Genetic Identification of *Pseudomonas aeruginosa* Virulence Genes among Different Isolates

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Abstract

Background and objectives: *Pseudomonas aeruginosa* possesses a variety of virulence factors that may contribute to its pathogenicity. *P. aeruginosa* also has a large number of virulence factors such as exotoxin A, exoenzyme S, nan 1 and Las genes. The aim of this study was to evaluate *oprI*, *oprL* as reliable factors for rapid identification of *P. aeruginosa* and to detect *toxA, exo S, nan1* and *LasB* genes by Polymerase Chain Reaction (PCR).

Materials and methods: In this study 30 isolates of *P. aeruginosa* were recovered from burn, pulmonary tract and blood infections.

Results and conclusions: The *opr*I and *opr*L genes were detected in all of 30 *P. aeruginosa* isolates collected. The presence of *toxA* gene in isolates from burn and pulmonary tract was significantly higher than that from blood. All tested isolates harbored *LasB* gene. However, difference between *exoS* prevalence in isolates from pulmonary tract and burn isolates was statistically significant higher than that from blood. The prevalence of *nan1* gene was significantly higher in isolates of pulmonary tract and burn specimens than isolates from blood. Molecular methods have been reported to be superior to the phenotypic methods for identification of *P. aeruginosa* by designing a multiplex PCR assay based on *oprI* and *oprL* genes for molecular detection of *P. aeruginosa* Determination of different virulence genes of *P. aeruginosa* isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity. Significant correlations between some virulence genes and source of infections indicates implementation of infection control measures will help in controlling the dissemination of virulence genes among *P. aeruginosa* isolates.

Keywords: *Pseudomonas aeruginosa*; Polymerase chain reaction

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting virtually all tissues, can infect immunocompromised individuals and responsible for hospital acquired infections [1].Burn patients, mechanically ventilated patients, and cystic fibrosis (CF) patients are particularly susceptible to *P. aeruginosa* infections. It is a major cause of morbidity and mortality in patients with cystic fibrosis [2]. *P. aeruginosa* infections in hospitals mainly affect the patients in intensive care units and those having catheterization, burn, and/or chronic illnesses [3].

Pseudomonas aeruginosa possesses a variety of virulence factors that may contribute to its pathogenicity. *P. aeruginosa* also has a large number of virulence factors such as exotoxin A, exoenzyme S, nan 1 and Las genes [1] The outer membrane proteins of *P. aeruginosa* OprI *and OprL* play important roles in the interaction of the bacterium with the environment as well as the inherent resistance of *P. aeruginosa* to antibiotics where the consequence of the presence of these specific outer membrane proteins that have been implicated in efflux transport systems that affect cell permeability [4]. As these proteins are found only in this organism, they could be a reliable factor for rapid identification of *P. aeruginosa* in clinical samples [5].

Pseudomonas aeruginosa can cause pulmonary damage by different mechanisms. Exoenzyme S, encoded by the *exoS* gene, is an ADP ribosyltransferase that is secreted by a type-III secretion system directly into the cytosol of epithelial cells [6]. Exotoxin A, encoded by the *toxA* gene, inhibits protein biosynthesis. LasBelastase, a zinc metalloprotease encoded by the *LasB* gene, has an elastolytic activity on lung tissue [7]. In addition, the phospholipids contained in pulmonary surfactants may be hydrolysed by two phospholipases Cencoded by PLC-H and

PLC-N, respectively [8].The gene called *nan1* encodes a sialidase that is responsible for adherence to the respiratory tract [9]. An extracellular neuraminidase is thought to play an important role in implantation of the bacterium but the genetic basis of this process is still unknown [10].

Although conventional microbiological methods for identifying *P. aeruginosa* from clinical and environmental samples are reliable, they require several days to be completed. Rapid detection of isolates causing hospital infections is very important for consequent treatment decision of patients. PCR has the potential for identifying microbial species rapidly by amplification of sequences unique to a particular organism [11].

In this study, our aim was to evaluate *oprI*, *oprL* as reliable factors for rapid identification of *P. aeruginosa* isolated from pulmonary tract, blood and burn samples based on PCR amplification of I lipoprotein (*oprI*) for detection of genus and L lipoprotein (*oprL*) for detection of species of this organism and to detect *toxA, exo S, nan 1and Las* genes by PCR in different isolates of *P. aeruginosa* in order to find out any relation between these virulence factors and special manifestation of

P. aeruginosa infections, we detected virulence factors among these isolates by using PCR.

Patients and Methods

A total number of 30 clinical isolates of *P. aeruginosa* were obtained from different samples from the Department of Clinical microbiology Ain shams University hospitals in the period between October 2014 and February 2015. Ten *P. aeruginosa* isolates were burn infections, 10 isolates from pulmonary tract and 10 isolates from blood infections. Twelve of the isolates were isolated from community acquired infections and eighteen isolates were obtained from Hospital acquired infections. The isolates were identified based on the colony morphology on Nutrient agar, blood agar and MacConkey agar and were confirmed using IMViC biochemical tests.

Detection of Virulence Genes by PCR

Genomic DNA was prepared from overnight cultures grown on Tryptose Soy broth, 10 ml of broth was centrifuged, 360 μl ATL+50 μl protease were added and incubated for 1 h at 55°C, 400 μl AL buffer for 10 min at 55°C were added, then 400 μl ethanol were added; 700 μl were transferred to spin column, centrifuged at 8000 rpm/1 min. The rest of the sample was transferred to spin column and centrifuge. Then 700 μl of wash 1 buffer was added centrifuged. Transfer to new wash tube and 700 μl wash 2 was added. Spin column transferred to new wash tube, centrifuged at 13000/3 min, then transfer to elution tubes and add 100 μl preheated elution Buffer (70°C). Incubate 3 minutes at room temperature; centrifuge 7000 rpm/1 min [10].

PCR amplification was carried out using thermal cycler (BioRad, USA) with specific primers for *oprI, oprL, toxA, exoS, nan1* and *lasB*.

PCR was carried out in 50 μl volume reaction mixtures containing 1 μl of each primer, 10 μl of crude template DNA and 25 μl Qiagen master mix. The annealing temperature was 55°C for *oprI, oprL, toxA* and 58°C for *exoS, nan1* and *lasB*. PCR products were separated by gel electrophoresis on 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Statistical Analysis

Data has been collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 17; Inc., Chicago. IL). Descriptive statistics has been done, where qualitative data has been expressed as frequency and percent. Chi square test has been used to detect significance of the results; P value was calculated at significant level (0.10). Bar charts have been plotted.

Results

The *opr*I and *opr*L genes were detected in all of 30 *P. aeruginosa* isolates collected. However, presence of *tox A* gene in clinical samples was different. According to (Table 1) the presence of *tox A* gene in isolates from burn and pulmonary tract was significantly higher than that from blood (P<0.001). Our results showed that all tested isolates harbored *lasB* gene. However, difference between *exoS* prevalence in isolates from pulmonary tract and burn isolates was statistically significant higher than that from blood (P<0.1). The *nan1* gene, other virulence factor studied in this research, was found in 4 (40%) of 10 isolates from pulmonary tract, 4 (40%) of 10 from burn however it wasn't found in isolates from blood infections. The prevalence of *nan*1 gene was significantly higher in isolates of pulmonary tract and burn specimens than isolates from blood (P<0.1).

Amplified gene	Specific Primer	Amplified region
oprI	PS1, 5'-ATG AAC AAC GTT CTG AAA TTC TCT GCT-3'	250
	PS2, 5'-CTT GCG GCT GGC TTT TTC CAG-3'	
oprL	PAL1, 5'-ATG GAA ATG CTG AAA TTC GGC-3'	500
	PAL2, 5'-CTT CTT CAG CTC GAC GCG ACG-3'	
lasB	lasB lasf 5' GGA ATG AAC GAA GCG TTC TC 3'	300
	las r 5' GGT CCA GTA GTA GCG GTT GG 3'	
toxA	toxA toxf 5' GGT AAC CAG CTC AGC CAC AT 3'	352
	tox r 5' TGA TGT CCA GGT CAT GCT TC 3'	
exoS	exoS exo f 5' CTT GAA GGG ACT CGA CAA GG 3'	504
	exo r 5' TTC AGG TCC GCG TAG TGA AT 3'	
nan1	nan1 nan f 5' AGG ATG AAT ACT TAT TTT GAT 3'	1316
	nan r 5'TCA CTA AAT CCA TCT CTG ACC CGA TA	

Table 1: Specific primers *oprI, oprL, toxA, exoS, nan1* and *lasB*.

Twelve (40%) of the isolates were isolated from community acquired infections (CAI) and 18 (60%) isolates were obtained from hospital acquired infections (HAI). The isolates were then divided mainly into four genetic groups depending on the presence of virulence genes and the relation between these groups and the type of infections (Tables 2 and 3) (Figures 1 and 2). This study shows that the presence of two or three virulence genes is significantly higher among nosocomial infections than community acquired infections (P<0.001). However, most of the isolates from CAI do not contain any virulence genes or may harbor only one virulence gene.

Discussion

Identification of *P. aeruginosa* has traditionally relied on phenotypic methods. This still is the most accurate standard when dealing with typical isolates of *P. aeruginosa*. In cystic fibrosis (CF) patients, *P. aeruginosa* isolates display unusual phenotypic reactions. Moreover, biochemical testing takes long time to perform and requires extensive hands-on work by the technologist, both for setup and for ongoing evaluation. Molecular methods have been reported to be superior to the phenotypic methods for identification of *P. aeruginosa* [5,12] by designing a multiplex PCR assay based on *oprI* and *oprL* genes for molecular detection of *P. aeruginosa* showed that the specificity and sensitivity of the PCR assay were 74 and 100%, respectively. Lavenir [13] also noted that all of *P. aeruginosa* strains contained the **oprI** and *oprL* genes (sensitivity=100%, specificity=80%). Similarly in this study, all of the 30 isolates were remarkably positive for both *oprI* and *oprL* genes.

Pathogenicity of *P. aeruginosa* is clearly multifactorial. *LasB* is one of the most important proteases of *P. aeruginosa* [14]. In this study all isolates examined harbored *lasB* gene. This finding is in agreement with previous reports [10]. Mutation of *lasB* gene reduces markedly *P. aeruginosa* invasion. Prevalence of the *lasB* gene in all the environmental and clinical isolates implies the importance of *LasB* factor to survival of *P. aeruginosa* in various settings [15] *P. aeruginosa* isolates generally express cytotoxicity or invasion phenotypes which is correlated with presence of *exoU* (encoding exotoxin U) or *exoS* (encoding exotoxin S) respectively. In our study difference between *exoS* and *tox* prevalence in the isolates from pulmonary tract, burn and blood infections was significant (P<0.1) (Table 1). About the *nan1* gene, the other virulence factor studied in this research, we found that the prevalence of *nan*1 was significantly higher in isolates from pulmonary tract and burn than isolates from blood (P<0.1). These results correlate with those studied by Nikbin [10] and Lanotte [11] *nan1* gene has probably a role in CF
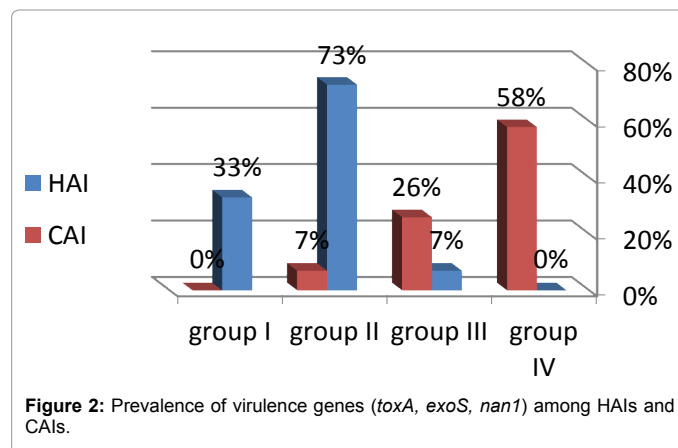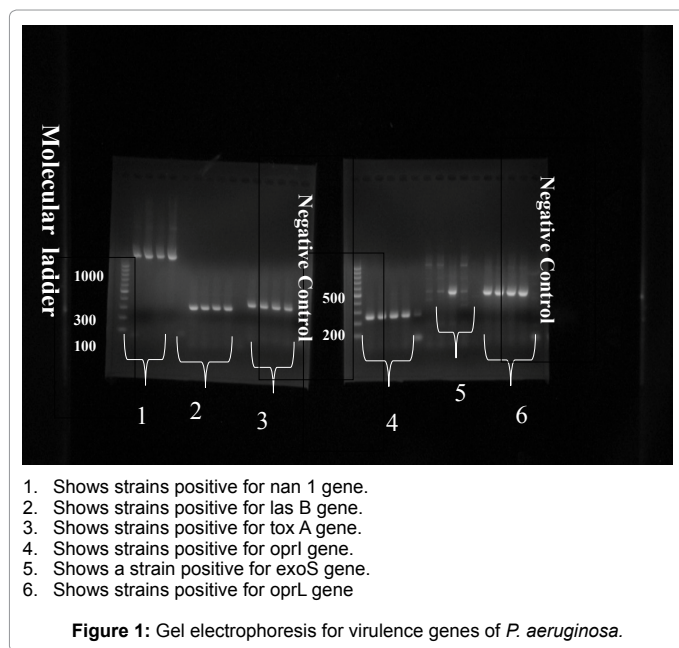
Virulence gene	Samples			P value (chi square)
	Blood % (n=10)	Burn % (n=10)	Pulmonary tract % (n10)	
oprI	100% (10)	100% (10)	100% (10)	
oprL	100% (10)	100% (10)	100% (10)	
LasB	100% (10)	100% (10)	100% (10)	
Nan1	Zero (zero)	40 % (4)	40% (4)	0.06 (5.45)
ExoS	10% (1)	70% (7)	60% (6)	0.015 (8.30)
tox	20% (2)	100% (10)	70% (7)	< 0.001 (14.06)

Table 2: Prevalence of virulence genes among *P. aeruginosa* obtained from various sources.

Genetic groups	HAI (18) n (%)	CAI(12) n (%)	P value
Group I	6 (33%)	zero	P < 0.001
Group II	11(73%)	1 (7%)	P < 0.001
Group III	1(7%)	4(26%)	P < 0.001
Group IV	zero	7(58%)	P < 0.001

group I: presence of *toxA, exoS and nan1*
group II: presence of any 2 virulence genes
group III: presence of only one virulence gene
group IV: non- existence *toxA, exoS and nan1*

Table 3: Prevalence of virulence genes among *P. aeruginosa* among HAIs and CAI.

1. Shows strains positive for nan 1 gene.
2. Shows strains positive for las B gene.
3. Shows strains positive for tox A gene.
4. Shows strains positive for oprI gene.
5. Shows a strain positive for exoS gene.
6. Shows strains positive for oprL gene

Figure 1: Gel electrophoresis for virulence genes of *P. aeruginosa.*

Figure 2: Prevalence of virulence genes (*toxA, exoS, nan1*) among HAIs and CAIs.

pulmonary disease evolution as previously described Lanotte [11]. The low prevalence of this factor among isolates from blood infections may show that the role of this gene in the blood infections is less important than burn and pulmonary tract infections. The differences in the distributions of virulence factor genes in the populations strengthen the probability that some *P. aeruginosa* strains are better adapted to the specific conditions found in specific infectious sites, and thus virulence genes expression differs according to site and severity of infection [10].

This study also showed that *P. aeruginosa* isolates are more virulent in HAIs than that in CAIs. Most of the isolates that were isolated from HAIs contain either three or two virulent genes while most of the isolates that were isolated from CAIs do not contain any virulent gene. These results correlate with those proved by Bradbury [16], who proved that *P. aeruginosa* isolated from nosocomial infections were found to have an increased prevalence of virulence genes. Most of the HAIs isolates were collected from Intensive care units (ICU) and the ICU environments are subject to more stringent disinfection protocols

than other environments within the hospital, which may facilitate and promote the acquisition of virulent genes by horizontal transfer between nosocomial strains of *P. aeruginosa*. It may be speculated that acquisition of other resistance mechanisms which may assist survival in the ICU environment might also positively modify the virulent genes acquisition rate [16].

In conclusion, it seems that simultaneous use of *oprI*, *oprL* and *LasB* genes provides more confident detection of *P. aeruginosa* by PCR. Determination of different virulence genes of *P. aeruginosa* isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity. This may have different consequence on the outcome of infections. Significant correlations between some virulence genes and source of infections obtained in this research indicate implementation of infection control measures will help in controlling the dissemination of virulence genes among *P. aeruginosa* isolates.

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