tests according to the methods described by (11) some isolates were confirmed using by API 20E and Vitic2 instrument.

B. Disinfectants

Savlon(Chlorohexidine/Cetrimide5%) Teeba Co. /Iraq, Dettol (Chloroxylenol 5%) SDI Co. / Iraq, Povidone-Iodine 10% (Golden Square Pharma Co./Lebnan) and Hexatane(Chlorohexidine/ Gluconate 4%) Al-rahma pharma. Co./ Jorden were used.

C. Antimicrobial Susceptibility test

All isolates were tested for susceptibility to antibiotics by disc diffusion method according to the National Committee for Clinical Laboratory Standard now called Clinical Laboratory Standard Institute (CLSI) (12). Twelve commonly used antibiotic discs were tested in this study, Ampicillin, Amoxicillin, Cloxacillin, Cephalin, Cefotaxime, gentamycin, Erythromycin, Trimethoprim, Chloramphenicol, Rifampin, Tetracycline and Doxycycline.

D. Determination of MIC of disinfectants for *P. aeruginosa* isolated

A loopfull of each stock culture (*P. aeruginosa*) was subcultured into nutrient broth and incubated at 35°C for 24 hrs. Serial dilution prepared in nutrient broth as followings: 2-32762, 2-16384, 2-16384 and 2-1024 according to the (13) respectively. Then 50 µl of bacterial broth was inoculated in each dilution and incubated at 35°C overnight. After incubation the growth of each dilution were subcultured by striking on MacConky's plates and incubated at 35°C for 18-24 hours, the results were read to the end of visible growth (14).

E. Sensitivity of disinfectant exposed *P. aeruginosa* to antibiotic

Coloneis from subminimal inhibitory concentration (SMIC) of each isolate were tested for their susceptibility to antibiotic according to (10).

F. SEM of *P. aeruginosa* isolated before and after disinfectant exposure

One hundred µl of *P. aeruginosa* suspensions which prepared from subMIC of different disinfectants were fixed with 100 µl formalin 10% in phosphate buffer (pH 7.4) for 10min. then a loopfull of the mixture were mounted on a glass slides and allowed to dry for 40 min. In final steps, Stubs were coated with pure gold by sputter coater and examined using SEM (15, 16).

After preparation of exposed isolates, they were mounted on a glass slide exhibited through the screen SEM by mechanical and computerized techniques. Then pictures were taken by the technician for further interpretation.

III. RESULTS

A. Isolation and identification of *Pseudomonas aeruginosa*

Fifty isolates of *P. aeruginosa* were identified from burn, wound, ear and urine in percentages 30%, 13%, 20% and 6%, respectively.

B. Susceptibility of *P. aeruginosa* isolates to antibiotics

It has been found that all *P. aeruginosa* isolates were resistant to doxycycline, tetracycline, chloramphenicol, ampicillin, amoxicillin, cloxacillin, cephalixin, rifampin, cefotaxime, trimethoprim and erythromycin, while one burn isolate was susceptible to gentamicin, chloramphenicol and trimethoprim, and 15 of burn, 6 of wound, 5 of ear, and all urine isolates were susceptible to gentamicin.

C. MICs of disinfectant for isolates for *P. aeruginosa* isolates

The results showed that the MICs of chloroxylenol (dettol) for burn, wound, urine and ear isolates were ranged from (2048–16384), (2048, 4096, 16384), (2048–8192) and (4096–16384) µg/ml, respectively. The MICs of Povidone-Iodine for burn, wound, urine and ear isolates ranged from (8192–32768), (8192–32768), (80192–16384) and (80192–16384)µg/ml, respectively, and the MICs of Chlorohexidine gluconate (hexatane) for burn, wound, urine and ear isolates were ranged from (128–512), (128–512), (256–512) and (128–512)µg/ml, respectively, while the MICs of chlorhexidine/ cetrimide (savlon) for burn, wound, urine and ear isolates were ranged from (40960:4096) – (327680:32768), (40960:4096) – (163840:16384), (81920:8192) – (327680:32768) and (40960:4096) – (163840:16384)µg/ml, respectively.

D. Antibiotic resistance *P. aeruginosa* sensitivity after exposure to antimicrobials

The results showed that the sensitivity of all *P. aeruginosa* isolates (100%) resistant to ampicillin, amoxicillin, cloxacillin, cephalixin and erythromycin didn't changed after disinfectants exposure, while 36(72%) of *P. aeruginosa* isolates lost their resistance to each of tetracycline and doxycycline, 34(68%) of them lost their resistance to rifampin, 18(36%) to trimethoprim, 14(28%) to cefotaxime and 11(22%) to chloramphenicol as shown in below table.

Degree of sensitivity of six antibiotics	Antibiotics					
	Tetracycline	Doxycycline	Rifampin	Trimethoprim	Cefotaxime	Chloramphenicol
Sensitive %	72	72	68	36	28	22
Variabl e %	6	6	10	46	48	46
Resistan t %	22	22	22	18	24	32

Table: Susceptibility to antibiotics pattern of *P. aeruginosa* isolates after disinfectants exposure.

E. Morphological changes on disinfectants exposed P. aeruginosa using Field Emission-SEM

Four *P. aeruginosa* isolates exposed to studied disinfectants from subMICs and before subMICs were chosen to study the morphological changes which may be related to the lose of their resistance to antibiotics using FE-SEM, one of them was exposed to dettol, other to savlon, the third to povidone-iodine, and the fourth was exposed to hexatane .

FE-SEM results on figure (1), show the normal rod shaped *P. aeruginosa* before disinfectant exposure which measured 1.5-3.0 μm .

Figure (2) show FE-SEM image of dettol exposed *P. aeruginosa*. The results showed normal morphology with small sized bacteria and observed nub formation on some bacterium also figure (3) showed membrane immobilization and shape changed bacteria, while figure (4) shows changes in morphology and shapes during the stress on surface of bacterium after exposed to savlon (chlorohexidine/cetrimide) which is elongated at low concentration and became rounded at subMICs also figure(5) shows deformation of *P. aeruginosa* cells when exposed to hexatane (chlorohexidine/gluconate).

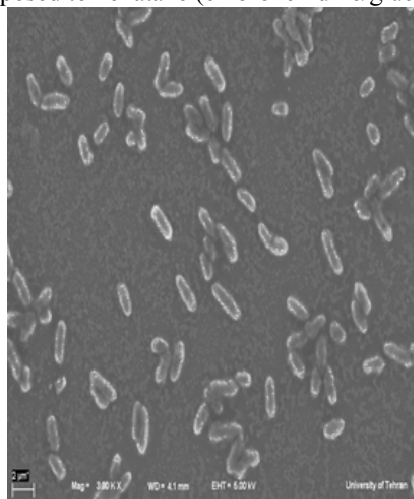


Figure: (1) FE-SEM micrograph of normal rod shaped *P. aeruginosa* before disinfectant exposure, with different sizes.

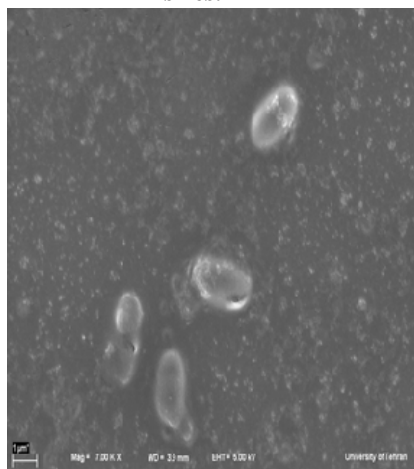


Figure: (2) FE-SEM micrograph of *P. aeruginosa* exposed to dettol; normal shaped but small sized bacteria with nub formation on some bacterium.

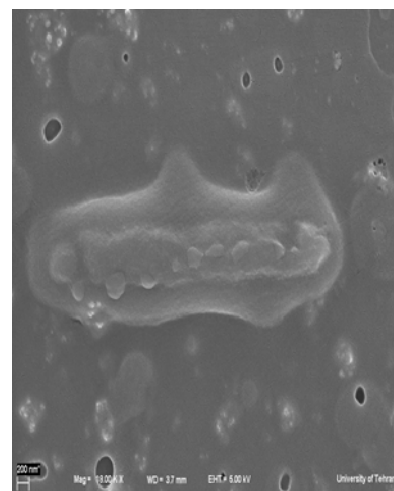


Figure: (3) FE-SEM micrograph of *P. aeruginosa* exposed to Povidone-Iodine; show bulging of cells (18000X).

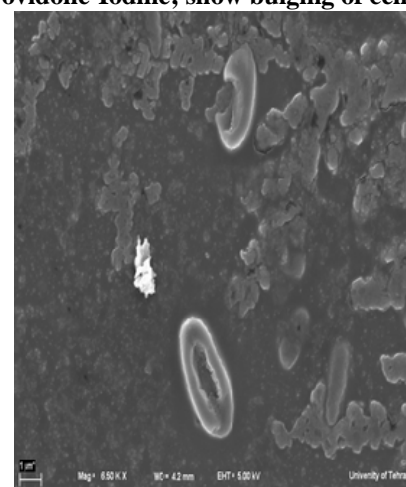


Figure: (4) FE-SEM micrograph of *P. aeruginosa* exposed to Savlon; show cell elongation at low concentration and rounded cell (6500X) at subMIC.

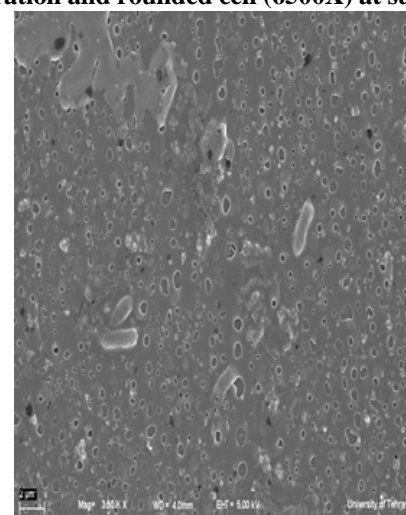


Figure: (5) FE-SEM micrograph of *P. aeruginosa* exposed to Hexatane; show cell deformation (3500X) and spheroplast formation.

IV. DISCUSSIONS

It is universally agreed that *P. aeruginosa* is an important pathogenic microorganism in burn, wound, ear and urine. This may be due to the fact that these samples were not taken regularly, but can therefore be considered as reflections of the actual situation of *P. aeruginosa* of the patients in these hospitals.

In a study conducted by Hama-Salih, in Iraq, it has been found that *P. aeruginosa* were isolated from 36%, 12% and 12% of burn, wound and urine infections, respectively (17). Another study in Iraq by Al-Grawi, et al., who revealed that the low susceptibility is attributable to concerted action of chromosomally-encoded multidrug efflux pumps genes. These genes are often controlled by regulatory gene located on the same operon of efflux pump. One of the particular significances is the MexAB - OprM efflux system, which is expressed constitutively, thereby contributing to the well-known intrinsic resistance of this organism to multiple antimicrobials (18).

A study in Italy by Bonfiglio, et al., who revealed the frequent mechanism of resistance was β -lactamase-independent (intrinsic resistance), which was founded in 183 isolates and was probably due to impermeability and /or efflux mechanism. They was also demonstrated β -lactamase-mediated resistance in 111 strains (11%), and detected class C chromosomal β -lactamase in 64 isolates whereas secondary plasmid-encoded β -lactamases were detected in 34 isolates(19).

Generally, the effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, the type and the number of microorganisms present. The results of MICs of each disinfectant showed that urine and burn isolates were more resistant than wound and ear isolates for each disinfectant. These resistance may be due to the high production rate of slime layer in burn and urine isolates in comparison with wound and ear isolates. The results also showed that hexatane more effective than dettol, savlon and povidone-iodine which may be due to disorder of protein structure and nucleic acid through change of oxidation of active group in these molecules (20) as well as the action of dettol may be due to poisoning of protoplasm and disruption of cell wall and its proteins.

Lose of resistance to antibiotics after exposure to disinfectants may be due to the morphological changes of cell membrane and cell wall which control the permeability in addition changes of plasmid mediated and extra-cellular chromosomally.

Another study reported survival of *S. enterica* serovar Typhymurium following exposure to various disinfectants at low concentration changing in antibiotic profile (21). They concluded that growth of *Salmonella* with sub-inhibitory concentrations of biocides favors the emergence of strains resistant to different classes of antibiotics. Fraud et al.,) found that *Pseudomonas aeruginosa* overexpressing multi-drug efflux systems during exposure to chlorhexidine (22).

Nikaido, reported changes in cell envelope such as reduction in porins and changes in LPS and other lipids when

some strain of Gram negative exposed to phenol-based disinfectant (23), also produced a change in protein expression consistent with the expression of an efflux pump system (24).

The bulging and shape changed bacteria when exposed to povidone-iodine that causes disorder of protein structure, oxidation of (-SH) group in amino acid, and membrane immobilization, while the elongation and deformation of bacteria exposed to savlon and hexatane may be due to inhibition of cross-linking of peptidoglycan cell wall and maybe there is a leakage of cell components. A recent study in Hong Kong by Cheung et al., hypothesized that the action of chlorhexidine may be more specific on certain lipids in the cell membrane of the bacteria after observed changes on cell wall by scanning electron microscope (25). Our results is agreed with Bulgaria study conducted by Shalamanov, who reported holes in the cell wall and deformation in *P. aeruginosa* treated with chlorhexidine gluconate which was observed by scanning electron microscope(26).

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