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Original Research Article

Virulence Factors of *Pseudomonas aeruginosa* Isolated from Wound and Burn Infections

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Abstract

Fifty number of Gram-negative non-fermenting lactose bacteria grown on MacConkey agar were isolated from burns and wounds of the samples collected from Al-Sadar Medical City in Al- Najaf Province, during 10/9/2010-10/12/2010. The bacterial isolates were identified according to cultural characteristics and biochemical activities. The results have revealed that 40(80%) isolated bacteria were identified as *Pseudomonas sp*. All of them 40(100%) belong to *Pseudomonas aeruginosa*. The virulence factor of *Pseudomonas* isolates were tested, using different methods .The result revealed that 40(100%) of isolates contain capsule and adhesion factors, while 40(100%) of isolates did not produce amylase, 28(70%) of them produce protease. The results also revealed that 36 isolates (90%) gave positive results for phospholipase.

Keywords

Burn infections

Pseudomonas isolates

Virulence factors

Wound and burn infections

Introduction

Pseudomonas is a genus of Gram-negative bacteria, belong to the family Pseudomonadaceae. The normal habitat of Pseudomonas is soil, plant and animal tissue .These bacteria are opportunistic pathogens. Cause Cystic fibrosis, burn and wound infections. *Pseudomonas* has several virulence factors that oppose host defenses which depend mainly on impair phagocytosis by polymorphonuclear granulocytes and the bactericidal effect of serum (Ben et al., 2011). These virulence factors include capsule which protect bacteria from phagocytosis, fimbriae responsible for adhesive factor, which help the microorganism in adhering to epithelial host cells, and exoenzyme like phospholipase, this enzyme usually act on the animal cell membrane by forming a pore in cell and cleaved phospholipids. Also *Pseudomonas* is producing proteases which causes bleeding and tissue necrosis (Ben et al., 2011).

Many studies reported that the virulence factors have been examined phenotypically in clinical isolates. However, the simplest and most common methods that has been used to detect the presence of virulence factors, the study attempts to reach the aim of detecting the virulence factors and this aim is achieved at by the following objectives:

(1) To isolate and to identify *Pseudomonas* species from burn and wound infections, and (2) Phenotypic detection of virulence factors of bacterial isolates.

History and classification

The genera Pseudomonad like other bacteria were lived hundreds of millions years ago. This bacterium was classified at the end of the 19th century and it was identified first time by Walter Migula as Greek *pseudes* "false" and "monad". These terms were used in the early history of microbiology to indicate single-celled organisms which means false unit (Palleroni, 2010). *Pseudomonas* was defined as a genus of Gram-negative bacteria, rod-shaped polar-flagella and non-spore forming.

Later, a very large number of species were assigned to the genus *Pseudomonas* matching to Migula's original description and isolated from many natural niches while other species were previously classified in the genus *Pseudomonas* now classified in the genera *Burkholderia* and *Ralstonia* (Cornelis, 2008). Later the *Pseudomonas* was classified in the first edition of Bergey's Manual of Systematic Bacteriology, comprising 61 species, perhaps *Pseudomonas*, has been described in the second edition of Bergey's and separated to subdivision of the genus on the basis of rRNA (Chen, 2009).

General characteristics

Pseudomonas are Gram-negative rod-shaped bacteria, 2-4 μm long, motile with polar flagella, aerobic and non-spore forming. It is a member of the family Pseudomonaceae (Willcox, 2007). Many species of *Pseudomonas* can produce extracellular pigments, like pyocyanine (green-blue); pyorubrin (yellow-green) is produced by *Pseudomonas aeruginosa* and fluorescence is produced by *P. fluorescens* (Franzetti and Scarpellini, 2007). All species are oxidase positive, distinguishes them from other Gram-negative bacteria. Colonies are usually white to cream to yellow and typically beta hemolytic on blood agar and are negative for indole, methyl red and Voges–Proskauer tests (Mena and Gerba, 2009).

Pathogenicity

Pseudomonas is one of the important opportunistic pathogens, always invades the host tissue and cause many infections including endocarditis, osteomyelitis, urinary tract infections, gastrointestinal infections, meningitis commonly, septicaemia and bacteremia in immunocompromised hosts (Banerjee and Stableforth, 2000).

P. aeruginosa is the most common agent associated with infection and inflammation during contact lens wear. The bacteria colonize on lenses and produce proteases to kill or invade corneal cells. *P. aeruginosa* virulence factors counteract host defences and can cause direct damage to host tissues (Willcox, 2007). *P. aeruginosa* can always colonize on open burn wounds, causing, abscesses, and sepsis. Other *Pseudomonas* species are opportunistic and cause rare infection (Japoni et al., 2009).

Virulence factors

Capsule: Capsule is an important structure of some bacterial cells. It lies outside the cell wall of bacteria. and made as a barrier between the cell wall and the environment it cause of various diseases (Hassett et al., 2002). Capsule of *Pseudomonas* contains exopolysaccharide layer called alginate associated with the outer membrane. This layer serves to protect the bacteria from adversity and also enhances adhesion to solid surfaces. The alginate layer of *P. aeruginosa* which helps it to escape from host defenses and resist the antimicrobial action of antibiotics, and increase attachment of the bacteria away from the surface, allowing them to spread and colonize new sites (Palleroni, 2010).

Adhesion factors

The physicochemical interactions between bacteria and surfaces adhesion.is due to various outer membrane features such as pili, flagella,and lipopolysaccharides (LPSs) (Walker et al., 2004).The pili of Pseudomonas aeruginosa will adhere to the epithelial cells of the upper respiratory tract. These adhesins appear to bind to specific galactose or mannose receptors on epithelial cells .Pseudomonasaeruginosa has many type of fimbriae, known as CupA, CupB and CupC fimbriae, (Vallet et al., 2001). The fimbriae, CupA, have been play an important role for adherence to abiotic surfaces, and formation of biofilm in *P. aeruginosa* biofilm protects the bacteria from the host defenses such as lymphocytes, phagocytes and antibodies (Ruer et al., 2007).

Exotoxin

The ability of *P. aeruginosa* to invade tissues depends on many extracellular enzymes and toxins that break down physical barriers and damage host cells, like exoenzyme S is produced when the bacteria growing in the burned tissue and may be detected in the blood before the bacteria are present (Deng et al., 2007); while exotoxin A has the same mechanism of action as the diphtheria toxin. This toxin binds to a specific receptor on animal cells, and inhibition of protein synthesis in the affected cell (Wolf and Elsässer-Beile, 2009).

Also *P. aeruginosa* produces proteases and elastase has been associated with virulence that exerts their activity at the invasive stage: Elastase cleaves collagen, IgG, IgA, and complement. Together, elastase and protease destroy the ground substance of the cornea (Jorgensen et al., 2008). The other exotoxins produce from *P. aeruginosa* are phospholipase and lipase. They appear to act synergistically to break down, lecithin and lipids that effects on neutrophils, lymphocytes and other eukaryotic cells (Moller et al., 1994).

Materials and methods

Chemical materials

Chemical materials used in this research include: HCl, NaOH, NaCl, Ethanol, Na_2HPO_4 and KH_2PO_4.

Apparatuses

Apparatuses used in this research include: Autoclave, Incubator, and Water bath, Sensitive Balance Distillator, Compound Microscope and Centrifuge.

Reagents

Oxidase reagent: It has been prepared by dissolving 1 g of tetramythel praphenylene diamine dihydrochloride (Mast, UK) in100 ml of distilled water (Baron and Finegold ,1991).

Kovacs reagent: It has been prepared by dissolving 5 g of P- dimethylaminebenzylaldehyde in 75 ml of amyl-alcohol and then 35 ml of concentrated HCl was added, it was used to detect indole production (Baron and Finegold, 1991).

Methyl red reagent: It has been prepared according to MacFaddin (2000) by dissolving 0.1 g of methyl red powder in 300 ml of (95%) ethanol, then the volume was completed to 500 ml with distilled water.

Voges –Proskaur reagent: It has been prepared according to MacFaddin (2000) by mixing two solutions: Solution A: prepared by dissolving 5 g of alpha - naphthol in 100 ml of ethyl alcohol (absolute). Solution B: prepared by dissolving 40 g of KOH in 100 ml distilled water, it has been used to detect the partial hydrolysis of glucose.

Iodine reagent: It has been prepared by dissolving 20g of KI and 12.7g of Iodine in 1000 ml of distilled water. It has been used to detect the ability of bacteria to produce amylase enzyme (MacFaddin, 2000).

Dyes

Gram's stain (Collee et al., 1996): It has been used to stain bacterial isolate to distinguish between Gram negative and Gram positive bacteria.

Indian ink (B.D.H, Germany): It has been used for capsule detection (Cruickshank et al., 1975).

Culture media

Blood agar medium: It has been prepared according to Difico Manual (1953) by dissolving 40 g of Blood Agar base (Himedia , India) per one litter of distilled water and autoclaved at 121°C for 15 min.; 8% human blood was added after cooling the medium to 50-55°C, it is used for primary cultivation and to detect the type of hemolysis.

MacConkey agar medium (Himedia, India): It has been used for the isolation and differentiation between Gram negative and Gram positive bacteria, and differentiation between lactose fermented and non-lactose fermented bacteria.

Pepton water medium (Himedia, India): It has been used to detect the ability of bacteria to produce indol (MacFaddin, 2000).

MR-VP medium (Himedia, India): It has been used to detect the partial and complete hydrolysis of glucose (MacFaddin, 2000).

Simmon citrate medium (Himedia, India): It has been used for determining the ability of bacteria to utilize Citrate as the sole source of carbon (MacFaddin, 2000).

Triple Sugar Iron agar "TSI" (Himedia, India): It has been used to test the ability of bacteria to ferment carbohydrates and produce acid with or without gas formation as well as H_2S production (MacFaddin, 2000).

Skim milk agar medium: Skim milk agar has been prepared according to the recommendation of manufacturing company (Hi media), by dissolving 10g of skim milk in 100 ml distilled water and heating to 50°C, autoclaving in 121°C for 15 min. In another flask a 2% agar was prepared and autoclaved in 121°C for 15 min, then the two media were left at 50°C and well mixed in a sterilized condition and kept in Refrigerator after putting it in sterilized Petri dish for using. It was used to detect the ability of bacteria to produce protease (the enzyme lyse protein) (Benson, 2002).

Starch medium: It has been prepared by dissolving 2.5g of starch in 100 ml of basal medium (Nutrient agar). The mixture was heated to dissolve the starch completely. The pH was adjusted at 7.2 autoclaved in 121°C for 15 min. It was used to detect the ability of bacteria to produce amylase enzyme that liquefy the starch (MacFaddin, 2000).

Phospholipid medium: It has been prepared by dissolving 1 g of NaCl in 100 ml Nutrient agar, the medium was heated until dissolving, then autoclaved at 121°C for 15 min, let to cool at 50°C, one of egg yolk was added to it in sterilized conditions and mixed well, after that, the medium was poured to sterile plates and maintained at 4°C. It was used to detect the ability of bacteria to produce phospholipase enzyme (lecithinase) (Atlas et al., 1995).

Methods

Specimens collection

Fifty Gram– negative bacteria non-fermented lactose were collected from Al-Sadar Medical City in Al-Najaf province, the specimens were collected from 15/8/2013 to 27/10/2013 and represented 20 (40%) isolates from hospitalized patients from burns and 30 (60%) isolates from outpatients from wounds, represented 27(54%) from females and 23 (46%) from males The specimens were transferred immediately to laboratory for culturing and identification.

Cultivation and identification of clinical isolates

The samples were cultured on MacConkey and blood agar, incubated at 37°C for 18-24h. The bacterial isolates were identified according to MacFaddin (2000).

Typical characteristics

After the incubation period , the typical characters of *Pseudomonas* spp. were used in identification of bacterial isolates, the *Pseudomonas* isolates were distinguished by producing large smooth, with flat edges of beta hemolysis, a metallic sheen, and green pigment colonies on blood agar while on Macconkey agar , they would not ferment the lactose and produce irregular, colorless to pink colonies (Franzetti and Scarpellini, 2007).

Biochemical tests

The following biochemical tests were performed for distinguishing *Pseudomonas* spp. isolates from other related isolates (MacFaddin, 2000).

Indole test: Peptone water was inoculated with young culture and incubated at 37°C for 24h, 3-5 drops of Kovacs reagent (0.5 ml) was added, forming a red colour ring in the alcohol layer, indicating a positive result

Methyl Red test: MR-VP was inoculated with a single colony of young culture and incubated at 37°C for 24 h, 5 drops of Methyl red reagent were added, mixed, and the result was read immediately. The development of a bright red colour indicates positive test.

Voges–Proskaur test: MR-VP was inoculated with a single colony of young culture and incubated at 37°C for 24 h, 3ml of reagent A (5% alpha naphthol) and 1 ml of reagent B (40% KOH) were added, a positive reaction was indicated by the development of a pink color in 2-5 min.

Citrate utilization test: Simmon citrate medium was inoculated with a single colony of young culture and

incubated in 37°C for 24 h, a blue color and streak of growth appearance indicated a positive result.

Triple Sugar Iron test: Heavy inoculums were stabbed into the butt and streaked over the surface of the slant medium and incubated at 37°C for 24 h, results were recorded.

Slant/butt	**Color**
Alkaline/Acid	Red/Yellow
Acid/Acid	Yellow/Yellow
Alkaline/ Alkaline	Red/ Red
H_2S	Black precipitate

Investigation of virulence factors

Capsule

The negative staining of Indian ink was Inquired to detect the presence of bacterial capsule, according to Atlas et al. (1995):
- Mixing one colony of young culture with a drop of normal saline on clean slide.
- A drop of Indian ink was added and mixed with loop carefully , then spread the mixture by edge of another slide (angle 45 degree).
- The slide was examined directly under the oil lens, the formation of clear zone around bacterial cell indicated the production of capsule.

Protease test: Skim milk medium was inoculated with pure young colonies grown on MacConkey agar with sterilized stick by pick and patch, incubated in 37°C for 48 h. The positive result was recorded when a clear zone appeared around colonies (Harrigan and MacCancel, 1987).

Amylase test: Starch medium was inoculated with pure young colonies grown on MacConkey agar with sterilized stick by pick and patch, incubated in 37°C for 48 h, then iodine reagent was added. The positive result shows clear zone around the colony with purple-blue color. It refers to complete hydrolyzation of starch (MacFaddin, 2000).

Lecithinase test (phospholipase): Phospholipid medium was inoculated with pure young colonies grown on MacConkey Agar, incubated at 37°C for48 h. The precipitation of white to brown colour halo around colony indicated positive test results (Baron et al., 1994).

Adhesion factors

Mid –stream urine of healthy females was centrifuged at 3000 rpm for 5 minute ,then the precipitation of epithelial cells was washed with buffer solution for 3 times and centrifuged with each one, then the cells were suspended in buffer solution.
- Mixed 0.5 ml of implanted bacterial broth age 18 h with 0.5 ml of suspension prepared in last step. It was added to 1ml of buffer solution, then centrifuged at 5000 rpm to remove non adhesion bacteria.
- The precipitation was re-suspended with the buffer solution.
- One drop of the final mixture was put and spread on clean slide and dried at room temperature, stained the smear with Gram stain , then washed with distillated water, let to dry ,examined under microscopic oil lens to notice the adhesion of epithelial cells (Lomberg et al., 1986).

Results and discussion

Collection and identification of bacterial isolates

Specimen collection: Fifty number of non-lactose fermenting isolates have been collected from wound and burn infections. Two groups of patients were included: 20 (40%) isolates from hospitalized patients from burns and 30 (60%) isolates from outpatients from wounds, represented 27 (54%) from females and 23 (46%) from males (Table 1).

Table 1. Distribution of bacterial isolates according to patients setting at AL-Sadaar hospital.

Nature of infection	Hospitalized patients				Outpatients				Total
	Positive		Negative		Positive		Negative		
	Male	Female	Male	Female	Male	Female	Male	Female	
Burn	9	6	2	3	-	-	-	-	
Wound	-	-	-	-	14	11	2	3	
Total	15		5		25		5		50
	20				30				50

Bacterial identification

Identification of bacterial isolates based on cultural and morphological characteristics: The characters of the bacterial colonies grown on MacConkey agar were studied; irregular, colorless to pink colonies were not fermenting the lactose. The blood agar produced large smooth, with flat edges of beta hemolysis, a metallic sheen and green pigment colonies (Franzetti and Scarpellini, 2007).

Biochemical tests: All Gram-negative isolates that grown on MacConkey agar undergo biochemical tests in order to distinguish *Pseudomonas* from other members of bacteria that non fermenting lactose , all biochemical tests have been carried out according to MacFaddin (2000). Forty (80%) isolates have given positive results for oxidase test. Pseudomonas gave positive result for oxidase. While 10 (20%) of isolates give negative results for oxidase; 40 (100%) of isolates have given negative results for indole test. The breakdown of tryptophan for nutritional leads to the release of the indole that can be detected through the use of Kovacs' reagent, which reacts with indole and produces a red color on the surface of peptone water gave positive result (Macfaddin, 2000).

The results have been indicated that 40 (100%) isolates were negative for methyl red test, *P. aeruginosa* gave negative result ,the positive result belong to glucose fermentation with acid accumulation in MR-VP medium, causing decreased of pH, leading to the formation of red colour when methyl red reagent was added (Sylvia, 2009). Citrate utilization test were positive in 40 (100%) isolates, the *P. aeruginosa* gave positive results; the changing the color of Simmon citrate slants to blue as a result of utilizing citrate as a carbon source (MacFaddin, 2000). Triple sugar iron agar (TSI) 40 (100%) isolates gave alkaline/alkaline without gas. If an organism is capable of using neither glucose nor lactose, the slant of the tube will be red and the color of the butt will remain unchanged. The results revealed that 40 (100%) of isolates were *P. aeruginosa*.

Clinical isolates of *P. aeruginosa*

The *Pseudomonas* spp. clinical isolates have been accounted to be 40 (100%). The isolates obtained from two infections site were represented by 15 (37.5%) isolates from burn infections, 25(62,5%),isolates from wound infections. The finding may be true, because *Pseudomonas* is one of the most important opportunistic pathogens which is commonly predominant in hospital (Japoni et al., 2009).

P. aeruginosa is the most common cause of infections of burn injuries, The study of Ryan et al. (2003) found *Pseudomonas* was to be the most common agent (57%) followed by *Acinetobacter* (17%), *Escherichia coli* (12%), *Staphylococcus aureus* (8%) and other organisms (6%). *P. aeruginosa* is a colonizer of medical devices (e.g., catheters) (Williams et al., 2007). *Pseudomonas* in burn can be transmission from person to person. And found in contaminate floors, bed rails, and sinks of hospitals, also cultured from the hands of nurses (Japoni et al., 2009).

Virulence factors

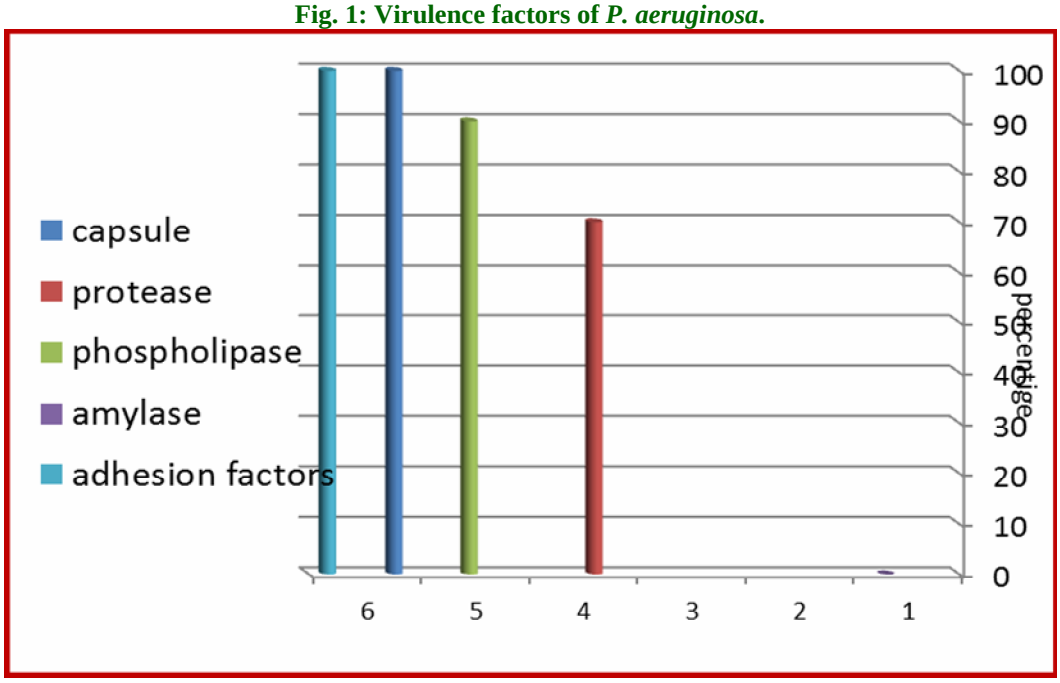
Capsule

By using negative staining of Indian ink, the result of direct examination showing that all bacterial isolates 40 (100%) have capsule (Fig. 1 and Appendix 1). The study of (Ilham et al., 2013) found *P. aeruginosa* produce capsule (100%). Capsules are one of important virulence factor of *P. aeruginosa* that inhibit ingestion and killing by phagocytes and protect cells from antibodies and lysozyme and adhere to cell surfaces and structures such as medical implants and catheters (Sridhar Rao, 2009).

Protease production

The result of the study appeared after 48 h incubation the bacterial isolates were gave positive result 28 (70%) because clear zone was apparent around the colony.While12 (30%) of isolates gave negative results (Figs. 1, 2 and Appendix 1). The results of the study of Jagger et al. (1983), and Gupta et al. (2006) found *P. aeruginosa* produced 65% and 80% of protease respectively. The proteases was responsible for many features of the pathogenesis of *Pseudomonas* in the eye Proteases are degrade the integrity of the host's physical barriers by splitting proteins and amino acids (Lyczak et al., 2000) proteases are considered important virulence factors in burn infections were contributed to the invasiveness of the organisms which damage host tissues and interfere with host antibacterial defense mechanisms (Anzai et al., 2000).

Fig. 1: Virulence factors of *P. aeruginosa*.

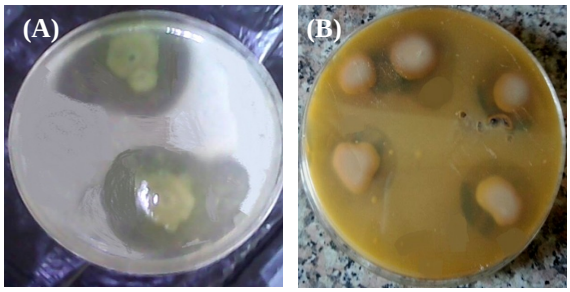


Lecithinase production (phospholipase)

The results revealed that only 36 isolates (90%) give positive results. Fatty acid resulted from break down of phospholipids around colony as a white precipitation, resulting from production of phospholipase enzyme (lecithinase), as a result of phospholipase production 4(10%) gave negative result (Fig. 2 and Appendix 1).

The study of Anne et al. (2003) found 70% of *P. aeruginosa* produce phospholipase. This virulence factor is important in burn wound infections (Espinosa et al., 2000) and acts on the animal cell membrane by insertion into the membrane forming a pore in cell and cleaves phospholipids, also lyses red blood cells, phagocytes and their granules (Edward et al., 2006).

Fig. 2: Clear zone produced by the isolate in skim milk (A) and phospholipid medium (B).



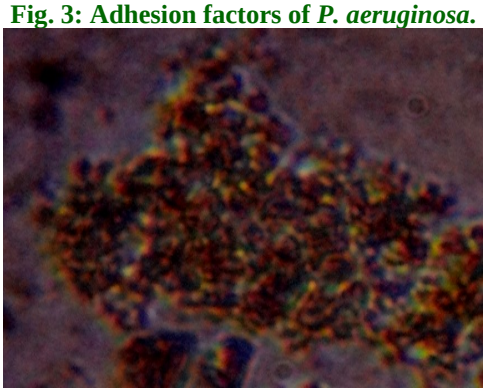
Amylase production

P. aeruginosa isolates (100%) gave negative result in starch medium (Fig. 1). Growth of *P. aeruginosa* on a starch agar plate and addition of iodine did not form clear zone surrounding the bacterial growth indicating starch hydrolysis. The study of Kanmani et al. (2005) found *P. aeruginosa* produce 5% amylase.

Adhesion factors

The epithelial cells obtained from urine of healthy females were used to inquire adhesion factors of locally isolated *Pseudomonas* isolates, and the results revealed that all *Pseudomonas* isolates (100%) have the ability of adhesion to human epithelial cells (Fig. 3 and Appendix 1). The study of Dang et al. (2003) found that the adhesion of *P. aeruginosa* was 100%. *P. aeruginosa*, an important opportunistic pathogen, has numerous factors for initial attachment to the host, stainless steel and a biotic surface (Scharfman et al., 2001). The major bacterial adhesions are pili and flagella. Flagella are used by the bacteria for various purposes-to adhere to cells, swimming motility, and biofilm formation. Pili are allowing the bacteria to move over a solid surface in a process termed twitching motility which contribute to early steps in biofilm formation, serve as adhesion with mammalian cells and resisting antibiotics (Emam et al., 2006).

Fig. 3: Adhesion factors of *P. aeruginosa*.



Conclusion

P. aeruginosa is the one of important agents of wound and burn infections. The clinical isolates of *P. aeruginosa* posses a number of virulence factors such as capsule, protease, phospholipase and adhesion factors. These virulence factors promote adherence of bacteria to host cells and damage host tissue, elicit inflammation and disrupt defense mechanisms due to impairment of the skin barrier in burn patients and frequent scrubbing and debridement. In the hospital, more careful attention to routine infection control practices must be followed, especially hand hygiene and environmental cleaning, to substantially lower the risk of infection.

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Appendix 1. Performance of virulence factors in clinical isolates of *P. aeruginosa.*

Virulence factors					
No. of isolates	**Capsule**	**Protease**	**Adhesion factors**	**Phospholipase**	**Amylase**
Sample 1	+	_	+	+	_
Sample 2	+	+	+	+	_
Sample 3	+	+	+	+	_
Sample 4	+	+	+	+	_
Sample 5	+	+	+	+	_
Sample 6	+	+	+	+	_
Sample 7	+	+	+	+	_
Sample 8	+	_	+	+	_
Sample 9	+	_	+	+	_
Sample 10	+	+	+	_	_
Sample 11	+	+	+	_	_
Sample 12	+	+	+	+	_
Sample 13	+	+	+	+	_
Sample 14	+	+	+	+	_
Sample 15	+	_	+	+	_
Sample 16	+	+	+	+	_
Sample 17	+	+	+	+	_
Sample 18	+	+	+	+	_
Sample 19	+	+	+	+	_
Sample 20	+	+	+	+	_
Sample 21	+	_	+	+	_
Sample 22	+	_	+	+	_
Sample 23	+	+	+	+	_
Sample 24	+	_	+	+	_
Sample 25	+	+	+	_	_
Sample 26	+	+	+	_	_
Sample 27	+	+	+	+	_
Sample 28	+	+	+	+	_
Sample 29	+	+	+	+	_
Sample 30	+	_	+	+	_
Sample 31	+	+	+	+	_
Sample 32	+	+	+	+	_
Sample 33	+	+	+	+	_
Sample 34	+	_	+	+	_
Sample 35	+	_	+	+	_
Sample 36	+	+	+	+	_
Sample 37	+	+	+	+	_
Sample 38	+	+	+	+	_
Sample 39	+	_	+	+	_
Sample 40	+	_	+	+	_