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Molecular detection of virulence genes in *Pseudomonas aeruginosa* isolated from children with Cystic Fibrosis and burn wounds in Iran

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ABSTRACT

Pseudomonas aeruginosa possesses various virulence factors which contribute to the bacterial invasion and toxicity. Moreover, children suffered from Cystic Fibrosis (CF) and burn wounds are at a high risk of various bacterial infections. The aim of this study was to determine the prevalence of virulent genes in *P. aeruginosa* isolated from children with CF and burn wounds and comparing their virulence genes to figure out the role of every virulent factor in the infections.

P. aeruginosa were isolated from sputum, oropharyngeal swabs, and broncho-alveolar lavage (BAL) specimens from CF and burn wounds between June 2013 and June 2014 in Tehran's hospitals. Bacterial genomic DNAs were extracted and uniplex, duplex and multiplex PCR were performed for detection of *toxA*, *algD and plcN*, *exoS, lasB, plcH* genes, respectively.

The prevalence rate of virulence genes in *P. aeruginosa* isolated from CF was; *toxA* (63.1%), *algD* (64.6%), *plcH* (87.7%), *plcN* (60%), *lasB* (95.4%) and *exoS* (70.8%) and virulence genes in *P. aeruginosa* from burn patients were: *toxA* (36.9%), *algD* (70.1%), *plcH* (79%), *plcN* (63.1%), *lasB* (82%) and *exoS* (21.1%).

The prevalence of three virulent genes in *P. aeruginosa* was higher in CF comparing to burn wound infections. We found that the number of *toxA*, *lasB* and *exoS* were significantly higher in the bacteria which were isolated from children with CF. This finding shows that these virulence factors play an important role in CF infections by *P. aeroginosa*.

1. Introduction

Pseudomonas aeruginosa is a gram negative bacterium which is an ubiquitous microorganism and could survive in hospital environments [1]. Meanwhile, there are many sources for *P. aeruginosa* such as sinks, toilets, respiratory therapy equipment and ever disinfectant solutions. These bacteria are present in intestine and skin of some carriers [2].

P. aeruginosa is leading to various infections such as pneumonia, keratitis and Folliculitis in immune-deficient and healthy individuals [3]. The bacterium possesses many virulence factors which contribute to the bacterial invasion and toxicity [4]. Moreover, the bacterium is responsible for nosocomial infections. Immune-compromised patients are the most susceptible ones to infection. Children with Cystic Fibrosis (CF) and burn wounds are at a high risk of getting infection with *P. aeruginosa* [3].

Cystic fibrosis is a life-threatening, genetic disease that causes persistent lung infections and progressively limits the ability to breathe. Children with CF have a defective gene which influences sodium chloride transportation. The deficiency is caused by mutations in the genes encoding the protein Cystic Fibrosis Transmembrane conductance Regulator (CFTR) [5]. Therefore, mutations in these genes cause increased viscosity and secretion disorder in the mucous secretion in the airways, pancreas and liver. Thus, the defect leads to elevate the amount of salt in sweat gland secretions [6]. Meanwhile, this deficiency causes a sticky mucus layer, thickened digestive juices and salty sweat [7]. Additionally, children with CF have a high risk to get air way infections by *P. aeruginosa*. Although there is a strong antibody response in serum, saliva and secretions from the lungs, the immune system is unable to eradicate the infection [8]. Children with CF usually are

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colonized with *P. aeruginosa* by three years old, which form a biofilm in their airways [9].

Burn wounds are another attractive niche for *P. aeroginosa*. Cellular damage to the skin and local infections can suppress local cell-mediated and humoral (IgA) immune responses [10]. The bacteria in wound infections may be originated from gastrointestinal microbiota as endogenous and environmental *P. aeroginosa* as exogenous routes of infections [11].

It has been proved that pathogenicity of the bacterium completely depends on its virulence factors. Virulence factors of *P. aeruginosa* can be classified in factors involved in the acute and chronic infection. Pili, exoenzyme S, exotoxin A and Phospholipase C are important for acute phase of disease. While, siderophores (pyoverdin and pyochelin), and pseudocapsule of alginate are essential for chronic phase of *P. aeruginosa* infections [12]. Several studies have also shown that there is a relative contribution of some virulence factors in *P. aeruginosa* infections. For instance, type 3 secretion system and elastase are the most important virulence factors in *P. aeruginosa* isolated from pneumonia and proteases most likely play a role in localized pseudomonas infections such as keratitis, pneumonia and burn infection [13,14].

The present study aimed to determine the prevalence rate of virulence genes in *P. aeruginosa* among bacteria which isolated from children with CF and burn wounds in Tehran's hospitals.

2. Materials and methods

2.1. Bacterial isolates

One hundred and fourteen *P. aeruginosa* were isolated from sputum (n = 17), oropharyngeal swabs (n = 14), broncho-alveolar lavage (BAL) (n = 26) and burn wounds specimens (n = 57) between June 2013 and June 2014 from children with CF and burn wounds in Tehran's hospitals. All of the isolates were confirmed with the conventional microbiology laboratory tests [15].

2.2. DNA extraction

Bacterial genomic DNAs were extracted with the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's protocols. The DNAs were preserved at −20 °C until used for polymerase chain reaction (PCR) tests.

2.3. PCR method

After DNA extraction, uniplex, duplex and multiplex PCR were performed in a total volume of 25 μL containing 1 × PCR buffer, 1 μmol/L of each primer, 1 μL of genomic DNA (approximately 150 ng), 200 μmol/L of dNTPs mix, 2 mmol/L of $MgCl_2$, and 0.05 U/μL Taq DNA polymerase. PCR amplifications were performed in an automated thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: Uniplex PCR of *toxA*: 30 cycles of 1 min at 94 °C, 1.5 min at 63 °C, and 1 min at 72 °C. Duplex PCR of *algD and plcN*: 30 cycles of 1 min at 94 °C, 1.5 min at 60 °C, and 1 min at 72 °C. Multiplex PCR of *exoS, lasB, plcH,*: 30 cycles of 1 min at 94 °C, 1.5 min at 60 °C, and 1 min at 72 °C. The amplified genes were detected by electrophoresis in a 1% agarose gel stained with ethidium bromide (Table 1) [16].

2.4. Statistical analysis

Chi-square statistical analysis test was used to determine whether or not there is any significant difference in the virulence factors between *P. aeroginosa* isolated from CF and burn wound infections. P value was set at 0.05.

Table 1
Nucleotide sequences of primers used for amplification of *P. aeruginosa* virulence factors genes [16].

Gene	Primer sequence (5′–3′)	PCR product (size) (bp)	Number of pair G+C
alg D	F:CGTCTGCCGCGAGATCGGCT	313	14
	R:GACCTCGACGGTCTTGCGGA		13
las B	F:GGAATGAACGAAGCGTTCTCCGAC	284	13
	R:TTGGCGTCGACGAACACCTCG		13
tox A	F:CTGCGCGGGTCTATGTGCC	270	13
	R:GATGCTGGACGGGTCGAG		12
plc H	F:GCACGTGGTCATCCTGATGC	608	14
	R:TCCGTAGGCGTCGACGTAC		12
plc N	F:TCCGTTATCGCAACCAGCCCTACG	481	14
	R:TCGCTGTCGAGCAGGTCGAAC		13
exo S	F:CGTCGTGTTCAAGCAGATGGTGCTG	444	14
	R:CCGAACCGCTTCACCAGGC		13

2.5. Ethical consideration

The research proposal was approved by the Pediatric Infections Research Center (PIRC) of Shahid beheshti University review board ethics. Informed consent was obtained from parents or guardians before children were enrolled.

3. Results

In the current study, clinical isolates were screened for the prevalence of different virulence genes of *P. aeruginosa*. Interestingly, *toxA*, *lasB* and *exoS* genes had higher occurrence in *P. aeroginosa* isolated from patients with CF. Forty one (63.1%) and 21 (36.9%) of *P. aeroginosa*, which were isolated from CF and burn wound infections, possessed *toxA* gene (Fig. 1A). Sixthly two (95/4%) and 47 (82%) of the isolated bacteria from CF and burn wounds had *lasB* gene (Fig. 1C) and Forty six (70/8%) and 12 (21/1%) of *P. aeroginosa* isolated from CF and burn wounds possessed *exoS* gene (Fig. 1C).

The results showed that there is no significant difference in *algD*, *plcH* and *plcN* genes in *P. aeruginosa* strains. Forty two (64.6%) and 40 (70.1%) of the isolated bacteria from CF and burn wounds had *algD* gene (Fig. 1B). Fifty seven (87.7%) and 45 (79%) of isolated *P. aeroginosa* from CF and burn wounds had *plcH* gene (Fig. 1C). Thirty nine (60%) and 36 (63.1%) of *P. aeroginosa* which were isolated from CF and burn wound infections, possessed *plcN* gene (Fig. 1B).

4. Discussion

Pseudomonas aeruginosa as an opportunistic pathogen has different virulence factors which aid the bacteria to colonize different niches in their host and the bacteria are a leading cause of nosocomial and community-acquired infections worldwide [17]. *P. aeruginosa* is one of the most important bacteria which can infect airways in CF and burn wounds in children [18]. Moreover, the bacteria have a number of virulence factors such as exotoxin A, alginate, exoenzyme S, elastase and phospholipase which are regulated via particular signaling systems [19]. In epidemiologic investigations, distribution of highly virulent *P. aeruginosa* is a major problem of world health system [20]. Clinical outcomes of *P. aeruginosa* infection depend on both the proper response of the host and bacterial virulence factors that fundamentally operate to neutralize the host response [21].

In the present study, we aimed to determine the prevalence of virulence genes in *P. aeruginosa* such as exotoxin A, exoenzyme S, alginate D, phospholipase N, phospholipase H, and elastas B by