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ISOLATION AND IDENTIFICATION OF MULTI DRUG RESISTANT BIOFILM PRODUCER PSEUDOMONAS AERUGINOSA FROM PATIENTS WITH BURN WOUND INFECTION IN BASRA PROVINCE/IRAQ

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ABSTRACT

Clinical specimens were collected from 120 patients, 44 of them are males and 76 females with age range 1-70 years. A total of 49(40.8%) isolates were diagnosed as *P. aeruginosa* based on a number of tests and confirming by using 16SrDNA gene specific for *P. aeruginosa*. Results showed that the highest antibiotic resistance was in 40(81.6%) isolates resistant to Amikacin (AK), while 26(53.1%) isolates were sensitive to Colistin (CT). All isolates were resistant to more than three antibiotics, therefore classified *P. aeruginosa* as MDR .Also, some selective isolate showed the ability to form biofilm by using Cong Red method.

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INTRODUCTION

Burns are necrosis and damage to the skin and tissue caused by several factors including chemicals, electricity, heat, sunlight or nuclear radiation. Burn wound infections is an extensive health problem in many countries of the world (Mabrouk *et al.,* 2016). It is one of the most common wound infections. A Nosocomial infection is one of a very serious complication in burn units and associated with approximately 10% of the hospital acquired infections (NNIS, 2004). Its cause either by internal source that caused by microorganisms that exist as part of normal flora or caused by external source through exposure to the hospital environment, equipments and medical staff. The development of this infection among patients depends mainly on the source of the presence of the pathogens and the way it was transferred to the hospital and the susceptibility of the patient to the infection (Samuelsen *et al.,* 2010). One of the most common pathogens that are multidrug resistant and colonize burns wounds is *Pseudomonas aeruginosa*, they are found everywhere in water, soil and moist environment and have the ability to adapt to different environmental conditions (Singh *et al.,* 2010).

Until 1984, more than 100 species of *Pseudomonas*, including human and animal pathogens, were detected. These human pathogens were opportunistic (Collins and Lyne, 2004). *P. aeruginosa* is an opportunistic pathogen that can cause serious diseases and toxigenic infections in patients. It can infect nearly any external site or organ and its strains can caused nosocomial infections such as pneumonia, bacteremia, urinary tract infection, as well as skin infections. In addition to their involved in chronic and acute infections, especially in immunocompromised patients. It is common in hospitals, especially in intensive care units and it's found as dominant colonizer of the burns wound and quickly propagate within the damaged tissues often leading to distributed infections (Markou and Apidianakis, 2014).

MATERIALS AND METHODS

Sample collection

A total of 120 (44 male and 76 female) clinical samples of patients suffering from moderate to severe burns wound infection categorized according to age, gender, degree, and

percentage of burn were collected from February 2016 to December 2016 from Al-Fayhaa general hospital/Burns unit.. The samples were collected using sterile cotton swabs from the purulent burned skin (clean the place of burn wounds before took samples by normal saline) and submerged in 5 mL sterile nutrient broth, then transferred immediately to the laboratory in the Department of Biology/College of Science.

Identification and purification of MDR *Pseudomonas aeruginosa*

After transporting specimens to the laboratory, the swaps were streaked on MacConkey's agar and incubated at 37°C for 24 h. After incubation, blue-green pigmented colonies and those gave oxidase reaction positively were subcultured on *Pseudomonas* agar base, then the pure single colony subcultured on agar to diagnosis by morphological characteristics for colonies and microscopic examination by using gram stain and different biochemical tests including oxidase test, gas production, indole production, citrate utilization, H_2S production and fermentation tests (glucose, sucrose, and lactose) according to Kirby Bauer method in accordance with NCCLs guidelines (NCCLS, 1998).

Extraction of bacterial DNA

The bacterial DNA was extracted from 49 bacterial isolates by using DNA extraction kit, based on the manufacture protocol (Presto™ Mini gDNA Bacteria Kit, Geneaid -Taiwan). The extracted DNA was subjected to electrophoresis in 1% agarose gel stained with ethidium bromide. By using PCR technique, the 16 SrDNA gene of *P. aeruginosa* was amplified by using universal bacterial 16 SrDNA primers (Spilker *et al.,* 2004). Amplification of targeted DNA was done in a total volume 20 µl reaction (table 2) by using PCR reaction condition (table, 3)

Table 1. 16Sr DNA primers

Primers	Sequence
Forward	5′GGGGGATCTTCGGACCTCA-3′
Reverse	5′TCCTTAGAGTGCCCACCCG-3′

Table 2. The reaction mix (25 µl)

Chemicals	Volume (µl)
DNA template	1 µl
Forward primer	1 µl
Reverse primer	1 µl
GoTaq Thermo Master Mix	12.5 µl
Nuclease-free water	9.5 µl
Total	25 µl

Table 3. The PCR Condition of bacteria

	Steps	Temperature(^{0}C)	Time
1	Denaturation	94	4 min.
	1-Denaturation	94	30 sec.
2	2-Annealing	60	30 sec.
	3-Extension	72	2 min.
3	Elongation	72	7 min.

Antibiotic Susceptibility Test

The sensitivity of *P. aeruginosa* isolates and their resistance to antibiotics were tested by using disc diffusion method on Mueller-Hinton agar plates and using 13 antibiotic-containing discs commercial including (Amikacin, Azteronam, Cepfepime, Ceftazidime, Ciprofloxacin, Colistin, Gentamicin, Imipenem, Levofloxacin, Meropenem, Piperacillin, Ticarcilin, Tobramycin). The diameter of the zone of inhibition was interpreted as sensitive, resistance and intermediate patterns of *P. aeruginosa* against different antibiotics based on criteria published by CLSI guidelines (CLSI 2014).

Biofilm formation tests

Congo Red Agar (CRA) Method was to examine qualitative ability of *Ps. aeruginosa* for biofilm formation. The Congo red pigment have the ability to stain polysaccharides black. This medium was prepared by using sucrose and congo red stain (50g/L and 0.8g/L) were added to Brain Heart Infusion agar. The examined bacteria was streaked on congo red agar and incubated at 37 ^{0}C for 24 hours. Biofilm producers form black colonies on CRA, whereas non-producers form red colonies (Freeman *et al*, 1989).

RESULTS

Characterization of study population

Clinical specimens were collected from 120 patients, 44 of them are males and 76 females with age range 1-70 years, the mean age (Mean ± SD) was 21.3 ± 15.8 years. As shown in Table (3-3), the results indicated that the main cause of the burns was the flame in 92 (76.6%) patients, followed by hot water burned in 23 (19.1%) patients, then the electricity in 5(4.1%) patients. The percentage of burns caused by flame in females was higher than in males, where it reached to 69(57.5%) patients, and burns caused by hot water and electricity in males were higher than in females, where it reached to 17(14.16%) and 4(3.3%)respectively. The degrees of burns were varied but mostly over to 15%, also it was noted that most of the burns were higher in rural areas, where it reached to 82 (68.34%).

Isolation and Identification of *Pseudomonas aeruginosa*

A 120 isolates were swabbed on MacConkey agar, the colonies that appeared small and pale and gave positive results for oxidase test were subcultured on *Pseudomonas* agar base, where they produced dyes including green, fluorescent, and brown pigments and all other isolates were excluded. A total of 49(40.84%) isolates were diagnosed as *P. aeruginosa* based on a number of tests including gram stain and some biochemical tests such as oxidase test, gas, H_2S production, lactose fermentation, citrate utilization and indole test. Of 120 swabs, 19/120(15.83%) gave no growth in which patient with mortality rates 8/19(42.1%), also 52/120(43.33%) patients were infected only with bacteria other than *P. aeruginosa* with mortality rates were 13/71(18.3%). A 49/120(40.84%) patients were infected with *P. aeruginosa*, 22 of them are males and 27 females with age range 1-48 years, the mean age (Mean ± SD) was 21.9 ± 16.6 years.

Furthermore, of 49 patients, 28/49(57.14%) patients were infected with only *P. aeruginosa* with mortality rates were 28/28(100%), while 21/49(42.86%) patients were infected with mixed infections including *P. aeruginosa* and other bacterial species with mortality rates were 19/21(90.48%), while the proportion of recover patients was 2/21(9.52%). Overall, mortality rates due to *P. aeruginosa* were 47/49(95.92%).

Confirming identification of *P. aeruginosa*

Extracted DNA was subjected to PCR technique, amplified 16SrRNA gene was then subjected again to gel electrophoresis. PCR products gave a sharp band on agarose gel corresponding to a 956 bp when compared to the molecular ladder 2000 bp, thus identifying the isolates as *Pseudomonas aeruginosa* (Figure 1).

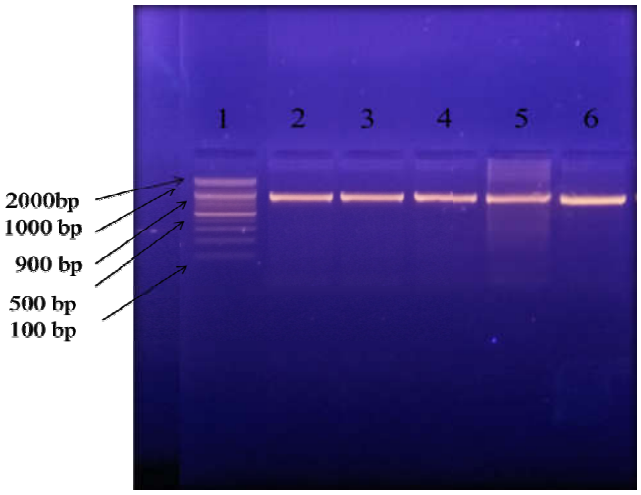

Figure 1. PCR product electrophoresis

Lane 1: ladder (100-2000bp) Lane 2,3,4,5, 6 isolates of *P. aeruginosa*

Antibiotic sensitivity of MDR *P. aeruginosa*

Table (6) showed that the highest antibiotic resistance was in 40(81.6%) isolates resistant to Amikacin (AK), while 26(53.1%) isolates were sensitive to Colistin (CT). All isolates were resistant to more than three antibiotics, therefore classified *P. aeruginosa* as MDR according to the criteria published by clinical and laboratory standards institute CLSI, 2014.

Table 6. Antibiotic sensitivity of MDR *P. aeruginosa* isolates

Antibiotic	No. of resistant *p. aeruginosa*	No. of sensitive *p. aeruginosa*
Amikacin (AK)	81.6%	18.4%
Azteronam (ATM)	61.2%	38.8%
Cepfepime (FEP)	69.4%	30.6%
Ceftazidime (CAZ)	59.2%	40.8%
Ciprofloxacin (CIP)	40.8%	59.2%
Colistin (CT)	46.9%	53.1%
Gentamicin (GN)	42.9%	57.1%
Imipenem (IPM)	65.3%	34.7%
Levofloxacin (LEV)	53.1%	46.9%
Meropenem (MEM)	59.2%	40.8%
Piperacillin (TPZ)	67.3%	32.7%
Ticarcillin (TIC)	57.1%	42.9%
Tobramycin (TOB)	71.4%	28.6%

Results of the present study showed the ability of the MDR *Ps. aeruginosa* to from biofilm as it revealed in figure (?) included the isolate which used for bacteriophage study .Results of Congo Red showed that isolates which produced black colonies were strong biofilm producers. Dark colonies without dry crystalline colony morphology considered as moderate biofilm producers. Weak production of biofilm gave dark pink colonies. Non-slime producers mostly turned out as dry red colonies.

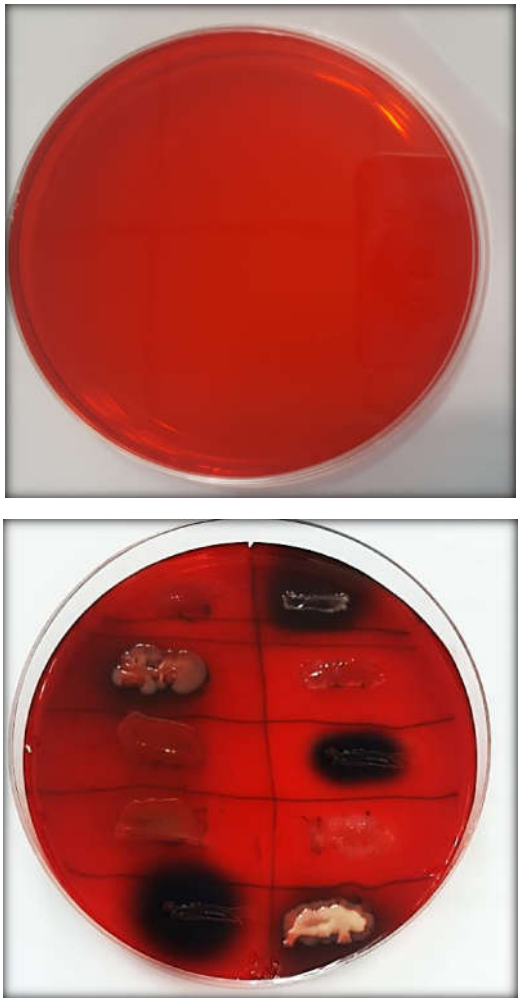

Figure 2. Biofilm formation of MDR *Ps. aeruginosa* by using Congo Red method .Left uncultured medium and the right culture of different isolates of bacteria showing the positive results (the black culture) and the negative (the red one)

DISCUSSION

Pseudomonas aeruginosa is the main pathogen that causes infection in burn units in Iraqi hospitals. The surface of moist wounds provides an environment suitable for their survival, This indicates that it is the ideal environment for its colonization and it will participate in the spread of diseases in the burns unit (Naqvi *et al.,* 2005). *Pseudomonas aeruginosa* is involved in a significant proportion of mortality rates. In current study, out of 40.8% patients infected with MDR *P. aeruginosa* but other studies have been recorded in Iraq and Tunisia 27% (Chahed *et al.,* 2014; Othman *et al.,* 2014) but South Africa 14.5% (Coetzee and Kahn, 2013) and Egypt 19.5 patients infected with *MDR P. aeruginosa* (Hassuna *et al.,* 2015). In the current study, a total 95.9% died within days after diagnosed, but in a study conducted in Pakistan, mortality rates were 18.9% (Al-Ibran *et al.,* 2011) and In Iran, the mortality rate was 30% (Anvarinejad *et al.,* 2014), but in Brazil, mortality rates were higher 51.1% (Millena *et al.,* 2008), while the proportion of recover patients was 0.98%, compared with other studies recorded 15% or less (Appelgreen, 2002) while in Pakistan has recorded a cure rate of 23.1% (Naqvi *et al.,* 2005). The present results showed that the proportion of burn in females was higher than males (57.5% vs. 19.16%) and the highest groups is burns of Flame 76.6%, Followed by burning with scald (Hot water) 19.1% then burning with electricity 4.1%, most of them attributed to

suicides as well as household chores such as cooking and heating. Compared to a study conducted in Sulaimania, the male infection rate was 56.5% and the highest groups scald (Hot water) 72.5%, flame 22.8% then electrical 1.8% (Rashid *et al.,* 2017). The antibiotic susceptibility test is one of the important tests to determine resistance and sensitivity of bacteria that are important for clinical purposes. The resistant of bacteria results from acquired many of mechanisms such as horizontal gene transfer and gene dissemination by horizontal transfer also due to the high use of these antibiotics as Amikacin, Gentamicin, Ciprofloxacin, and beta-lactam antibiotics. These bacterial strains make it difficult to treat patients (Aruna *et al.,* 2010). The study was recorded the highest resistance to bacteria for Amikacin 81.6%, Tobramycin 71.4% was the second effective drug against the isolates, Cepfepime 69.4%, Piperacillin 67.3%, Imipenem 65.3%, Azteronam (monobactam β-lactam) 61.2%, Ceftazidime 59.2%, Meropenem 59.2%, Ticarcillin 57.1% and Levofloxacin 53.1% respectively, while it was more sensitive to Ciprofloxacin 40.8%, Gentamicin 42.9% and Colistin 46.9%, although it was widely used in the treatment of patients with *P. aeruginosa* infection in the burns unit.

In a study conducted by Kamaria *et al.,* 2016 noted all the isolates were sensitive to Colistin. As compared to studies conducted by Naqvi *et al.,* 2005 that showed 70.5% isolates *P. aeruginosa* were resistant to Amikacin, 32.7% to Imipenem, 6.8% to Azteronam, 18.2% to Piperacillin and as recorded 77.3% sensitive against Imipenem and 81.8% for Piperacillin and recorded that Ciprofloxacin the most effective against 54.5% of isolates, but in the study by Zafer *et al.,* 2014 showed 72% of isolates were resistant to imipenem, 91% resistant to Ceftazidime. Also, in a study done Hassuna *et al.,* 2015 was recorded no resistance for imipenem and Piperacillin and 30.6% was sensitive to Cefepime while showed high resistance 86% for Ceftazidime and the results displayed 80 % *P. aeruginosa* isolates sensitive to Gentamicin.. Either Lockhart *et al.,* 2007 was exhibited that Ceftazidime sensitive against 4.5% and 28% in USA and Brazil respectively. The high resistance to antibiotics is due to many mechanisms, such as efflux pumps, β-lactamase, change of target sites for drugs and other mechanisms which means the difficulty of accessing the drugs to its target (Tavajjohi *et al.,* 2011). One of the main reasons for this resistance in the burns unit is due to the frequent use of antibiotics, especially the broad spectrum, in addition to the existence of strains resistant to treatment continuously and moving from one patient to another. Staff are also the main source of contamination and the spread of bacteria from one patient to another (Beheshti and Zia, 2011).
Bacteria that produce biofilm have the responsibility of different types of infections and difficult to eradicate. They are resistance to various antibiotics by various methods such as restricted penetration of antibiotic into biofilms, growth rate reduction and resistance genes expression. Different methods were used for biofilm detection. The present study showed that some isolate of bacteria were strong producer of biofilm by using Congo red Agar which published by Freeman *et al.,* 1989 that detect by diffusion of black pigment in the agar with growth of black pigmented colonies.

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