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Detection of some virulence factors of *Pseudomonas aeruginosa* isolated from burns and wounds

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Abstract

Pseudomonas aeruginosa is a pathogenic microbe that has a wide variety of opportunistic infections, including surgical site, urinary tract, pneumonia and bloodstream, wounds and burns, ear, nose, and throat infections, as nosocomial infections in the hospital intensive care units (ICUs). Nineteen isolates of *P. aeruginosa* from burns and wounds were collected. All isolates also were subjected to the cultural, biochemical tests as well as vitek 2 system. Moreover, these isolates had been tested for susceptibility to (10) antibiotic discs and also detection of some virulence factors included hemolysin, pyocyanin, swarming motility, and biofilm formation. Most isolates were resistant to tetracycline, imipenem, and doxycycline, whereas they showed more sensitive to amikacin, tobramycin, and aztreonam. *P. aeruginosa* had been showed different range for hemolysin activity, pyocyanin and swarming motility, furthermore they varied (moderate and weak) for biofilm formation.

Keywords: *Pseudomonas aeruginosa*, Virulence factors, Wounds and burns

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Introduction

In a wide range of immunosuppressed patients, *Pseudomonas aeruginosa* is a significant human pathogen that causes serious infections [1]. More significantly, it is a widespread multidrug-resistant (MDR) gram-negative pathogen that causes pneumonia in hospitalized patients and is a highly prevalent pathogen of nosocomial infections worldwide [2]. *P. aeruginosa* is a significant human pathogen that causes serious infections. Furthermore, it is a common multidrug-resistant G-ve bacterium which tends to cause pneumonia in hospitalized patients and is a common cause of nosocomial infections across the world. *P. aeruginosa* is a bacterial pathogen related to higher morbidity, mortality, and worse value of living in a number of human illnesses, involving burns, ulcers, and lung damage [3].

In wound infection, it's one of the most common pathogens, it's also renowned for forming difficult to remove antimicrobial-resistant biofilms, as well as chronically wounds, and consistent infection, severe inflammatory processes, and a significantly delayed healing process are a tremendous burden on patients and healthcare systems around the world [4]. A set of cellular structures and extracellular molecules, enzymes, and toxins such as exotoxin A, exoenzyme S, elastase, alkaline protease, hemolysin, phospholipase C, pigments (pyocyanin, pyoverdin, pyomelanin, and pyorubin) and other forms of virulence factors that play an important role in pathogenicity are produced by *P. aeruginosa* based on-stage pathogenicity to trigger different types of diseases [5, 6].

P. aeruginosa develops as colonies or communities enclosed in a matrix of extracellular polymeric substances (EPS), known as bacterial biofilm, in the natural environment and during hosts' infection [7]. The current study aimed to detection of some virulence factors and antibiotic susceptibility of P. aeruginosa isolated from burns and wounds.

Materials and Method

Collection of bacterial isolates

Bacterial isolates were obtained from Mustansiriyah University/College of Science/ Department of biology, they were obtained from wounds and burns swabs.

Identification of *Pseudomonas aeruginosa*

Cultural Examination

Nineteen isolates had been cultivated on blood agar and nutrient agar plates. The colonies in these media subcultured on MacConky agar, and pseudomonas agar plates to make sure of those isolates belong to *P. aeruginosa*. The recovered isolates were subjected to morphological and biochemical test for re-identification.

Pseudomonas aeruginosa was identified depending on the morphological features on blood agar, MacConkey agar, Pseudomonas agar were described according to their shape, color, diameter, oder and other characteristics [8].

Microscopical Examination

According to Holt [9], one isolated colony was transferred to a microscope slide, which was then fixed and stained with gram stain. Gram reaction, cell arrangement, and cell morphology were all noted.

Identification using Vitek 2 System

Analyst automated diagnostic apparatus confirmatory used for *P.aeruginosa* isolates using the GN ID card. Streak the surface of Pseudomonas agar by isolated bacteria to had been diagnosed and incubated at 37oC for 24 h. The GN ID Cards are removed from their

covers, and the model number is entered on the device's record, these steps of inoculated cards were done.

Antibiotic Susceptibility Test

Kirby–Bauer disc diffusion method is generally used for antibiotic sensitivity testing.

For (10) antibiotic discs including (Imipenem, Tetracycline, Doxycycline, ceftriaxone, Ceftazidime, Gentamicin, Amikacin, Cefepime, Tobramycin and Aztreonam).

Hemolytic activity

Plate Method (agar medium)

Bacterial suspensions in sterile saline matching to 1.5 x108 CFU/ml were done from 18 h cultures of *P.aeruginosa* isolates. 10µl of each suspension was dropped on the surface of the blood agar media and was incubated at 37°C for 16h.

After 16 h, the hemolysis was examined 10.

Spectrophotometric Method (liquid medium)

The hemolysin production was detected in liquid medium by spectrophotometric method described by Di Venanzio et al. (2014) with some modification [11]. The hemolysis percentage was calculated as equation described by Hertle et al. (1999) [12]: Hemolysis%=(A_571 (sample with hemolysin)- A_571 (control without hemolysin))/(A_571 (total lysis caused by Triton X100)-A_571 (control without hemolysin))×100

Biofilm Formation

Bacterial isolates from burns and wounds were tested for biofilm forming capabilities using the Micro titer plate technique, as reported by Mathur et al., (2006) and Ali (2012) [13,14]. According to the absorbance values, the biofilm formation of each isolate was classified into the following [15].

OD ≤ ODc (None),

ODc < OD ≤ 2 ODc (Weak),

2ODc < OD ≤ 4 ODc (Moderate),

4ODc < OD (High)

Pyocyanin production

This test has been used to detect bacterial isolates' capacity to produce pyocyanin, P. aeruginosa isolates was inoculated in King A agar, incubated at 37C°for 24 h and observed for color change by visually observing of agar medium. The blue-green pigment indicated positive result [16].

Swarming motility assay

Swarming assay was done according to the method described by Ugurlu et al. (2016) with some modification in incubation period 18-48hrs. The capacity to swarm was measured in 24 h by measuring the distance swarming from the primary inoculation site [17].

Results

The total isolates (19) of *P. aeruginosa* from burns and wounds were obtained (Mustansiriyah University/College of Science/Biology Department). *P.aeruginosa* isolates had been re-identified using cultural characteristic, biochemical tests and vitek 2 system.

Identification of *Pseudomonas aeruginosa*

Cultural Examination

The isolate of *P. aeruginosa* from burns and wounds were grown on blood agar, MacConkey agar, Pseudomonas agar, and nutrient agar plates to ensure identification, which was subsequently followed by further differential identification tests.

The bacterial colonies looked pale yellow on MacConkey agar because they not fermented lactose, which was agreed with Forbes et al, 2016.

The growing colony of *P. aeruginosa* on pseudomonas agar exhibited as pale yellow smooth spherical colonies with green pigment production that diffused throughout the agar, the growth of *P. aeruginosa* colonies on Nutrient agar was studied in terms of colors and odor generation (grape like odor). The colonies of *P.aeruginosa* isolates create a clear zone on blood agar media due to hemolysis production and also have a grape-like or tortilla-like odor. These results are agreed with Brooks et al. (2013).

Identification using Vitek 2 System

The Vitek-2 GN ID system had been a novel and promising highly automated method for the fast identification of G-ve bacterium species. Nineteen isolates of *P.aeruginosa* were identified, which (7) isolated from wounds, while (12) isolated from burns.

Antibiotic susceptibility test

The results revealed that all the *P.aeruginosa* isolates obtained from this study showed variable resistance to the tested ten antibiotics used. *P.aeruginosa* isolates showed varied levels of resistances to antibiotics. Also results revealed that the 15 isolates had been resistant to tetracyclin with 78.95 %, where the 14 isolates of *P.aeruginosa* from 19 had been resistant to imipenem (73.68%) and 13 isolates had been resistant to doxycycline (68.42%). Ceftriaxone had a resistance rate of 47.36%, Ceftazidime and Gentamicin had a resistance rate of 26.32 %, Aztreonam had a resistance rate of 21.05%, Cefepime had a

resistance rate of 15.79%, Amikacin had a resistance rate of 10.53% and, finally Tobramycin had a resistance rate of 5.26% (Figure 1).

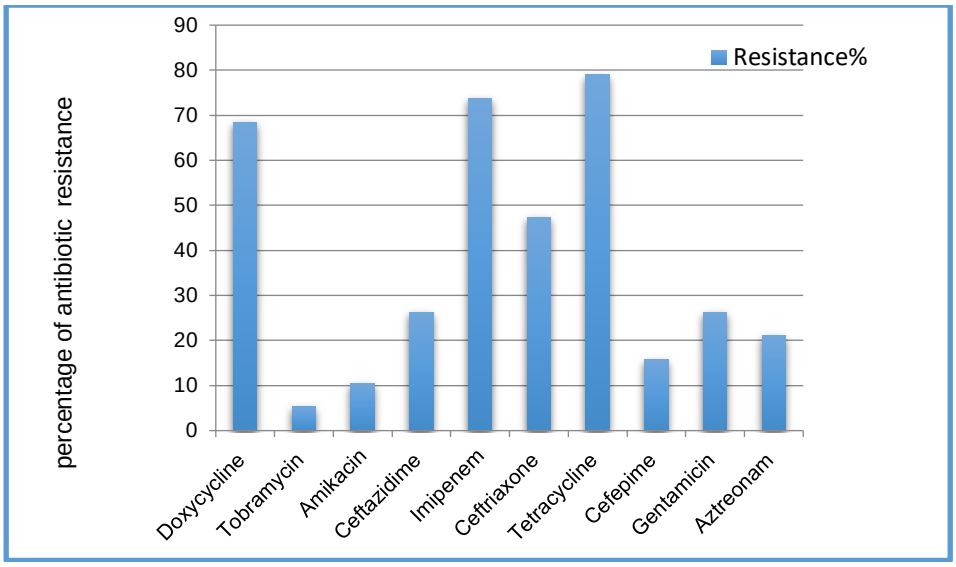

Figure 1.

Percentage of antibiotic resistance for *P.aeruginosa* isolates

Hemolysin production

The current study gave the results that highest hemolysis occurred in (9) out of (19) isolates of *P.aeruginosa*, including (4) isolates from burns (pb7, pb9, pb14, pb15) and (5) isolates from wounds (pw3, pw6, pw11, pw16, and pw17. The highest of hemolysis (91.89%) was recorded in *P.aeruginosa* (w6) isolated from wounds, and 90.65% in P.aeruginosa (b9) isolated from burns, while the lowest hemolysis (9.63) was investigated in P.aeruginosa (b19) isolated from burns, and 19.18% in *P.aeruginosa* (w1) isolated from wounds (Table 1).

Table 1.

Percentage of hemolysis of wound and burn infection *P.aeruginosa*

Isolates	Hemolysis (%)
P. aeruginosa 1(w)	19.18
P. aeruginosa 2(b)	15.94
P. aeruginosa 3(w)	91.22
P. aeruginosa 4(b)	18.7
P. aeruginosa 5(b)	17.46
P. aeruginosa 6(w)	91.89
P. aeruginosa 7(b)	87.59
P. aeruginosa 8(b)	26.04
P. aeruginosa 9(b)	90.65
P. aeruginosa 10(w)	22.9
P. aeruginosa 11(w)	90.17
P. aeruginosa 12(b)	12.40
P. aeruginosa 13(b)	29.10
P. aeruginosa 14(b)	89.03
P. aeruginosa 15(b)	85.50
P. aeruginosa 16(w)	87.30
P. aeruginosa 17(w)	85.78
P. aeruginosa 18(b)	11.55
P. aeruginosa 19(b)	9.63

Biofilm formation

The biofilm biomass that adheres to the microtiter plate walls was quantified in this experiment. The results revealed that 14/19 isolates (73.68%) of *P.aerugiosa* exhibited the capability to produce biofilm. 7/19 isolates recorded as former weak biofilm and 7 isolates moderate biofilm formation, and 5 isolates had no biofilm formation (Table 2).

Table 2.

Detection of biofilm formation of *P.aeruginosa* isolated from burns and wounds.

Bacterial isolates	Biofilm formation
P. aeruginosa 1(w)	Non
P. aeruginosa 2(b)	Non
P. aeruginosa 3(w)	Moderate
P. aeruginosa 4(b)	Weak
P. aeruginosa 5(b)	Weak
P. aeruginosa 6(w)	Moderate
P. aeruginosa 7(b)	Moderate
P. aeruginosa 8(b)	Moderate
P. aeruginosa 9(b)	Moderate
P. aeruginosa 10(w)	Weak
P. aeruginosa 11(w)	Moderate
P. aeruginosa 12(b)	Weak
P. aeruginosa 13(b)	Weak
P. aeruginosa 14(b)	Moderate
P. aeruginosa 15(b)	Non
P. aeruginosa 16(w)	Weak
P. aeruginosa 17(w)	Weak
P. aeruginosa 18(b)	Non
P. aeruginosa 19(b)	Non

Pyocyanin production

Only 10 *P.aeruginosa* isolates proved capable to produce pyocyanin (a blue green pigment) when streaked on King's A medium, including *P.aeruginosa* (pb2, pb4, pb5, pb7, pb8, pb9, pb12 and, pb14) 8/19 (42.11 %) isolated from burns and *P.aeruginosa* (pw3, and pw11) 2/19 (10.53%) wounds isolates (Table 3).

Table 3.

Pyocyanin production of *P.aeruginosa* isolated from burns and wounds

Bacterial isolates	Pyocyanin
P. aeruginosa 1(w)	-
P. aeruginosa 2(b)	+
P. aeruginosa 3(w)	+
P. aeruginosa 4(b)	+
P. aeruginosa 5(b)	+
P. aeruginosa 6(w)	-
P. aeruginosa 7(b)	+
P. aeruginosa 8(b)	+
P. aeruginosa 9(b)	+
P. aeruginosa 10(w)	-
P. aeruginosa 11(w)	+
P. aeruginosa 12(b)	+
P. aeruginosa 13(b)	-
P. aeruginosa 14(b)	+
P. aeruginosa 15(b)	-
P. aeruginosa 16(w)	-
P. aeruginosa 17(w)	-
P. aeruginosa 18(b)	-
P. aeruginosa 19(b)	-

Swarming motility

There were only 13 of 19 *P.aeruginosa* isolates which grown on swarming media appeared able to induce swarming. Amongst 13 isolates, 10 were burns isolates and 3 were wounds isolates. The highest swarming zone (40mm) was recorded in *P.aeruginosa* (b9) isolated from burns, and 30mm in *P.aeruginosa* (w3 and w11) isolated from wounds, while the lowest swarming zone (15mm) was identified in *P.aeruginosa* (b4) isolated from burns (Table 4), demonstrated that 76 (95%) of *P. aeruginosa* isolates formed biofilms [29].

The presence of biofilms has been confirmed in infected chronic wounds, however, whether biofilms in wounds hinder wound healing is a subject of debate. Increasing evidence has shown that the presence of biofilms in wounds leads to delayed healing [30]. Trøstrup et al. (2018) reported that Biofilms of *P. aeruginosa* impede central wound healing by diminishing vascular epithelial growth factor [31].

The result of Shariati et al. (2019) indicated that one of the main aspects that leads to delay the treatment process is high rate of biofilm formation of *P. aeruginosa* in burns and wounds infections [32].

The most prevalent virulence factor produced by *P.aeruginosa* was pigment [26]. Sismaet et al. (2017) identified that 75.6 % of isolates tested positive for pyocyanin after 24 hours of *P.aeruginosa* growth [33].

Our results had been agreed with finding of Al-shamary, (2018) that reported 22/63 (34.92 %) burns isolates, 8/63 (12.7%) wounds isolates, all fluids isolates 4/63(6.35%), and keratitis isolates 3/63(4.76%) were capable to produce pyocyanin from 63 *P.aeruginosa* isolates cultivated by streaking on King's A medium [28].

According to research of Robitaille et al. (2020), swarming motility as a compelling microbial cooperative behavior, the generation of LasR-deficient clones under swarming circumstances with *P. aeruginosa* [34].

Evidence indicated that swarming is much more than a way for bacteria to move, it was also a complicated living adaption in response to a variety of environmental cues that resulted in major metabolic alterations [35]. Several genes have been dysregulated during swarming motility, leading in a hard multi-antibiotic-resistant phenotype with accelerated virulence factor synthesis and iron scavenging [36]. As a result, research into the main molecules and mechanisms that control swarming motility becomes critical for the development of a therapy for these bacteria [37], demonstrated that 76 (95%) of *P. aeruginosa* isolates formed biofilms [29].

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Conclusion

Most isolates of *P. aeruginosa* were resistant to antibiotics and had been showed different range for production of virulence factors.

Ethical Approval

The study was approved by the Ethical Committee.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors shared in conception, design of the study, acquisition of data, and manuscript writing, the critical revising and final approval of the version to be published.

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