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Molecular Characterization of *Pseudomonas aeruginosa* Isolated from Wound Infections of Iranian Children

Shahram Parvin[1], Amir Masoud Hashemian[2], Amir Farshid Fayyaz[3], Marzieh Miar[4] and Eslam Ghezelsofla[5]*

[1]Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
[2]Department of Emergency Medicine, Imam Reza Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
[3]Department of Legal Medicine , AJA University of Medical Sciences , Tehran , Iran.
[4]Department of Microbiology , Tonekabon Branch , Islamic Azad University , Tonekabon, Iran.
[5]Iranian Blood Transfusion Organization, Shahr-e-Kord, Iran.

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***Pseudomonas aeruginosa*, a ubiquitous opportunistic pathogen is one of the main causative agents of human wound infection. Wound infection due to this bacterium are difficult to heal and caused serious economic issues. Its putative virulence genes are responsible for causing wound infection. This investigation was aimed to study the prevalence of *P. aeruginosa* and its virulence factors in the wound infection samples of children. Totally, 150 swab samples were collected from children hospitalized due to wound infection. Samples were cultured and their positive results were subjected to PCR to identify the distribution of virulence factors. Total prevalence of *P. aeruginosa* in the swab samples were 62.6%. Totally, 65% of boys and 60% of girls were infected with *P. aeruginosa*. The highest prevalence of *P. aeruginosa* was achieved in less than 2 years old children (80.9%). The most commonly detected virulence genes were *exoS* (91.4%), *plcH* (86.1%), *algD* (80.8%) and *exoU* (60.6%). High prevalence of virulent strains of *P. aeruginosa* in Iranian hospitals should be controlled carefully and modern therapeutic agents should be applied especially in the less than 2 years old children.**

Key words: *Pseudomonas aeruginosa*, virulence factors, Wound infection, Children, Iran.

Wound infection is an important cause of health care associated infections among damaged or surgical patients. Wound infections caused longer hospital stays, more expensive hospitalizations, and increased mortality[1]. The annual wound care products market is projected to reach $15.3 billion by 2010[1].

Pseudomonas aeruginosa contributes substantially to wound-related morbidity and mortality worldwide. *It* is widely distributed, mostly in the hospital field and is one of the most important agents of hospital-ac-quired wound infections, ecthyma gangrenosum and black necrotic lesions [2, 3]. Recent study showed that of the 300 bacterial isolates from human wound infections, 89 (29.6%) were *P. aeruginosa*[2]. Its total prevalence as a causative agent of human wound infections through 2004 to 2009 was 18-32 percent which is entirely high[4-6].

P. aeruginosa produces several substances that are thought to enhance the colonization and infection of skin tissue[7]. These substances together with a variety of virulence factors, including those participate directly or indirectly in physical interactions with the host cell and/or host proteins, including secretion systems (type II, type III, type VI) and associated effectors (including exoenzyme T (*exoT*), *exoU*,

* To whom all correspondence should be addressed.
Tel : +98383334343;
E-mail: mohghe@gmail.com

exoS, *toxA*, phospholipase C, and alkaline protease), flagella, and structures involved in attachment to host cells such as type IV pili. Other recognized virulence factors include those involved in quorum sensing (PQS and AHL systems), iron acquisition (pyoverdine, pyochelin), small molecule synthesis (phenazines, hydrogen cyanide and rhamnolipid), lipopolysaccharide, alginate (*algD* gene), and biofilm[8, 9]. A zinc metalloprotease called *lasB* has an elastolytic activity on skin tissue[7]. The gene called *nan1* encodes a sialidase that is responsible for adherence to the human tissues[7].

In the recent years, the growing incidence of *P. aeruginosa* has been of particular concern. The incidence of *P. aeruginosa* in wound infection is becoming more serious in developing countries like Iran (7, 10). This issue has comparatively higher concern in children due to their relatively lower levels of immune system. Therefore, the present study was carried out in order to study the prevalence of *P. aeruginosa* and its putative virulence factors in the wound infection samples of Iranian children.

MATERIALS AND METHODS

Samples and identification of Pseudomonas aeruginosa

From March to May 2014, a total of 150 swabs were collected from hospitalized children suffered from wound infections. Samples were collected during a three month period of time from hospitalized pediatrics of some educational Hospitals, Tehran, Iran. All samples were transmitted to the Microbiology and Infectious Diseases Research Center of Tehran in a cooler with ice-packs. Samples were placed on the selective medium *Pseudomonas* cetrimide agar (PCA) (LABOBASI, Mendrisio, Switzerland) using a spreading technique. Plates were incubated for 18-24 hours and observed for suspected colonies of *P. aeruginosa*. *P. aeruginosa* was identified done by colony pigmentation, grape-like odor, motility and biochemical tests including carbohydrate fermentation (-), citrate assimilation (+), lysine decarboxylase (-), indol (-), oxidase (+), beta-hemolysis on blood-agar (+) and DNAse (-). Inocula from pure colonies on PCA were cultured on nutrient agar slants and kept at 4ºC.

DNA extraction and PCR confirmation

Chromosomal DNA was extracted from each *P. aeruginosa* isolate by DNA extraction kit (Fermentas, Germany) according to manufacturer's instruction.

The bacteria were confirmed using the PCR method for *nanI* gene of the *P. aeruginosa* (11). PCR was carried out with 2 µL template DNA, 0.25 µM of each primer (F: 5'-ATGAATACTTATTTTGATAT and R: CTAAATCCATGCTCTGACCC-3'), 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Fermentas) in a total volume of 25 µL. The DNA was amplified using the following protocol: initial denaturation (94 ºC for 5 min), followed by 25cycles of denaturation (94 ºC for 35 s), annealing (53 ºC for 45 s) and extension (72 ºC for 1 min), with a single final extension of 7 min at 72 ºC.

Identification of virulence factors

The multiplex PCR assay was used in order to amplification of various virulence genes. The programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device was used in all PCR reactions.

The PCR reaction based on the method of Mitov et al. (2010)[12] was used in order to detection of *algD*, *pilB*, *nan1*, *lasB*, *plcH*, *exoS* and *exoU* virulence genes. List of primers is shown in Table 1.

PCR was carried out in a total volume of 25 µl including 2 µl template DNA, 0.25 M of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 Mm MgCl2 and 1.5 U Prime Taq DNA polymerase (GeNet Bio). The DNA was amplified using the following protocol: initial denaturation (94 ºC for 5 min), followed by 25–30 cycles of denaturation (94 ºC for 35–45 s), annealing (53–62 ºC, from 45 s to 1 min) and extension (72 ºC, from 45 s to 1 min 35 s), with a single final extension of 7 min at 72 ºC.

Gel electrophoresis

Fifteen microliters of PCR products were resolved on a 1.5% agarose gel containing 0.5 mg/ml of ethidium bromide in Tris–borate–EDTA buffer at 90 V for 1 h, also using suitable molecular weight markers. The products were examined under ultraviolet illumination.

Statistical analysis

The results were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA) for analysis. Statistical analysis was performed using SPSS/16.0 software (SPSS Inc., Chicago, IL) for significant relationship between incidences virulence genes of *P. aeruginosa* isolated from clinical samples. A $Ç^2$ test and Fisher's exact 2-tailed test analysis were performed in this study. Statistical significance was regarded at a P value < 0.05.

RESULTS

Prevalence of *P. aeruginosa* in the wound infections of 105 children has been studied in the present study. Total distribution of *P. aeruginosa* in studied samples is shown in Table 2. Ninety four out of 150 (62.6%) swab samples were positive for *P. aeruginosa*. In the other hand, total distribution of bacterium in the swab samples of boys and girls of our investigation was 65% and 60%, respectively. There were no statistically significant differences for the prevalence of *P. aeruginosa* between boys and girls. Senile distribution of *P. aeruginosa* in the swab samples of children suffered from wound infections is shown in Table 3. Results showed that the less than 2 years old children had the highest incidence of *P. aeruginosa* (80.9%), while the 6-8 years old children had the lowest incidence (50%). There were significant differences (P =0.037) in the incidence of *P. aeruginosa* between younger and older children.

Figure 1 to 4 show the results of gel electrophoresis of PCR products for various virulence factors. Table 4 shows the distribution of putative virulence factors in the *P. aeruginosa* isolates of wound infections. The *exoS* was the most commonly detected virulence factor (91.4%), while the *nan1* was the less commonly detected (40.4%). The total distribution of *algD, pilB, lasB, plcH* and *exoU* virulence genes among 94 *P. aeruginosa* isolates were 80.8%, 42.5%, 55.3%, 86.1% and 60.6%, respectively. We found statistically significant association between the incidence of *exoS* and *nan1* (P =0.015), *exoS* and *pilB* (P =0.020), *plcH* and *lasB* (P =0.042), *plcH* and *nan1* (P =0.036) and *exoS* and *exoU* (P =0.048).

Table 1. Oligonucleotide primers used for detection of virulence genes of *Pseudomonas aeruginosa* (11, 13).

Gene	Sequence (5'–3')	Size of product (bp)
algD	F: ATGCGAATCAGCATCTTTGGT	1310
	R: CTACCAGCAGATGCCCTCGGC	
pilB	F: ATGAACGACAGCATCCAACT	826
	R: GGGTGTTGACGCGAAAGTCGAT	
nan1	F: ATGAATACTTATTTTGATAT	1317
	R: CTAAATCCATGCTCTGACCC	
lasB	F: GGAATGAACGAGGCGTTCTC	300
	R: GGTCCAGTAGTAGCGGTTGG	
plcH	F: GAAGCCATGGGCTACTTCAA	307
	R: AGAGTGACGAGGAGCGGTAG	
exoS	F: CTTGAAGGGACTCGACAAGG	504
	R: TTCAGGTCCGCGTAGTGAAT	
exoU	F: GGGAATACTTTCCGGGAAGTT	428

Table 2. Distribution of *Pseudomonas aeruginosa* in the wound infection samples of children

Type of samples	No. samples	Sex of pediatrics		No. positive samples (%)		
		Male	Female	Male	Female	Total
Swabs from wound infection	150	80	70	52 (65)	42 (60)	94 (62.6)

DISCUSSION

The results of the present study showed that *P. aeruginosa* has a higher prevalence in the children wound infections. Totally, 62.6% of the swab samples of children were positive for *P. aeruginosa* to our best knowledge, the finding are the highest prevalence of *P. aeruginosa* in swab samples of children wound infection. Lower prevalence rate of *P. aeruginosa* in the human wound infections have been reported previously by Ranjan et al. (2010) (27.7%)[2], Anupurba et al. (2006) (32%)[4], Oguntibegri and Nwobu (2004) (33.2%)[5], Masaadeh and Jaran, (2009) (27.78%)[6] and Siguan et al., (1990) (18.8%)[14]. Higher prevalence of *P. aeruginosa* in the clinical samples of our study maybe due the fact that type of samples (swabs samples of children) and health care managements are different with those of other investigations. Children have the lower levels of immune systems and are more likely to get *P. aeruginosa* wound infection. Our results also showed that the less than 2 years old children had the highest incidence of *P. aeruginosa* (80.9%) and this is also due to the fact that they have lower levels of immunity. Higher prevalence of *P. aeruginosa* in the less than 10 years old children has been reported recently by Srinivas et al., (2012)[15] (4.9% in male and 0.59% in female). Low levels of healthcare managements in Iranian healthcare units and hospitals has been recognized from the results of our study and the results of various previous Iranian investigations [7, 10, 16, 17].

Statistical analysis showed that there were no significant differences for the prevalence

Table 3. Distribution of *Pseudomonas aeruginosa* among various age groups of children

Age groups (Year)	No. samples	No. positive samples (%)
<2	21	17 (80.9)
2-4	29	20 (68.9)
4-6	42	28 (66.6)
6-8	58	29 (50)
Total	150	94 (62.6)

Table 4. Distribution of putative virulence factors among *Pseudomonas aeruginosa* isolates of wound infections

No. positive results	Distribution of virulence factors (%)						
	algD	*pilB*	*nan1*	*lasB*	*plcH*	*exoS*	*exoU*
Male (52)	45 (86.5)	22 (42.3)	21 (40.3)	28 (53.8)	46 (88.4)	49 (94.2)	32 (61.5)
Female (42)	31 (73.8)	18 (42.8)	17 (40.4)	24 (57.1)	35 (83.3)	37 (88.0)	25 (59.5)
Total (94)	76 (80.8)	40 (42.5)	38 (40.4)	52 (55.3)	81 (86.1)	86 (91.4)	57 (60.6)

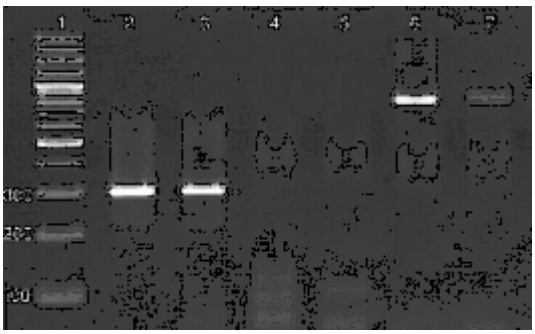

Fig. 1. Results of the gel electrophoresis for identification of *lasB and pilB* genes of *P. aeruginosa* isolates of wound infection, 1: 100 bp DNA ladder (Fermentas, Germany), 2: Positive control for *lasB* gene, 3: Positive sample for *lasB* gene (300 bp band), 4: Negative sample, 5: Negative control; 6: Positive sample for *pilB* (826 bp band) and 7: Positive control for *pilB* gene.

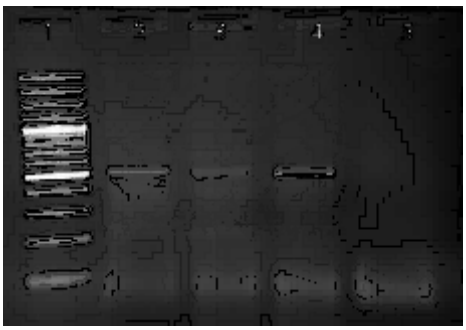

Fig. 2. Results of the gel electrophoresis for identification of *exoS* gene of *P. aeruginosa* isolates of wound infection, 1: 100 bp DNA ladder (Fermentas, Germany), 2: Positive control for *exoS* gene; 3 and 4: Positive samples for *exoS* gene (504 bp band) and 4: Negative control.

of *P. aeruginosa* between boys and girls but our results indicated that total prevalence of *P. aeruginosa* in boys and girls were 65% and 60%, respectively. Al-Hassan et al., (2008)[18] reported that 62.80% of *P. aeruginosa* strains were isolated from hospitalized males. Also, gender-wise prevalence showed 61.78% male and 38.22% females of the study of Khan et al., (2008)[19] were infected by *P. aeruginosa* which was similar to our results.

The most commonly detected virulence factors in the *P. aeruginosa* strains of our study were *exoS* (91.4%), *plcH* (86.1%) and *algD* (80.8%). Nikbin et al., (2012)[7] showed that the total distribution of *toxA*, *exoS* and *nan1* genes in the *P. aeruginosa* isolates of wound infection were 90%, 62% and 30%, respectively which was entirely similar to our results. In a study conducted by Mitov et al., (2010) (12) the incidence of *algD*, *pilB*, *nan1*, *lasB*, *plcH*, *exoS* and *exoU* in the *P. aeruginosa* strains of wound infection were 84.6%, 26.9%, 15.4%, 100%, 96.2%, 65.4% and 46.2%, respectively.

The *exoS* gene is directly translocated into eukaryotic cells by the contact-dependent type III secretory process and, as such, it provides the bacterium with a mechanism for manipulating the eukaryotic cells it encounters. In support of *exoS* contributing to *P. aeruginosa* pathogenicity, bacterial translocation of *exoS* into epithelial cells results in a general inactivation of cellular function, as recognized by the inhibition of DNA synthesis, loss of focal adhesion, cell rounding, and microvillus effacemen[20, 21]. The *plcH* gene is responsible for proinflammatory activities, virulence to animal models and inhibition of oxidative burst of neutrophils[22, 23].

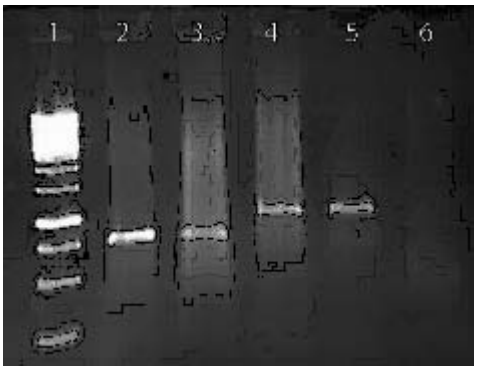

Fig. 3. Results of the gel electrophoresis for identification of *plcH and exoU* genes of *P. aeruginosa* isolates of wound infection, 1: 100 bp DNA ladder (Fermentas, Germany), 2: Positive control for *plcH* gene, 3: Positive sample for *plcH* gene (307 bp band), 4: Positive sample for *exoU* (428 bp band) and 6: Negative control.

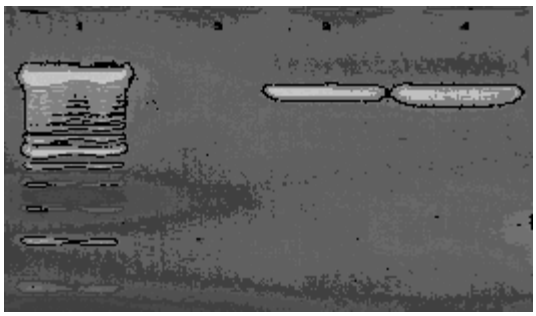

Fig. 4. Results of the gel electrophoresis for identification of *algD* gene of *P. aeruginosa* isolates of wound infection, 1: 100 bp DNA ladder (Fermentas, Germany), 2: Negative control, 3: Positive sample for *algD* gene (1310 bp band) and 4: Positive control.

CONCLUSION

The present study is one of the most widely prevalence reports of *P. aeruginosa* and its putative virulence factors in the wound infection samples of children in Iran. Boys less than 2 years old had the highest prevalence of bacteria. *ExoS*, *plcH* and *algD* were the most commonly detected virulence factors in bacterial isolates. With respect to the high prevalence of virulent strains of *P.*

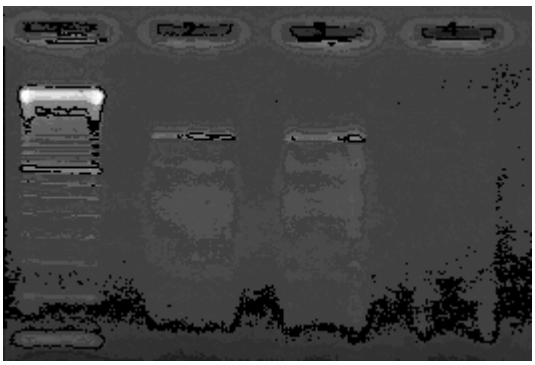

Fig. 5. Results of the gel electrophoresis for identification of *nan1* gene of *P. aeruginosa* isolates of wound infection, 1: 100 bp DNA ladder (Fermentas, Germany), 2: Positive control, 3: Positive sample for *nan1* gene (1317 bp band) and 4: Negative control.

aeruginosa in the wound infection samples of Iranian children, wide ranges of healthcare, treatment and control strategies should performed in majority of Iranian hospitals. It is important to identify the prevalence status of *P. aeruginosa* and its virulence factors in each province, city and even hospital.

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