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DETECTION OF PATHOGENICITY MARKERS PRODUCED BY *PSEUDOMONAS AERUGINOSA* CAUSING SKIN INFECTION

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Abstract

This study aimed to determine the *P.aeruginosa* that causes skin infection by detecting of pathogenisity markers. Bacterial isolated and identificated phenotypically and genotypically, as well as detects the pathogenicity markers encoding genes producing from *P.aeruginosa* by using PCR techniques. One hundred twenty clinical specimens were collected from patients suffering from different clinical infections during the period from September 2014 to January 2015 in AL-Sadder Medical city. The identification of the *P.aeruginosa* isolates depended on colonial morphology, microscopic examination and biochemical tests. *P.aeruginosa* had the ability to produce capsule, biofilm , adhesion, protease, bacteriocin, haemolysin, β-lactamase and gelatinase. Regard to molecular study the outcome showed that 24 (100%) of *P. aeruginosa* isolates carrying *lasB* gene, 24(100%) carrying *aprA*, 23(95%) carrying *plcH* while 13(54%) carrying *algD*. To conclusion that *P. aeruginosa* able to produce sever pathogenicity markers which responsible for pathogenicity of *P.aeruginosa* infections.

Key words : *Pseudomonas aeruginosa*, skin infection, virulence factors encoding genes, PCR assay.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium and an important opportunistic pathogen in the healthcare setting. *P.aeruginosa* particularly affects those with impaired host immunity and has a broad range of presentations, including respiratory infections in cystic fibrosis and mechanically ventilated patients, bloodstream infections in premature neonates and wounds in burns injuries (Pier and Ramphal, 2005). 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 signalling systems (Morales and Wiehlmann, 2004). Protein biosynthesis is inhibited by exotoxin A and virulence factor exoenzyme S is secreted by a type III section system (Lee *et al*., 2011). A zinc metallo protease called Las B has an elastolytic activity on lung tissue (Strateva *et al*., 2007). The gene called *nan1* encodes a sialidase that is responsible for adherence to the respiratory tract (Toder *et al*., 2006).

The study is aimed to detect the *Pseudomonas aeruginosa* that causes skin infection by the determination of the pathogenicity markers at molecular level by PCR technique.

Methods

Specimens collection

A total of 120 clinical specimens were collected from patients suffering from various clinical infection such as burn,wound,and skin during the period from September, 2013 to January, 2014, which attending to AL-Sadder Medical City. All specimens were cultured on the MacConkey agar plates then incubated at 37°C under

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aerobic condition for 18 - 24 hour (Wa'ad, 2011).

Isolation and identification of *P. aeruginosa*

All specimens were initially cultured on bacteria isolation media including blood agar and MacConkey agar, incubation of for 24 hr at 37ºC, the suspected colonies of pure cultures (gram negative bacilli with atypical macroscopic appearance) were investigated. They were sub cultured on blood agar plates 24 hr after incubation at 37ºC for heamolysis (MacFaddin, 2000).

Catalase test was preformed on all gram negative suspected of being bacteria,gram negative bacteria were tested for their ability to produce pigments on MacConkey and positive for Voges-Proskauer, Gram negative isolates giving the following reaction was considered as *P. aeruginosa*. Then the bacteria were confirmed by an additional biochemical test with VITEK-2 compact system (Collee *et al*., 2006).

Detection of pathogenicity markers

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 production (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 referred to β–hemolytic or green zone referred to α-hemolytic (Baron, 2001).

Molecular study

1. Isolation of Bacterial Chromosomal DNA

Total DNA was extracted from colonies grown on agar plates by boiling method according to (Yi *et al*., 2010). The suspension was heated for 15 min at 100 ºC followed by 5 min on ice rapidly. The suspension containing DNA was stored at -20ºC until used as template for PCR. DNA was determined spectrophotometrically by measuring its optical density at 260 nm .The purity of DNA solution is range between 1.8±0.2 for pure DNA (Svanborg *et al*., 1977).

2. Determination of DNA concentration

Concentration of DNA was determined spectrophotometer by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 µg/ml at 260 nm) the purity of DNA solution is indicated by ratio of OD260/OD280 which is in the range of 1.8±0.2 for pure DNA (Stephenson, 2003).

3. PCR assay

The PCR assay was performed to detect the (*lasB,aprA, algD, plcH*) genes for confirmation the identification of *P.aereuginosa*. All primers in this study were synthesized by Bioneer Company (Korea) in

lasB (F 5'-TTCTACCCGAAGGACTGATAC-3'), (R 5'-AACACCCATGATCGCAAC-3'). *aprA* (F 5'-ACCCTGTCCTATTCGTTCC-3'), (R 5'-GATTGCAGCGACAACTTGG-3'). *algD* (F 5'-ATGCGAATCAGCATCTTTGGT-3'), (R 5'-CTACCAGCAGATGCCCTCGGC-3'). *plcH* (F 5'-GAAGCCATGGGCTACTTCAA-3'), (R 5'-AGAGTGACGAGGAGCGGTAG-3').

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 94°C / 1min,annealing 55°C /1min and extention 72°C /2min.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 50°C 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).

Results

Identification of bacterial isolates

The bacterial isolates obtained as a pure and predominant growth from clinical specimens were only considered for the present study.

All these bacteria were identified on the 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) and Collee *et al*. (2006).

Pathogenicity markers determination

The results showed that all bacterial isolates producing capsule, biofilm, β-heamolysin and able to adhesion for all isolates (100%). The results of this study agreed with the findings of Nastaran and Hassan (2014), who found that the ability of *P. aeruginosa* to form capsule and disagree with Zawacki (2004). Biofilm is an important factor in the attachment of *P.aeruginosa* to surfaces and other cells; it is one of the virulence factors in many pathogenic such *as P.aeruginosa* strains (Strateva *et al*., 2007). This findings accept to Schouls *et al*. (2009), who reported ability of *P.aeruginosa* to form biofilm.It has been hypothesised that biofilm formation initially requires flagella-dependent association and binding to a surface to allow formation of a single cell monolayer (Sauer *et al*., 2002). 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. Heme may serve as a source of iron *in vivo* and the has gene involved in heme utilization was the most highly induced *in vivo* gene (Ochsner *et al*., 2002). These results seems to agree with Schwarzkopf *et al*. (2010), who said that *P.aeruginosa* produce β-heamolysin on blood agar and not produce α and δ-heamolysin. These observations of *P.aeruginosa* adhesion seemed to agree with the findings of Itah and Essien (2005) and Pier and Ramphal (2005), who reported the high ability of *P. aeruginosa* isolates to adherence to epithelial cells of skin. PiliA, play a major role in the adhesion of *P.aeruginosa* to host cell membranes. The findings showed that 6(25%) of *P. aeruginosa* produce gelatinase, these observations

Table 1 : Virulence factors of Studied bacteria specimens.

Virulence factors \ Bacteria		No. 24 *P. aeruginosa*
Capsule		24 (100%)
Gelatinase		6(25%)
Biofilm		24(100%)
Adhesion		24(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%)

Pathogenecity marker: **E.coli*, ***K. pneumonia*.

Table 2 : Distribution of genotypic virulence determinants of *P. aeruginosa* according to the type of specimens.

Studied bacteria \ Type of specimen		Wound swab	Burnswabs	Skin swab	Total
P. aeruginosa	***lasB***	9(100%)	12(100%)	3(100%)	24(100%)
	aprA	9(100%)	12(100%)	3(100%)	24(100%)
	algD	4(44.44%)	8(66.66%)	1(33.33%)	13(54.16%)
	plcH	9(100%)	12(100%)	2(66.66%)	23(95.83%)

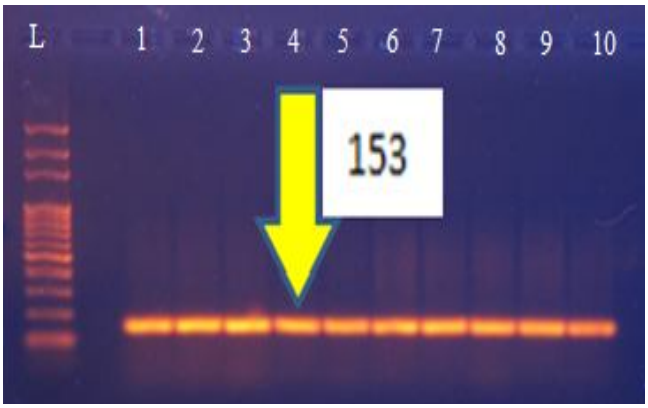

Fig. 1 : Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *P.aeruginosa* isolates that amplified with *LasB* gene primers with product 153bp for 1 hr. at 80volt/cm.

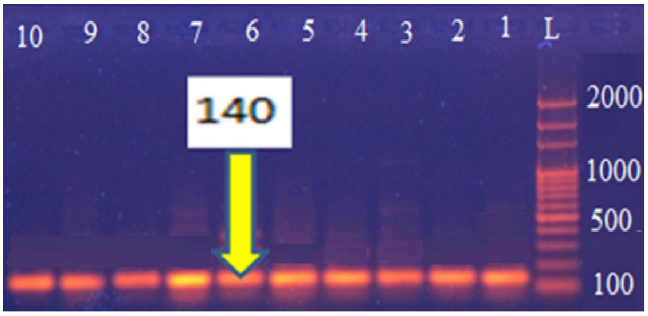

Fig. 2 : Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *P. aeruginosa* isolates that amplified with *AprA* gene primers with product 140bp for 1 hr. at 80volt/cm.

similar to the findings of Kun Zhao *et al*. (2013), who reported ability of *P. aeruginosa* to produce this enzyme. The results showed that the protease produced 21(87.5%) 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. The results of *P. aeruginosa* was active against *E. coli* (41.66%) and less active against *K. pneumoniae* (33.33%). The observations agreed with the findings of Forbes *et al*. (2007), who reported the ability of *P. aeruginosa* to produce bacteriocin and also, indicated that *P. aeruginosa* more produced it in comparative with other species in table 1.

Molecular detection of by PCR technique

Detection of pathogenicity markers encoding genes

A collection of 24 *P. aeruginosa* isolates recovered from different clinical specimens was molecular screened for the presence of virulence factors encoding genes of *P.aeruginosa*. The presence of genes that encode bacterial isolates genes were *LasB*, *AprA*, *AlgD* and *PlcH*

Fig. 3 : Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *P.aeruginosa* isolates that amplified with *AlgD* gene primers with product 1310bp for 1 hr. at 80volt/cm.

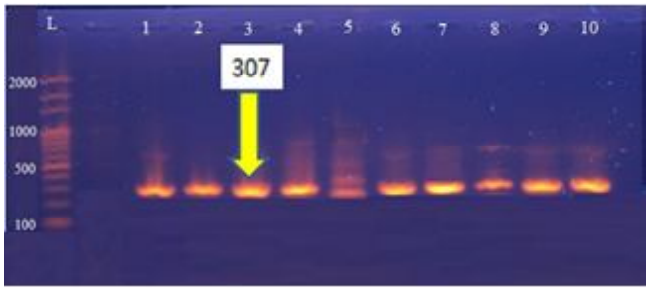

Fig. 4 : Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *P.aeruginosa* isolates that amplified with *PlcH* gene primers with product 307bp for 1 hr. at 80volt/cm.

of *P.aeruginosa* were investigated by PCR technique.

The results of this study to pathogenicity markers encoding genes of encoding genes of *P. aeruginosa* by using PCR technique showed that : *LasB* gene was 24 (100%) as in fig. 1. This is in accordance with a previous study in which the gene was present in medical isolates (Lee *et al*., 2011 and Wa'ad, 2011). *AprA* gene was 24 (100%) in fig. 2. It agree with Livermore (2002) who shows that all isolates of *P. aeruginosa* and their results showed that *AprA* and *LasB* genes were universally present. *AlgD* gene was 13 (54.16%) in fig. 3. This result was identical with Mathee *et al*. (2008), who was detected *AlgD* in (50%) of isolates, which was lower than the 58.9 prevalence of *AlgD* detected in *P. aeruginosa* isolates but disagree with Wa'ad (2011), who said that the results of PCR for *AlgD* gene (the gene responsible for alginate production) showed that all isolates 20(100%) possess this gene. *PlcH* gene was 23 (95.83%) as in fig. 4. These observations seemed to agree with the findings of Strateva *et al*. (2007), which was found that this virulence determinant was found at a high rate among clinical strains, with a tendency for it to be present more often among burn strains than in wound swab strains,and also accept with study conducted by Zeynab *et al*. (2012) on 36 strains of *Pseudomonas aeruginosa* found that *LasB*, *ToxA*, *PlcH*, *PlcN* were

prevalent and it was shown that these virulence factors could play important roles in pathogenesis of this bacterium.

Table 2 showed the distribution of studied *P. aeruginosa* isolates according to the type of specimens, the gene encode *LasB* in wound swab 9 (100%), 12(100%) burn swab, 3(100%) skin swab; gene encode *AprA* in wound swab 9(100%), 12(100%) burn swab, 3(100%) skin swab; gene encode *AlgD* in wound swab 4(44.44%), 8(66.66%) burn swab, 1(33.33%) skin swab; gene encode *PlcH* in wound swab 9(100%), 12(100%) burn swab, 2(66.66%) skin swab.

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