Effect of Salicylic Acid and Azithromycin on Pseudomonas aeruginosa

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Abstract: 10 clinical isolates of *pseudomonas* were collected from College of Science department of Biology/Al-Mustansiriyah University . This bacterium was able to grow on Pseudomonas agar as a selective medium for Pseudomonas genus .The ability of the isolates to produce biofilm were tested using congo red plate method .Results showed that 70% isolates were not biofilm producers while other isolates 30% produce it in different degrees .Screening of proteolytic bacteria All isolates tested for their proteolytic did not observe presence clear zones around the streak on the skimmed milk agar (an indication of protease production). But Screening the Collected Isolates for Phospholipase Production 80%(8/10) isolates were found to produce phospholipases on the surface of eggyolk agar plates.

P.aeruginosa isolates were tested for 8 antibiotics by using agar diffusion method, The results showed high resistance against Ceftriaxone100%,Ceftazidime 90%,Pipracillin 90%,Amikacin90% and Ticarcillin + glavulanic acid 90%. Moderate resistance against Ciprofloxacin 70% and Oflaxicin 70%. the Azithromycin was found to be the most effective antibiotic against these isolates and the resistance was noticed only in 50% of the P.aeruginosa isolates . Agar dilution method was used for the determination of minimum inhibitory concentration (MIC) for the the Azithromycin. The results showed that there were differences in the MIC values among the these isolates, most of the isolates were able to grow in concentrations of the Azithromycin antibiotic reached to (0.5-4) µg/ml), at the same time some isolates they were able to resist this antibiotic with MIC value reached to 8 µg/ml and 16 µg/ml. That all tested concentrations of SA resulted in profound inhibition of all tested bacteria. The zone of inhibition increased as the concentrations of SA are increased. that lethal effect against bacterial cells which resulted in complete death of bacterial growth laid between the 20 and 80 MM for SA.

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Introduction

P. aeruginosa is a gram negative, uniformly stained, straight or slightly curved rods, measuring 0.5 to 1.0 µm by 1.5 to 5.0 µm in length. They are aerobic,non-spore forming , motile by one or more polar flagella (1). They are either incapable of utilizing carbohydrates as sourceof energy or degrade them“oxidatively”rather than fermentative pathway (few species are asaccharolytic) ,without gas formation (2) . *Pseudomonas aeruginosa* is a common gram-negative bacterium found in a variety of nosocomial infections (3) . One of the key factors rendering treatment of infections difficult is the formation of biofilm. Biofilm formation in *P. aeruginosa* has been shown to protect the bacterium from antibiotics and environmental fluctuations and is characterized by an exopolysaccharide secretion (4) .

The success of *P.aeruginosa* in diverse environmentsis attributed to its impressive arsenal of virulence factors,which include multiple cell-associated factors alginate (an exopolysaccharide), lipopolysaccharide, flagella and pili, and secreted virulence factors, including toxins, elastases, protease, phospholipase, as well as small moleculesthat include phenazines,rhamnolipid, and cyanide (5) .

Salicylic acid is a nonsteroidal anti-inflammatory drug , that inhibits growth and biofilm formation (6). The laboratory has reported that salicylic acid reduces the attachment of *P.*

aeruginosa and *S. epidermidis* to human corneal epithelial cells in vitro. (7). Furthermore, several authors have referred to the antibiofilm properties of salicylic acid. (8) .

Although antibiotic therapy has great benefits in treatment, the emergence of multidrug resistance in *P. aeruginosa* has left clinicians with limited therapeutic options. Azithromycin (AZM), amember of the macrolide class of antibiotics, is used to cure certain bacterial infections, primarily caused by Gram positive bacteria but also some Gram-negative pathogens. However, many clinical and experimental studies have shown beneficial effects of AZM in the treatment of patients with diffuse panbronchiolitis and cystic fibrosis, which are associated with *P. aeruginosa* infection (9) .

The major objectives of this study were to identify virulence factors such as biofilm ,phospholipase and proteases production of *P.aeruginosa* isolates.Test the antimicrobial susceptibility of *P.aeruginosa* isolates using disk diffusion method. Determination the Minimum Inhibitory Concentration (MIC) for Azithromycin on multidrug resistance *P. aeruginosa*.The present study was undertaken to investigate the effect of salicylic acid on multidrug resistance *P. aeruginosa* .

Materials and Methods

2-1 Cultures media:

2-1-1 Congo red agar :

The CRA medium was prepared with 37 g/l BHI broth, 50 g/l sucrose, 10 g/l agar, and 0.8 g/l Congo red. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121 C for 15 min separately from other medium constituents, and was then added when the agar had cooled to 55 C. Plates were inoculated and incubated at 37 _C for 24 h. The plates were inspected for the color of the colonies at 24 and 48 h. A positive result was indicated by black colonies where as nonproducing strains developed red colonies The Congo red dye directly interacts with certain polysaccharides, forming colored complexes or more likely some metabolic changes of the dye to form a secondary product could play a more important part in the formation of dark colonies (10) .

2-1-2 Egg yolk agar medium

Primary Screening The solid medium used for screening the isolates was egg-yolk nutrient agar prepared by aseptically beating up one egg-yolk with 15 ml sterile saline using sterile magnet and a stirrer, then mixing with 85 ml sterile molten (60˚C) nutrient agar (Difco) and pouring in Petri dishes, 20 ml each (11).

2-1-3 Skim milk agar

Skim milk agar was prepared with Gram weight per liter 71.0gm/L , Dry Milk, Instant Nonfat 50.0gm , Pancreatic Digest of Casein 5.0gm , Yeast Extract 2.5gm , Glucose 1.0gm , Agar12.5gm (12) .

2-1-4: Pseudomonas isolation agar:

Pseudomonas agar was prepared by dissolving 24.2 gm of pseudomonas agar base in 100 ml then completed to 500 ml of distilled water containing 5 ml glycerol; pH was adjusted to 7 then heated until the constituent is completely dissolved.The media was sterilized by autoclaving at 15 bar/in² pressure (121°C) for 15 min. The media was cooled to 50 °C and aseptically add sterile rehydrated content of Cetrinix supplement then the media was mix well and poured into sterile petri plates (13) .

2-2 Isolation of Pseudomonas spp :

10 clinical isolates of *pseudomonas* were collected from College of Science department of Biology/Al-Mustansiriyah University .

2-3 Identification of pseudomonas spp.

To confirm this diagnosis the bacterial isolates cultured on MacConkey agar and Pseudomonas agar under aerobic conditions followed by other diagnostic tests .The pale colonies on MacConkey agar (lactose non fermenting). This bacterium was able to grow on *Pseudomonas* agar as a selective medium for *Pseudomonas* genus and also some isolates were able to produce pigments as pyocyanin and the fluorescent pigment pyovridin.

2-4 Biofilm Production Congo red test

(14) have described method for screening of biofilm formation. Plates were inoculated by pure single isolated colony and incubated aerobically for 24 - 48 hr at 37C°. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result .

2-5 phospholipase formation

we used egg yolk media for detection of phospholipase enzyme production of ***pseudomonas*** isolates, transport part of colony from nutrient agar media its aged 24 hours to egg yolk media and cultivation in incubator by 37c for 24h after incubation we can see inhibition zone about colony that's mean production of phospholipase (15) .

2-6: protease formation

we used Brain heart infusion broth to activation and cultivation the isolates we cultivation the media in 37c for 24h , Rejection of transplanted by cold centerfuge in 6000 cycle/min for 15min , and then take 100 mlt from filtration and put on pores on skim milk agar and cultivation by 37c for 24h and seen inhibition zone about pores that's mean production of protease (16) .

2-7: Antibiotic Susceptibility Testing

Susceptibility testing was conducted by disk diffusion according to the guidelines of the Clinical and Laboratory Standards Institute (17). Antibiotic discs (Becton Dickinson, USA) were placed on Mueller-Hinton agar plates, incubated at 37°C for 24 h, and the diameter of each zone was measured in millimeters. The following antibiotic discs were used:8 Antimicrobial disks tested included Azithromycin Azi(15ng) , Ceftriaxone:Cro(30 µg) , Ceftazidime:Caz(30 µg), Pipracillin; Pi(100 µg) , Amikacin AK (30 µg), Ticarcillin + glavulanic acid; tcc (75 µg) , Ciprofloxacin Cip(5ng) and Oflaxicin Ofx (5ng) . *P.aeruginosa* ATCC 27853 was used for quality control purposes in susceptibility testing.

2-8: Determination of the Minimum Inhibitory Concentration (MIC)

Stock solution of the antibiotics **Azithromycin** was prepared at final concentration 10 mg/ml according to Clinical Laboratory Standard Institute (17) (CLSI) recommendations using distilled water. They were sterilized by filtration and stored at -20°C in small container Minimum inhibitory concentration (MIC) was determined for all isolates according to the CLSI criteria by a standard agar dilution method as follows: Serial two fold dilution of each antibiotic being tested ranged from 0.05 to 1024 µg /ml were prepared. The following antibiotic were used (Azithromycin).Mueller-Hinton agar medium was prepared, sterilized by autoclave and cooled to 45°C, then the antibiotics were added in appropriate amount from their stock solution , mixed well and poured into the plates. Few colonies (2-4) from overnight culture were transferred to 2ml of normal saline in order to prepare the bacterial suspension and were adjusted to 0.5 McFarland turbidity equal to 1.5×10^8 CFU/ml .Five microliter of each inoculum was spotted on the agar surface by micropipette. The plates were left to dry for 5 minutes and then incubated at 37°C for 18-24 hr. Mueller-Hinton agar medium without any antibiotic was used as a positive control and the standard strain *P. aeruginosa* ATCC 27853 were used as a negative control. MIC results were read after 18-24 hr. The lowest concentration of antibiotic inhibiting the bacterial growth was recorded as the MIC.

2-9 : Effect Salicylic acid on *pseudomonas*

Stock solutions of SA was prepared in sterilized distilled water supplemented with 0.1% tween 80 to obtain certain concentrations. Serial dilutions of each compound were made using sterilized distilled water and I or nutrient broth medium (for bacteria) .Concentrations of 0,10,20,40 and 80 mM based on the molecular weight of each of was used.In vitro assay: The direct effect of SA and ASA at different concentrations on the growth of various bacterial isolates was in vitro evaluated. A- Effect on bacterial growth: Zone of growth inhibition technique (18) was followed in this test. Conical flasks each containing 150 ml of warm sterilized nutrient agar medium were artificially seeded before solidifying with 5 ml of a 48 hr old nutrient broth of tested bacteria, then poured into Peal dishes. Wells (0.5 cm diameter) were made up into solidified medium using sterilized cork borer. Equal volume (0.1 in]) of each SA concentration tested was poured into each well. Concentrations at 10,20,40 and 80 mM of each SA was evaluated.Five Petit dishes were used as replicates for each particular treatment as well as the control. All Peal dishes were incubated at 23*1°C for 72 hr then examined. Clear zone diameter of bacterial growth inhibition was measured

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and the inhibited growth area was calculated.Plate count technique(18) was used to determine the lethal dose of SA on bacteria.

RESULTS& DISCUSSION

3.1 :Collection and diagnosis of the isolates

The 10 collected isolates from College of Science department of Biology/Al-Mustansiriyah University .To confirm this diagnosis the bacterial isolates cultured on MacConkey agar and Pseudomonas agar under aerobic conditions followed by other diagnostic tests .The pale colonies on MacConkey agar (lactose non fermenting). This bacterium was able to grow on *Pseudomonas* agar as a selective medium for *Pseudomonas* genus and also some isolates were able to produce pigments as pyocyanin and the fluorescent pigment pyovridin.

3.2: Detection of biofilm formation

The ability of *P.aeruginosa* to adhere and form multilayered biofilms on host tissue and other surfaces is one of the important mechanisms by which they are able to persist in the diseases.An association is observed between multiresistance and biofilm production. The biofilm environment seemed to increase genetic exchanges and hence may contribute to multiresistance phenotypes (19). From this point the ability of *P. aeruginosa* isolates were tested to produce biofilm by Congo red agar method.

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3.2.1 Congo-red agar method (CRA)

The results of biofilm production showed that 30% (3/10) of isolates produced strong slime layer indicated by formation of black colonies, while 70% (7/10) of isolates did not produce slime layer indicated by formation of pink colonies(figure 3-1).This result disagree with (20) who indicated that the 90% of isolates gave black colour colonies on congo red agar while only 10% isolates gave pink colour colonies indicating that no biofilm production . As well as these results were in harmony with those reported by (21) ,who indicated that the 72.7% isolates gave black colour colonies on congo red agar plate while 27.2% isolates gave pink colour colonies indicating non biofilm production . In the same manner. The differences in the results could be due to the high number of tested isolates that be are used in the study (10 isolates) which compared with other studies that included low number of isolates that may affect the sensitivity and accuracy of test.

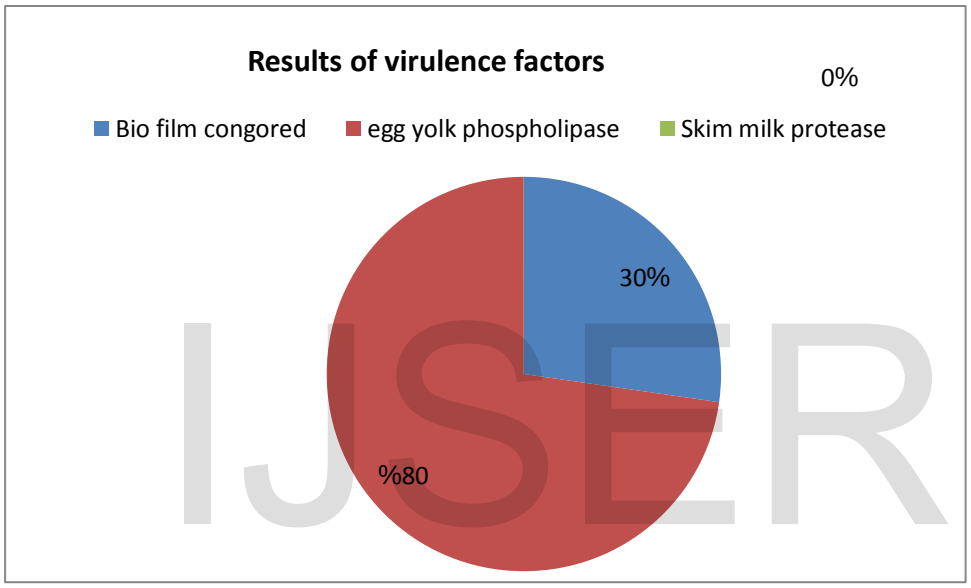
3.3: Screening of proteolytic

The screening of proteolytic 10 isolates tested for their proteolytic potential .Did not observe presence clear zones around the streak on the skimmed milk agar (an indication of protease production). This result disagree with (22) When tested for their proteolytic potential, 11 Isolates demonstrated clear zones around the streak on the skimmed milk agar (an indication of protease production). Among these isolates, demonstrated the highest zone of proteolysis (24 mm) . Less production of protease by clinical isolates from different

infections have also been related with deletion of QS components (23) .Various other workers have also documented low levels protease among QS deficient strains (24) .

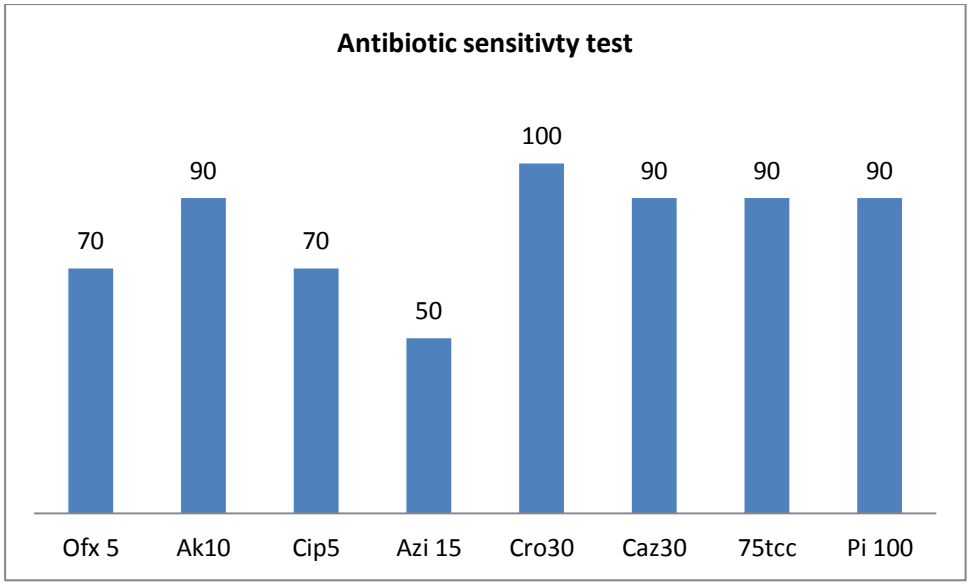
3.4: Screening Phospholipase Production

screening the Collected Isolates for Phospholipase Production 80%(8/10) isolates were found to produce phospholipases on the surface of eggyolk agar plates (figure 3 -1). the isolate (1,4,5,6) gave the highest Phospholipase Production and highly multidrug resistance.Therefore, these four isolates were selected for high virulence factor . This results which compared with (25) that included Screening the Collected Isolates for Phospholipase Production Of the collected 205 bacterial isolates, 43 bacterial isolates having different morphological characters were found to produce phospholipases .



3.5 .Antimicrobial susceptibility test for *P.aeruginosa*:

antimicrobial susceptibility test of all the isolates to different antibiotics was determined by the disk diffusion method .These isolates showed different susceptibility towards 8 antimicrobial agents used.All the *P. aeruginosa* isolates showed multidrug resistance to more than one antimicrobial agents. In this study some *P. aeruginosa* isolates were high resistance against Ceftriaxone100%, Ceftazidime 90%,Pipracillin 90%,Amikacin90% and Ticarcillin + glavulanic acid 90%. Moderate resistance against Ciprofloxacin 70% and Oflaxicin 70%. the Azithromycin was found to be the most effective antibiotic against these isolates and the resistance was noticed only in 50% of the *P.aeruginosa*



Figure(3-1): Azithromycin Azi(15ng) , Ceftriaxone:Cro(30 µg) Ceftazidime:Caz(30 µg), Pipracillin; Pi(100 µg) , Amikacin AK (30 µg), Ticarcillin + glavulanic acid; tcc (75 µg) , Ciprofloxacin Cip(5ng) and Oflaxicin Ofx (5ng) .

*According to CLSI Results of the high percentage of resistance in the current study against third and forth generation of cephalosporins (Ceftazidime and Ceftriaxone) ,this result agree with (26,27) who found that the resistance of *P.aeruginosa* to, Ceftazidime and Ceftriaxone,78% ,80% respectively. These differences could be explained to the increase resistance of Gram-negative bacteria to Cephalosporines .As well as *P. aeruginosa* isolates have low resistance of *P.aeruginosa* low resistance to Azithromycin 50% may explain their high activity of this antibiotics this partialy agree with (28) who found the rate of resistant to this antibiotic is 31.6% .Only four isolates (P1,P6,P7,P10) in this study were MDR to all antibiotics used .This high degree of antibiotic resistance may be due to the widespread usage of broad spectrum antibiotics leading to selective survival advantage of pathogen (29) .

3.6: Determination the Minimum Inhibitory Concentration (MIC) for Azithromycin.

The determination of the Minimum inhibitory concentrations for Azithromycin was done as complementary test to the previous sensitivity test to verify the amount of resistance . An isolate was characterized as resistant if the MIC was greater than the breakpoint MIC defined by (17) while it will be susceptible if it is less than the breakpoint. Five *P. aeruginosa* isolates showed complete resistance to Azithromycin ranged from (16) µg/ml and four isolates showed complete resistance to Azithromycin ranged from (0.5-4) µg/ml except one isolate showed MIC value equal to the break point (2) µg/ml. It has been observed by other worker (30) that The MIC for AZM for P. aeruginosa PAO1 was determined as 177 mg ml21. A sub-MIC (88.5 mg ml21) was used for all further experiments. Strain PAO1 produced 818 Miller units (MU) BHL and 137 MU OdDHL in absence of AZM (Fig. 1). The levels of BHL and OdDHL decreased to 372 MU (P,0.001) and 120 MU (P,0.01), respectively, in the presence of AZM. AZM is neither bactericidal

nor bacteriostatic in *P. aeruginosa*, but it inhibits QS signal production and may lead to attenuation of the virulence of *P. aeruginosa*.

3.7: Effect of Salicylic acid on *pseudomonas*

Different concentrations of SA was evaluated for their inhibitory effect against *P. aeruginosa* under laboratory conditions**.**The present work was conducted to study the direct effect of SA on the in vitro bacterial growth (figure 3-3)**.**

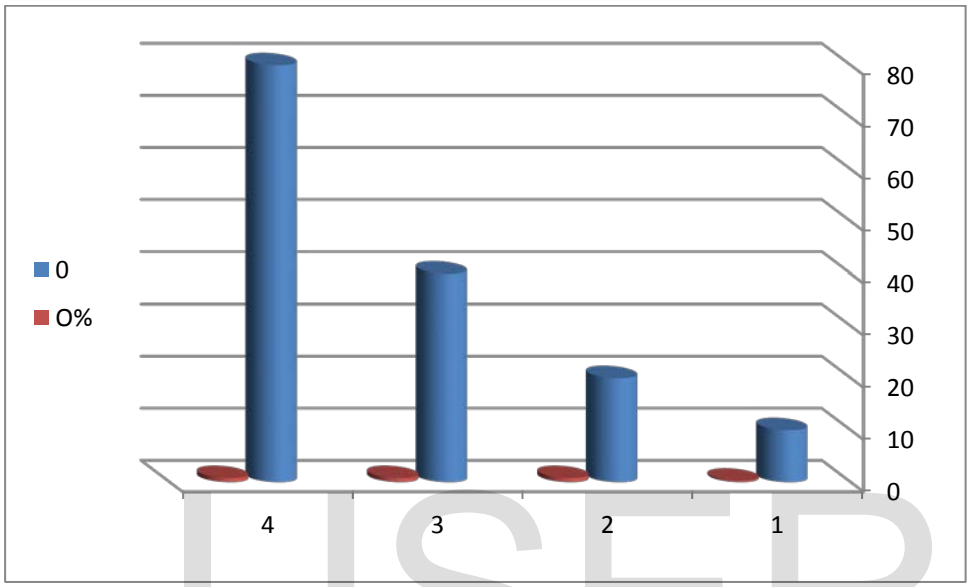


Figure 3-3: Percentage of effect different concentration of salicylic acid on isolates

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Data in Figure 3-3 show that all tested concentrations of SA resulted in profound inhibition of all tested bacteria. The zone of inhibition increased as the concentrations of SA are increased. It is interesting to note that SA showed significantly inhibitive effect against bacterial growth. Data also show that lethal effect against bacterial cells which resulted in complete death of bacterial growth laid between the 20 and 80 MM for SA .This result agree with (31) that show that lethal effect against bacterial cells which resulted in complete death of bacterial growth laid between the 25 and 30 MM for SA .

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