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Research Article

Effect of pigments of *Pseudomonas aeruginosa* on adhering and cytotoxicity of A549 cell line

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Abstract
Pseudomonas aeruginosa gram-negative, bacilli and facultative aerobic, *P. aeruginosa* cause cystic fibrosis patients, wounds, burns, and immunodeficienct patients, that have many virulence factors such as pyocyanin , cytotoxic ,biofilm formation and motility, Eighty-eight isolates belonging to *P. aeruginosa* were collected including the 66 clinical isolates obtained from different hospitals in Baghdad and were from different sources and 22 environmental isolates from previous studies of soil near oil fields. Microscopical and cultural characteristics were studied and diagnosed using biochemical tests, VITEC device, their ability to adhere to non-living (Polystyrene), living cell line (A549) and cytotoxicity of bacterial filtrate by MTT method. The results displayed that all isolates belonged to *P. aeruginosa*. The pigment-forming (pe26 – pc36) isolates and (PE33 – PC31) non-pigment -forming isolates were selected. That all selected bacteria were able to adhere to the Polystyrene and an epithelial carcinoma of lung (A549) was of more than 300 colony formation units in dilution (1:10) ,(1:1000), and (1:10000). The toxicity of the *P. aeruginosa* filtrate (pc36) isolated from clinical sources and producing pigments was 15.7, 34.5, 44 % at a concentration of 40, 60, 80 % respectively, while the isolate (pc31) that was isolated from clinical sources and non-producing pigment was 28.1, 75.2, 80.9 % at the same concentrations. As for the isolate (pe26),isolated from environmental sources and forming the pigment, the inhibition rate was 38.5, 83.1, 48.8 % at concentrations of 40, 60, 80) % respectively, and the isolate (PE33) that was isolated from environmental sources was 42, 73.4, 74.1 % at concentrations of 40, 60, 80 % respectively. The study will be helpful in evaluating the effect of pigment formation in *P. aeruginosa* on adhesion.

Keywords: A549 cell line, Adhesion, Cytotoxicity, Pigments, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is negative gram bacteria, bacilli, lengths ranging between 1-5 mm and widths of 0.5-1.0 mm, facultative aerobic, grow through aerobic respiration and anaerobic respiration with nitrates as a peripheral receptor of the electron and have the ability to grow on a medium of small growth of salts with a single source of carbon and energy (Diggle and Whiteley, 2020). *P. aeruginosa* is non-spore-forming and motile by polar flagella (Sawada *et al.,* 2022) and is positive for Catalase and oxidase (Abdali and Al-Attar, 2020). *P. aeruginosa* prefers to grow in a wide pH range from 5.6 to 8.0 but its optimum pH value is 6.6 – 7.0 (Urgancı *et al.*, 2022). Its grape-like smell characterizes it due to its production (2-Aminoacetophenone) (Al-Araji and Ali, 2012) and is the most common opportunistic pathogen that causes pathogenicity and mortality (AL-Fridawy *et al.*, 2020) and can produce four types of pigments including pyocyanin, pyoverdin, pyorubin, pyomelanin (Pier and Ramphal, 2010; Ezeador *et al.*, 2020). Pyocyanin causes cellular damage, such as inhibition of cellular respiration, epidermal cell growth and survival of *P. aeruginosa* (Mohammed *et al.*, 2014). It grows well at a temperature of 37°C to 42° C. It is widespread in nature, is widely found in humid environments in hospitals, and can colonize various body sites such as (GI tract, respiratory tract and mu-

cous membrane) (Riedel *et al.*, 2019). *P. aeruginosa* bacteria cause cystic fibrosis patients, wounds, burns, immunodeficiency patients, chronic obstructive pulmonary disorder and cancer (Qin *et al.,* 2022) and cause serious infections involving mainly all human organs, including lung infection, pneumonia and urinary tract infection, soft tissue infections in burn and open wound, diabetic foot ulcer and keratitis (Morin *et al.*, 2021), they are associated with serious diseases and infections such as pneumonia (Mohammed and Zgair, 2022). *P. aeruginosa* bacteria have many virulence factors such as protease, elastase and pyocyanin that cause tissue damage in the bloodstream (Jawad and Rasheed, 2022). as well as having pilli, which has a role in adhering to epithelium and the exoenzyme (S) that promotes adhesion to epithelium cell (Moissenet and Khedher, 2011) and exotoxin A, which is responsible for tissue necrosis (Al-Shwaikh and Alornaaouti, 2018), and has the ability to form a biofilm that has a role in the adhesion of *P. aeruginosa* bacteria to the living and non-living surface (Vetrivel *et al.*, 2021). In addition to possessing PorinF, it is an adhesive to alveolar epithelial cells (A549). It contributes to the adhesion of P. aeruginosa bacteria (Azghani *et al.*, 2002). That movement has a key role in the initial adhesion of bacterial cells on living and non-living surfaces (Khan *et al.*, 2020) and its secretion is exoenzyme U, which has a role in producing high levels of cytotoxicity (AL-Mayyahi *et al.,* 2018) as well as its possession of lipid A in the outer membrane of *P. aeruginosa* bacteria and is an endotoxin (Buré *et al.*, 2021). This work aimed to study the relationship between pigment formation and the ability of *P. aeruginosa* to adhesion.

MATERIALS AND METHODS

Identification of isolates

Eighty-eight isolates belonging to *P. aeruginosa* were obtained. Among them, 66 clinical isolates from different hospitals in Baghdad and were from different sources (burns/ wounds/ ea/ lung/ dialysis/ blood/ urine/ stool/sputum) and 22 environmental isolates from previous studies (soil near oil fields) and were cultivated on MacConkey agar, Cetrimide agar and Pseudomonas agar and all isolates were diagnosed by microscopy, biochemical tests and Vitek-2 device. (Moehario *et al.*, 2021). All the work was conducted as per the ethical committee of Ministry of Health in Iraq (Baghdad Teaching Hospital-International Center, for teaching laboratories) according to number 26416 on 29 June 2022.

Adhesion to polystyrene

A modified method (Fonseca *et al.*, 2004) was used to test the ability of *P. aeruginosa* bacteria to adhere to the polystyrene plate, selecting two clinical isolates, (pigment (pc36) and non-pigment (pc31), as well as for two environmental isolates, pigment (pe26) and non-pigment (pe33).

Adhesion to A549 cell line

A modified (Al-Shammari *et al.*, 2014) method was used to test the adhesion ability of *P. aeruginosa* bacteria in A549, selecting two clinical isolates, (pigment (pc36) and non-pigment (pc31), and the same case for two environmental isolates, pigment (pe26) and non-pigment (pe33).

Study of toxicity of *P. aeruginosa* filtrate on A549 cell line

El-Housseiny *et al.* (2013) method was followed with some modifications, where two clinical isolates were selected, pigment (pc36) and non-pigment (pc31), as well as two environmental isolates, pigment (pe26) and non-pigment (pe33). *P. aeruginosa* was inoculated in brain heart infusion broth, incubated at 37°C for 24 hours, then centrifuged at 2500 rpm for 5 minutes, and filtrated with milipore filters (0.22 µm). Cytotoxicity was measured by MTT method.

RESULTS AND DISCUSSION

P. aeruginosa isolates were diagnosed based on microscopic characteristics and biochemical tests and their growth on Cetrimide agar and Pseudomonas agar and the diagnosis (Table 1) was confirmed using a VITEC device.

Growth test on Pseudomonas agar and production of pigments

All clinical isolates obtained from hospitals cultured on the Pseudomonas agar medium appeared in dark green (37.87%), light green (16.66%), yellowish green (31.81%) and colorless colonies (13.63%). As for the environmental isolates obtained from previous studies, colonies formed were in dark green (36.36%), light green (9.09%) and yellowish green (18.18%) and colorless colonies were also formed (36.36%) and this is what was indicated by Seiffein and Ali (2021) that (40%) of the clinical isolates could grow in the pseudomonas agar medium and formed a fruit-like smell and all of them were due to the bacteria *P. aeruginosa*. Shamkhi and Khudaier (2020) indicated that only (32.5%) of its isolates grew on pseudomonas agar medium, producing a pyocyanin pigment, which spread in the medium and was due to *P. aeruginosa*.

Ability of *P. aeruginosa* isolates to adhere in an A549 cell line

The ability of selected *P. aeruginosa* isolates to adhere to an epithelial carcinoma of lung (A549) was tested. The results showed that the number of isolates (pc31,

Table 1. Source of *Pseudomonas aeruginosa* isolates and the number of isolates of each source.

Isolates source (Clinical)	Number
Burns	45
Wounds	2
Otitis Media	1
Lung	1
Dialysis	1
Blood	2
UTI	7
Stool	1
Septum	6
Isolates source (Environmental) Soil near oil fields	22
Total Number	88

pe26, pc36, pe33) that could adhere to the (A549) were more than 300 cfu in dilution (1:10) and (1:1000). In contrast, in dilution (1:10000), pc36 and pe26 isolates could adhere were 64, 62 cfu respectively and pc31 and pe33 isolates more than 300 cfu for both isolates (Table 3).

Ability of *P. aeruginosa* Isolates to adhere to the polystyrene

The ability of the selected *P. aeruginosa* isolates (four isolates, including two clinical isolates forming the pigment and two non-pigment-forming isolates, and two environmental) to adhere to the polystyrene is shown in Table 4. All the selected isolates (pc36, pe26, pc31, pe33) able to adhere to polystyrene were more than 300 cfu in dilution (1:10) and (1:1000) while at dilution (1:10000), the (pe26) and (pe33) were more than 300 cfu, while (pc36) and (pc31) were (155,136) cfu respectively.

It was observed through the results of the ability of *P. aeruginosa* isolates to adhere to a non-living surface polysterene and on the surface of a living cell through their adhesion to the cancerous line in the lung A549 , might be due to their possession of many adhesive agents. Qin *et al.* (2022) pointed out that the ability of *P. aeruginosa* to adhere was using clinical isolates due to its possession of porinf (oprf) in the outer membrane as an adhesive to epithelial cells, and the results showed that bacterial adhesion to the cancerous cell line (A549) and that the occurrence of mutations in oprf leads to reduced adhesion to A549 cells. While (Paulsson *et al.,* 2019) indicated that *P. aeruginosa* can adhere to the lungs using clinical isolates because it has four receptors, namely oprG, oprD, EstA and PA3923, and that bacteria that are free of these receptors do not adhere to the lungs. Lillehoj *et al.* (2002); Lindhout *et al.* (2009) and Wood *et al.* (2023) noted that *P. aeruginosa* can adhere to living and non-living surfaces for clinical isolates because they have flagella while Badaoui *et al.* (2023) noted that the change in the nitrogenous base sequence of guanine using clinical isolates led to the stimulation of vav3 which in turn may help the adhesion of *P. aeruginosa* in the tracheal cells.

Day *et al.* (2019) indicated that pilin and lectin are proteins that have an essential role in the adhesion of *P. aeruginosa* clinical isolates to cells, especially airways, while Patil *et al.,* (2022*)* showed that *P. aeruginosa* bacteria can adhere of clinical isolates because they have the adhesive protein LectinA (LecA). Beaussart *et al.* (2014) stated that Pili (IV) has an important role in the adhesion of P. aeruginosa bacteria environmental isolates to non-living surfaces and that the mutation leads to non-adhesion of the bacteria. Hernandez-Montelongo *et al.* (2021) indicated that pili (iv) and L.P.S have a key role in gluing and the results indicate that each enviroumental strain has the ability to adhere

Table 2. Growth of *Pseudomonas aeruginosa* isolates on pseudomonas agar medium

Isolates	Total number	Dark green		Light Green		Yellowish green		Colorless	
		No.	%	No	%	No.	%	No.	%
Total hospital isolates	66	25	37.87	11	16.66	21	31.81	9	13.63
Environmental isolates from previous studies	22	8	36.36	2	9.09	4	18.18	8	36.36

Table 3. Ability of *Pseudomonas aeruginosa* to adhere in the A549 cell line

Isolates	Source	Ability to pigment formation	Colony formation units (cfu)		
			1:10	1:1000	1:10000
Pc 36	Clinical	Green	>300	>300	64
Pe 26	Environmental	Green	>300	>300	92
Pc 31	Clinical	Transparent	>300	>300	>300
Pe 33	Environmental	Transparent	>300	>300	>300

according to their own characteristics, while Hoffman *et al. (2020)* indicated that monosaccharide increases the ability of *P. aeruginosa* bacteria of clinical isolates to adhere to the epithelial cells of the lung A549. Paulsson *et al.* (2019) also noted that the ability of *P. aeruginosa* bacteria to adhere to epithelial cells of clinical isolates because they possess glycoproteins. While Ahmed *et al.* (2014) indicated the ability of *P. aeruginosa* clinical isolates bacteria to adhere to the epithelial cells of the lung A549 and adhesion depends on factors such as differences in the type of nutrient medium or different development conditions. Whereas Hawdon *et al.* (2010) stated that clinical strains that lack flagella, cilia, pilli and lipopolysaccharide have a low ability to adhere to A549 cells compared to strains that have flagella, cilia and lipopolysaccharide as well as the exoenzyme, the ability of bacteria to adhere to epithelial cells depends on the presence of the main virulence factors. Laventie *et al.* (2019) indicated that *P. aeruginosa* clinical isolates when attached to surfaces increases the concentration of C-di-Gmp within a few seconds, leading to adhesion to the surface.

Cytotoxicity of *P.aeruginosa* filtrate concentrations on the A549 cancer line

The results on the effect of cytotoxicity of *P. aeruginosa* filtrate in the lung cancer line A549 are shown in Table 5. It was noted that the inhibition rates of bacteria filtrate (pc36) isolated from clinical sources and producing pigments were 15.7, 34.5, and 44 at a concentration of 40, 60, and 80 %, respectively, while that of the isolate (pc31) isolated from clinical sources and non-producing pigments was 28.1, 75.2, 80.9 at the same concentrations. As for the isolate (pe26), isolated from environmental sources and forming the pigment, the inhibition rate was 38.5, 83.1, 48.8 at concentrations of 40, 60, 80 %, respectively, and that of the isolate (PE33), isolated from environmental sources was 42, 73.4, 74.1 at concentrations of 40, 60, 80 % respectively.

It was noted from the results of the toxic effect of *P. aeruginosa* bacteria in the A549 cell line that the bacteria gave a different toxic effect, possibly due to the source of isolation or the type of cellular line, and Al-mashgab *et al.* (2020) indicated that the outer membrane vesicles in *P. aeruginosa* clinical isolates bacteria have toxic effects on the human keratin cell line HaCat and that the bacterial isolate which isolated from the hospital was more toxic than the bacterial isolate that isolated from the laboratory.

Pang *et al.* (2022) and Bernardes *et al. (2014)* noted that *P. aeruginosa* clinical isolates bacteria cause a toxic effect due to their possession of exotoxinA, exoenzymeT, azurin, cyclodipeplide, pacaspase and rhamnolipids possess strong cytotoxicity against various cancer cells. Weldon and Pastan (2011) and Pang *et al.* (2022) indicated that exotoxinA is the most toxic virulence agent in *P. aeruginosa* clinical isolates bacteria and is widely applied in synthesis immune toxins for targeted cancer treatment.

While (Hirakawa *et al.*, 2021) stated that the pigment pyocyanin in *P. aeruginosa* clinical isolates bacteria leads to cell damage and death as A549 cells, while O'Malley *et al.* (2004)*;* Hall *et al.* (2016) and Abdelaziz *et al.* (2022) indicated that pyocyanin had high activity in cell membranes by influencing ROS levels of clinical isolates, and El-Housseiny *et al.* (2013) showed that some clinical isolates have a cytotoxic effect within a short period (two hours) as well as same study indicates that one or more secretory system proteins are type III (Exou, Exos, ExoT, or Exoy) was responsible for the cytotoxic effects. While Du *et al.*, 2010 and Wood *et al.* (2023) displayed that *P. aeruginosa* clinical isolates

Table 4. Ability of *Psuedomonas aeruginosa* to adhere to the surface of polystyrene

Isolates	Source	Ability to pigment formation	Colony formation units		
			1:10	1:1000	1:10000
Pc 36	Clinical	Green	>300	>300	155
Pe 26	Environmental	Green	>300	>300	>300
Pc 31	Clinical	Transparent	>300	>300	136
Pe 33	Environmental	Transparent	>300	>300	>300

Table 5. Cytotoxicity of *Pseudomonas aeruginosa* filtrate on the A549 cancer line

Isolate symbol	Isolate source	Ability to pigment formation	Activity rate		
			40	60	80
Pc36	Clinical	Green	15.7	34.5	44
Pc31	Clinical	Transparent	28.1	75.2	80.9
Pe26	Clinical	Green	38.5	83.1	48.8
Pe33	Clinical	Transparent	42	73.4	74.1

secrete ToxA, which in turn stimulates the apoptotic pathway of cellular death by the Mcl-1 protein, while Chemani *et al.* (2009) indicated that the ability of *P. aeruginosa* clinical isolates to toxicity on A549 cells is increased compared to isolates that mutated and removed LecA and LecB, while Malinovská *et al.,* (2019) designated that Lectin (lecA) has an important role in cell toxicity. Diggle *et al.* (2020) noted that *P. aeruginosa* clinical and environmental isolates are toxic due to their possession of pyocyanin, exotoxinA and hydrogen cyanide.

Conclusion

Ability of *P. aeruginosa* isolated from both clinical and environmental sources in the formation of pigments and that they have the ability to adhere to the non-living surface (polysterene) and the living surface A549 and have a toxic effect on living cells A549. There was no difference between clinical and environmental isolations in their adhesion ability.

Conflict of interest

The authors declare that they have no conflict of interest.

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