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Molecular identification and Virulence factors of *Pseudomonas aeruginosa* isolated from operation hall

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Abstract

This study aimed to determine the *P.aeruginosa* that causes contamination to operation hall in hospitals by detecting of pathogenisity markers ..Moreover, the virulence factors of predominant species were detected phenotypically by using routine techniques, that responsible for pathogenicity.

Fifteen samples were collected from different sites of operation hall during two months 2022 in AL-Hussein hospital/ karbala City. The identification of *P.aeruginosa* isolates depended on colonial morphology, microscopic examination and biochemical tests as a primary identification. The final identification was confirm by PCR technique from different sites .The results obtained by the PCR tests were twenty two isolates of *P.aeruginosa* were detected , which distributed into :(9) earth, (8) wall, and (10) beds.

The study investigated the virulence factors of *P.aeruginosa*, which had the ability to produce capsule, biofilm , adhesion ,protease, bacteriocin ,haemolysin and β-lactamase and gelatinase .

The results revealed variation in the resistance of bacteria to some antibiotics,..*P.aeruginosa* exhibited high resistance (96%) to Cefotaxime, but absolute susceptibility to Ciproflaxacin and Gentamycin and high susceptibility to Amikacin.Ceftiaxone,and Azithromycin.

Keyword: *Virulence factors , P.aeruginosa, opportunistic pathogen*

Introduction:

Pseudomonas aeruginosa is a ubiquitous Gram negative bacterium and an important opportunistic pathogen in the healthcare setting. (Macfadin,2018).

Most of the strains of *P.aeruginosa* cultures give a characteristic fruity odor due to the production of aminoacetophenone from tryptophan . Many strains non-lactose fermenting pale coloured on MacConkey agar. oxidase positive (Colvin *et al*.,2012). Nosocomial *P. aeruginosa* outbreaks are frequently reported and associated with water sources such as taps, showers, mixer valves and flow straight teners, sink traps and drains (Sauer *et al*., 2002). Other potential routes of transmission include cross-infection, for example, through contaminated medical equipment such as endoscopic devices (Kun Zhao *et al*., 2013).

P. aeruginosa also has a large number of virulence factors such as exotoxin A, exoenzyme S, elastase and sialidase which are tightly regulated by cell-to-cell signaling systems. Protein biosynthesis is inhibited by exotoxin A and virulence factor exoenzyme S is secreted by a type III section system (Lee *et al*., 2011.

The production of a biofilm mediated adherence of the bacteria to a surface.The formation of a biofilm gives the bacteria several advantages: it immobilizes the cells, it creates a reservoir of nutrients from lysed cells including DNA,., it also

protects the cells from the host immune defense, antibiotics, ultraviolet radiation and oxidizing or charged biocides (Flemming and Wingender, 2010).

The virulence factors associated with cell structures as single polar flagellum that enables it to move through liquid media to acquire nutrients. **Alginate** is the sticky polysaccharide capsule that is over-expressed by mucoid variants of *P. aeruginosa* alginate appears to be a protective mechanism through host immunity and also in resistance to penetration by antibiotics (Mah, 2012).

Lipopolysaccharide9 LPS0 provides protection against antimicrobial activity of host peptides including defenses (Shen *et al*., 2006) and **Outer membrane**, (OM) which provides the bacterium with a hydrophilic surface and functions as a permeability barrier for many external agents (Nikaido, 2003).

P. aeruginosa maintains antibiotic resistant plasmids, and is able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against Pseudomonas, including some B-lactams, aminoglycosides and fluoroquinolones, (Kiska and Gilligan, 2003).The bacterium is naturally resistant to many antibiotics including tetracyclines and benzylpenicillin by mechanisms include: Membrane Permeability ,.Efflux Pumps , Enzymatic inactivation and Mutational Resistance .

The study is aimed to detect the *Pseudomonas aeruginosa* by PCR technique that causes operation hall contamination and determination of the virulence factors and antibiogram.

- Methodology:

-Specimens collection

A total of 50 clinical specimens were collected from various site of operation hall like earth , wall and beds during one month of the study in Al hussein hospital / Karbala City. All specimens were cultured on the deferential media as MacConkey agar plates then incubated at 37°C under aerobic condition for 18 - 24 hour (Wa'ad, 2011).

-Isolation and identification of *P. aeruginosa*

All specimens were cultured on media including blood agar and MacConkey agar, incubation of for 24 hr at 37ºC, the suspected colonies of pure cultures is bacilli with atypical macroscopic appearance. They were sub cultured on blood agar plates 24 hr after incubation at 37ºC for heamolysis (MacFaddin, 2000).Catalase test was preformed and their ability to produce pigments on MacConkey and positive for Voges-Proskauer, Then the bacteria were confirmed by PCR system (Collee *et al*., 2006).

-Molecular identification of *P. aeruginosa*.

The PCR assay was performed to detect the(16S rRNA) genes for confirmation the identification of P.aereuginosa. All primers in this study
were synthesized by Bioneer Company (Korea) in; F- 5 CGACG ATCCGTAACTGGTCT3 and R- 5CCGGTGCTTATTCTGTTGGT3 with 203 bp. According to Sambrook *et al.* (2001).

PCR program that apply in the thermocycler to *P. aeruginosa* with condition of initial denaturation 94°C/5 min and the cycling condition of denaturation 95°C / 1min,annealing 54°C /1min and extention 72°C /1min.The final extention was 72°C

/5min.The PCR products and the ladder marker are resolved by electrophoresis on 1.0 % agarose gel. The resolved band is indicative of the corresponding of studied genes. The molecular weight identification of resolved band is based on their correspondence to the ladder bands (Oho *et al*., 2000).

-Detection of DNA content by Agarose gel electrophoresis

Gel electrophoresis was used for detection of DNA by UV transilluminator (Pier, 2007). Agarose was weighted 1g, boiled in 100ml (1X) TBE buffer, left to cool at 50oC and 5 µl of ethidium bromide is added to agarose and poured on preparing tray. Comb was removed after hardening of agarose leaving wells. 5-10 µl of DNA sample was mixed with 1-2µl of loading dye.

-Agarose electrophoresis

TBE (1X) buffer was added to the electrophoresis tank, tray with agarose was immersed in electrophoresis tank. Each well is loaded with 7µl of DNA sample and standard molecular weight of DNA ladder (marker) is loaded in a first well. Electrophoreses run at 80 volt/cm for 1 hr. Gel was visualized with UV transilluminator and photographed by using digital Camera (Mishera *et al*., 2009).

-Detection of virulence factors.

.Capsule Production according to MacFaddin (2000). .**Gelatinase** Production according to MacFaddin (2000). Gelatinase activity was assessed using samples from single colonies inoculated onto agar containing 5% gelatin and incubated at 37°C for 24-48h.

.Biofilm Formation according to Di Rosa *et al*. (2006). Suspension of tested strain was incubated in the glass tubes containing brain heart infusion broth (BHI broth) aerobically at the temperature of 35°C for the period of 2 days. Then the supernatant was discarded, the glass tube has been stained by 0.1% safranin solution, washed with D.W. three times and dried. In the case of biofilm formation, a grainy red structure on the test tube bottom was found.

.Adherence activity was carried out according to Svanborg *et al*. (1977).

Protease Production detect by Collee *et al*. (2006).

.β-Lactamase production: A direct capillary tubes method was used for detection of β-Lactamase production (MacFaddin, 2000).

.Detection of bacteriocin production: Cup assay method was carried out for detection of bacteriocin (AL-Qassab and AL-Khafaji, 1992).

.Haemolysin production; The blood agar plates were inoculated with bacterial isolate, and then incubated at 37°C for 24-48 hr. Appearance of clear zone around the bacterial colony.

-Results and discussion:

-Identification of bacterial isolates

The bacterial isolates obtained basis of colonial morphology and comparison with biochemical characteristics with standard description in Bergeys manual of determinative bacteriology. The microscopic examination showed *P.aeruginosa* are gram negative bacteria bacilli in gram stained films and blue-green in color on Macchonkey agar that expresses the exopigment pyocyanin (Lee *et al*., 2011). The results were compared with referral reported by MacFaddin (2000; Collee *et al*.

(2006). According to the results obtained , a total of 50 isolates of studied bacteria were distributed as follow:20(54.16%)isolates from earth , 15(29.16%) from wall and 15(16.66%) from beds while *P.aeruginosa* were distributed into : 9(13.84%) from earth swabs, 10(34.28%) wall swabs, and3(15%) beds swabs, as in the table 1.

Table -1: Distribution of bacterial isolates from different sites.

Type of specimens	No. of specimens	No.of P.aeruginosa specimens
earth	20(54.16%)	9(13.84%)
Wall	15(29.16%)	10(34.28%)
bed	15(16.66%)	3(15%)
Total	50	22(20%)

The results of biochemical tests that recorded in table (2) were considered as a complementary of the initial identification of studied bacterial isolates.The isolates confirm to general characteristics, Regard to *P.aeruginosa* gave positive results for indol,Vogus Proskuer, simmon citrate test,oxidase,and catalase while gave negative results for methyl red test, also, the both bacterial isolates . *P.aeruginosa* gave positive results for biofilm formation as in figure 2. (Flemming and Wingender, 2010).

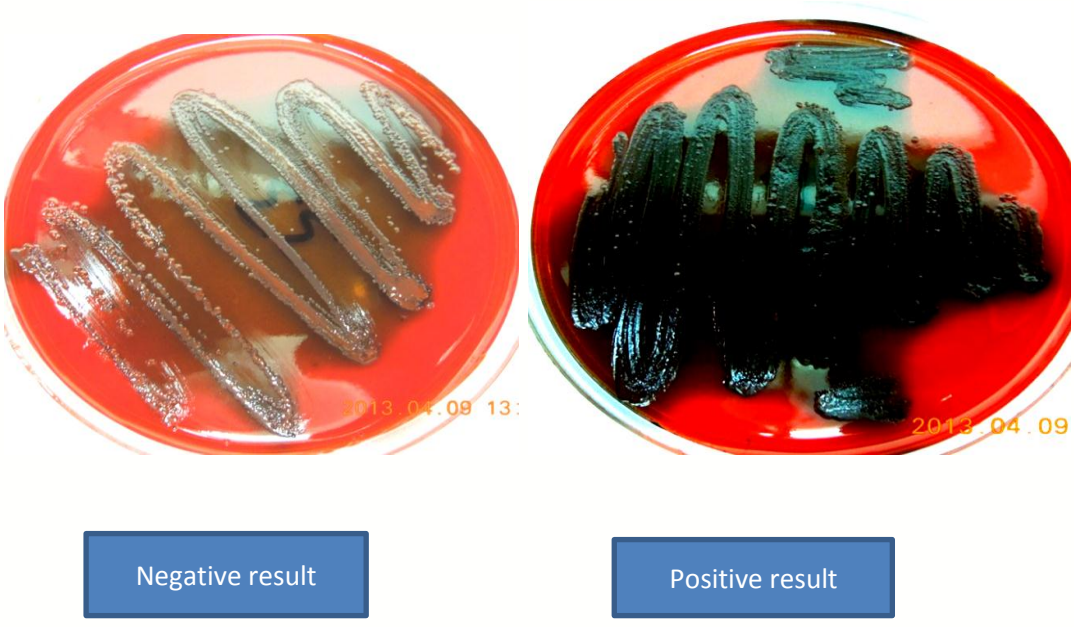

Figure- 1:Biofilm formation. Biofilm - producing isolates of *P.aeruginosa*

Table -2: Phenotypic characteristics & Carbohydrate ferment of *Pseudomonas aeruginosa* isolates.

Biochemical test	***P.aeruginosa No.22 Isolates***	**Biochemical est**	***P.aeruginosa No.22 Isolates***
Gram stain	_	**-Arabinose**	+
Catalase test	+	**D-Mannitole**	+
Oxidase test	+	**Raffinose**	_
Indole test	+	**- Glucose**	+
Methyl red	_	**Sucrose**	_
Vogas-Proskaures test	+	**Lactose**	_
Simmon Citrate test	+	**Motility**	+

-Molecular detection of by PCR technique:

Polymerase chain reaction technique of the *P. aeruginosa* isolates revealed one fragment with 230 bp that represented the 16S rRNA gene . The results show that all isolates of *P. aeruginosa* , carrying *16S r RNA* gene that is characteristic of *P. aeruginosa* as shown in figure(2).

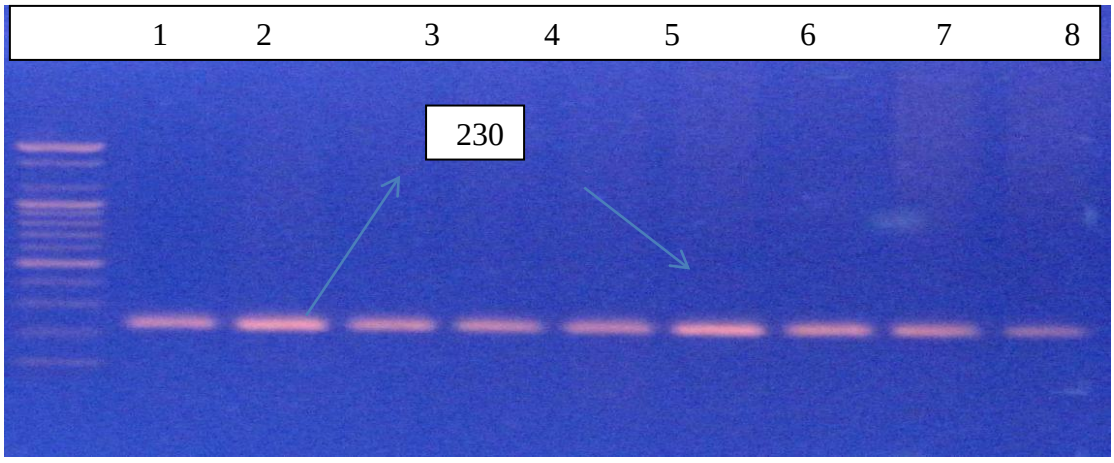

Figure 2 :PCR amplified products of *16S RNA* gene of the *P. aeruginosa* using the designed primers with expected size 230bp.

-Antibiogram for studied bacteria Isolates

The antibiotic sensitivity for antibiotics on bacterial isolates by using Kirby-Bauer disk diffusion method .The results were interpreted according to the diameter of inhibition zones and compared with inhibition zones determined by CLSI(2012).The results revealed that the *P.aeruginosa* isolates exhibited highly sensitivity to Amikacin 23(96%),to Ciprofloxacin 22(100%),and to Gentamycin

22(100%) respectively, while bacteria were resist to Cefotaxime as 21(96%).As in table -3.

Table -3: Antibiogram results of *Pseudomonas aeruginosa* isolates

Antibiotic / bacteria.		Amikacin	Ciprofloxacin	Ceftriaxon	Gentamycin	Meropenem	Azithromycin	Cefotaxime
P.aeruginosa	**R**	1(4%)	0(0%)	2(8%)	0(%)	1(4%)	2(17%)	21(96%)
	S	21(96%)	24(100%	20(92%)	24(100 %	21(96%	20(83%)	1(4%)

-Virulence Factors Determination

The results showed that all bacterial isolates producing capsule, biofilm, β-heamolysin and able to adhesion for all isolates (100) (Nastaran and Hassan 2014). Biofilm is an important factor in the attachment of *P.aeruginosa* to surfaces and other cells.

Haemolysin considered as one of virulence factors that associated with increased severity of infections (Tadashi *et al*., 2008). Most bacteria require iron for growth, and acquisition of iron is a significant challenge in most in vivo environments where little free iron exists (Ochsner *et al*., 2002). *P. aeruginosa* isolates to adherence to epithelial cells of tissue. The results showed that the protease produced 20(90.5%) by *P.aeruginosa*. Protease is hydrolysis of large polypeptides. Bacteriocin are antimicrobial peptides with different sizes, microbial target and mechanisms of action produced by large variety of bacteria as in table 4 (Forbes *et al*. (2007).

Table 4**.** virulence factors *P. aeruginosa* that associated with infection

BacterialVirulence factors		*P.aeruginosa*
Capsule		22(100%)
Gelatinase		6(25%)
Biofilm		22 (100%)
Adhesion		22(100%%)
Protease		21(87.5%)
β-Lactamase		22(91.66%)
Bacteriocin	*	10(41.66%)
	**	8(33.33%)
Haemolysin	β	24(100)%
	α	0(0%)
	δ	0(0%)

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