VIRULENCE FACTORS OF PSEUDOMONAS AERUGINOSA STRAINS ISOLATED FROM CLINICAL SAMPLES AND ROLE OF QUORUM SENSING SIGNAL MOLECULES IN THE PATHOGENESIS OF THE DISEASE

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ABSTRACT

Introduction*: Pathogenicity of Pseudomonas aeruginosa depends on multiple cell-associated factors and virulence factors including "Quorum sensing (QS)" molecules. QS system enables the bacterium to determine the population density around it and the bacterium uses this data to control the regulation of many of its genes. The aim of this study was to determine whether there was QS insufficient subspecies infective for humans, and to identify the relations between specific infection types and virulence factors.*

Materials and methods*: A hundred P. aeruginosa strains isolated from different clinical specimens were used in our study. Quantitative measurements of elastase, alkaline protease, pyocyanin, and biofilm formation were done. Strains were investigated to produce long and short chain signal molecules taken part in QS system.*

Results*: Elastase activities of the subspecies isolated from sputum and blood specimens were detected higher than those from other localizations. Pyocyanin values of the urine specimens were detected higher than those of blood, wound and sputum isolates. Biofilm formation level of the urinary isolates was significantly higher than the other isolates. All the subspecies produced long chain signal molecule, whereas 29 isolates produced short chain signal molecule. Productions of elastase, alkaline protease and pyocyanin in the short chain signal molecule producing strains were higher than those cannot produce.*

Conclusion*: These results suggested that the Quorum sensing could make the strains much virulent, and could play an important role in pathogenicity. To date, the QS system has not been clearly identified yet for many microorganisms, however, the agents targeting the QS system may be therapeutic alternatives in the treatment and prophylaxis of infections in the future.*

Key words*: Pseudomonas aeruginosa, Quorum sensing, pathogenicity, Virulence factors.*

Received *November 30, 2014;* ***Accepted*** *May 02, 2015*

Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen and a major cause of nosocomial infections. The pathogenicity of P. *aeruginosais* is multifactorial and associated with cell-related alginates, pili and lipopolysaccharides as well as extracellular virulence factors such as proteases, haemolysins, toxins and pyocyanins[(1-3)].

The adaptation of the bacteria to a new environment and their recognition and perception response to this environment is important in pathogenesis. The "Quorum sensing" (QS) system enables the bacterium to determine the population density around it and the bacterium uses this data to control the regulation of many of its genes[(4, 5)]. The expression of most of these virulence factors is made possible by two N-acyl-L-homoserine lactone (AHL) mediated intercellular communication systems[(2, 3)].

Intercellular communication occurs with the mediation of small AHL molecules called autoin-

ducers. P. *aeruginosa* has at least two systems such as las and rhl systems that relate to a well-defined QS system. These systems control the production of various virulence factors such as elastases (LasB and LasA), alkaline protease, pyocyanin, and superoxide dismutase[6-9]. Each QS system consists of two compounds known as transcriptional regulators (LasI and RhlI), which synthesize the signal molecules called "autoinducers" and which come from the same origin as these[6, 7].

Since the QS system controls various virulence factors, when one or both of these systems are lost, the capability of P. aeruginosa to cause infection in humans can be reduced considerably. This study aims at describing the QS-deficient strains that cause infections in humans, whether or not such strains exist and the relationships between specific infection types and virulence factors.

Material and Methods

A total of 100 P. *aeruginosa* strains were isolated from 30 urine, 21 blood, 19 wound, 16 sputum, 7 ear discharge and 4 catheter samples that were taken from patients hospitalized in the study. Their identification was carried out in the microbiology laboratory using the API ID 32E (BioMerieux, France) automatic system and conventional methods. Six colonies from each isolate that had been proliferated in the Laura Bertani (LB) agar were incubated in a 5 ml LB broth medium for 18 hours at 37oC for proliferation. All culture suspensions were standardized by adjusting them to 2.0 absorbance value at 540 nm. One ml of each suspension was taken and added into a 30 ml LB broth medium and incubated for 18 hours at 37oC. Then, they were centrifuged for 20 minutes at 4oC and 10000 g to set aside supernatants for enzyme measurements[10-12].

Pyocyanin assay

Pyoncyanin was measured by way of finding the absorbance values in an acidic solution at 520 nm wavelength[10].

Total protease assay

The proteolytic activity was measured using the method described by Lanotte et al.[11].

Elastase assay (Elastin Congo Red Trial)

The elastolytic activity in the supernatants that were obtained from the isolates was measured using the elastin congo red trial. P. aeruginosa PAO1 strain was used as positive control[12].

Biofilm measurement

They were standardized by being adjusted to 0.02 (OD 600) absorbance with an M 63 minimal medium after a night's incubation in a LB broth medium. One ml of each suspension was put into a polystyrene tube of 12 x 75. After incubated for 10 hours at 30 oC, the content of each tube was poured out slowly and the tubes were gently washed with distilled water. One ml of 1% crystal violet was put into the tubes. Staining continued for about 15 minutes. They were then washed again slowly with distilled water. 4 ml of 95% ethanol was poured into the tubes covered with stained biofilm to dissolve the biofilm layer. They were then taken to spectrophotometer vessels. Measurements were carried out at 495 nm wavelength.

Signal molecule assay

All strains were proliferated in LB agars. AHLs were identified using both a *Chromobacterium violaceum* strain CV026 and an *Agrobacterium tumefaciens* strain NT1[13-16].

Parallel Drawing Method for AHL Detection

The proliferation of AHLs was examined by observing pigment formation following an overnight incubation of the study strains and the CV026 and NT1 strains that were inoculated in parallel to the study strains. To test with the A. *tumefaciens* NT1 indicator strain, 50 μg/mL of X-Gal (5-Bromo-4-chloro-3-indolyl-β-Dgalactopyranoside) and 20 μg/ml of Gentamicin were added into the LB agar medium. The strains to be tested were incubated overnight at 30°C.

Statistical analysis

The statistical analysis was carried out on the software Statistical Package for the Social Sciences (SPSS) 6.0 for Windows 95 (IBM SPSS, Chicago, IL, United States of America) using the student's t test, Mann-Whitney U test, Spearman's correlation analysis and multiple regression analysis. Statistical significance was set at $p<0.05$.

Results

The elastase activity of the P. *aeruginosa* PAO1 strain used as positive control (0.973±0.0145) was found higher than that of the

strains in the study group. The elastase activities of the strains obtained from sputum and blood samples (0.499±0.35 and 0.46±0.396, respectively) were higher than those of the strains from other locations, but this difference was not statistically significant ($p>0.05$).

The alkaline protease activity was significantly higher in the P. *aeruginosa* PAO1 strain that was used as positive control (0.616±0.0197) than in the strains of all other regions ($p<0.05$). When the alkaline protease enzyme activities of all locations were compared with each other, no statistically significant difference was observed ($p>0.05$).

While the spectrophotometric measurement of pyocyanin production showed that it was significantly higher in the P. *aeruginosa* PAO1 strain (0.147±0.0025) than in sputum and blood isolates ($p<0.05$), the urine pyocyanin level was found close to that of the standard strain. The pyocyanin value of urine samples (0.0691±0.0654) was higher than those of the blood, wound and sputum isolates (0.0656±0.0614, 0.0488±0.0415 and 0.0492±0.063, respectively) and this difference was statistically significant ($p<0.05$).

When biofilm formation was examined, it was found significantly higher in the control strain P. *aeruginosa* PAO1 (0.845±0.0476) than in the blood and sputum isolates (0.414±0.265 and 0.403±0.447, respectively) ($p<0.05$). However, when the biofilm formations in urine, catheter, wound and tracheal aspirate were compared to that in the P. aeruginosa PAO1 strain, the difference was not significant ($p>0.05$). When the groups were compared within themselves, the level of biofilm formation in the urine isolates (0.719±0.441) was found significantly higher than the values in the blood, wound and sputum isolates (0.414±0.265, 0.467±0.31 and 0.403±0.447, respectively) ($p<0.05$).

The C. *violaceum* CV026 and A. *tumefaciens* NT1 indicator strains were used to observe the AHL production in all test strains and those that gave positive results were recorded. While all strains produced the long chain signal molecule, 29 of the strains produced the short chain signal molecule (Table 1). The elastase and alkaline protease values of the strains that produced short chain signal molecules were significantly higher than those of the strains that did not produce or produced small amounts of them. The pyocyanin values were also higher, but the difference was not statistically significant.

	CV026*		NT1**	
	+	-	+	-
Number of strains	29	71	100	0

Table 1: Response rates of short and long chain signaling molecules to NT1 and CV026 indicators in P. *aeruginosa* strains (n = 100).
**The standard Chromobacterium violaceum strain, **The standard Agrobacterium tumefaciens strain.*

Contrary to these, the biofilm values were lower in those that produced short chain signal molecules (Table 2). Correlation analyses were carried out between the levels of elastase, alkaline protease, pyocyanin and biofilm formation in all stains. There was a statistically significant and positive correlation between the elastase, alkaline protease and pyocyanin values. When a correlation analysis was performed between biofilm formation and the levels of elastase alkaline and protease, interestingly, a statistically significant and negative correlation was found. No significant relationship was found in the correlation analysis between pyocyanin and biofilm formation.

Signaling molecule	Elastase	Alkaline protease	Pyocyanin	Biofilm
Positive (n:29)	0.529 ± 0.304	0.389 ± 0.149	0.063 ± 0.041	0.409 ± 0.404
Negative (n:71)	0.315 ± 0.312	0.267 ± 0.184	0.0699 ± 0.079	0.606 ± 0.407
P value*	< 0.05	< 0.05	> 0.05	< 0.05

Table 2: Production levels of virulence factors in P. aeruginosa strains in samples positive and negative for signaling molecules.
** Levels of elastase, alkaline protease, and biofilm were found significantly higher in the samples positive for signaling molecule than the negative ones.*

Discussion

It was shown under in-vitro conditions that the QS system regulated the virulence-related genes in P. *aeruginosa*. For example, the Las system is responsible for the synthesis of exoenzymes such as elastase and alkaline protease, the production of exotoxin A and the formation of biofilm as well as for the formation of secretion apparatus, catalase, haemolysin and siderophore. The Rhl system, on the other hand, controls the production of elastase from exoenzymes and the formation of lectin, hydrogen cyanide, rhamnolipid, siderophore

and secretion apparatus[17, 18]. The effect of losing more than one virulence factor simultaneously on the colonization and reproduction capability of P. *aeruginosa* in various regions is still unknown. This possibility may arise if P. *aeruginosa* has a defect in a major regulator of multiple virulence factors such as QS systems.

We found the elastase and protease activities high in our study especially in the blood and sputum samples and this seems to support the data on the role of these enzymes in invasion in systemic infections. Similarly, we found the elastase and alkaline protease levels high in our study particularly in the strains that produced short chain signal molecules, indicating the role the QS system plays in virulence, pathogenicity and invasion capabilities.

Production of alkaline protease was found in sputum, wound, blood and urine samples in our study. However, values close to each other were obtained with respect to body regions. To further clarify this issue, broad series of studies need to be conducted.

A study carried out by Döring et al.[19] reported that protease production was 93% in P. *aeruginosa* isolates that were isolated from patients with chronic lung disease.

In a study by Tunc and Yagci, protease production was found in 96% of the P. *aeruginosa* strains that were isolated from various clinical samples[20]. The highest alkaline protease values were found in the sputum samples also in our study, but the difference was not significant ($p>0.05$).

Our results showed that the pyocyanin and biofilm levels in the strains obtained from the urine samples were higher than in those obtained from other body regions. Although most of the strains did not produce short chain signal molecules, they were able to produce virulence factors such as pyocyanin and biofilm formation. In P. aeruginosa, production of both las B and PCN is controlled by QS. Similarly, QS is also required for biofilm formation. The PCN and biofilm initiation mechanism in urine samples despite insufficient QS mechanisms is still unknown. Along with these factors, an investigation of the production of factors including rhamnolipids, alkaline phosphatises and lectins that are controlled by QS will play a key role for understanding such a mechanism.

It was reported in a study conducted by Tinaz et al. that 50 P. *aeruginosa* strains did not give a reaction to the C. *violaceum* CV026 strain, but gave a positive reaction to the A. *tumefaciens* NT1 indicator strains of all origins[16]. In our study, we observed short chain production in 29 of 100 P. *aeruginosa* strains. The lack of response to the CV026 strain in most of the strains suggests that there were no or very few short chain AHL molecules. Our results indicate that more complicated systems may be in action in the production of P. *aeruginosa* virulence factors during infections. The strains we proliferated are those that cause infections in a hospital environment and these strains usually produce virulence factors in considerable amounts. We found long chain signal molecules in all of these strains. However, we also found short chain signal molecules in 29 of the strains. This suggests that either the virulence factors are controlled by the QS system loosely or there are other control mechanisms other than QS that play a role or other factors that are induced in vivo and not yet discovered. If the QS system was the main cause of infections, most of the isolates would not have been QS defective. On the other hand, if the virulence factors that are not controlled by QS can compensate the loss of those that are QS dependent, then the QS defective strains will be able to cause infections.

Our results showed that elastase activity was slightly higher in blood and sputum isolates, although this was not statistically significant ($p<0.05$). Similarly, the alkaline protease levels were higher in blood, sputum and wound samples.

However, production of pyocyanin and biofilm formation was significantly higher in our urine samples than in blood, sputum or wound samples ($p<0.05$).

Biofilm formation was high in ear discharge, catheter and tracheal aspirate samples, close to the values in urine samples. However, we worked on a small number of isolates in these samples. For sound results and interpretations, we need studies on a larger number of samples.

While all of the strains produced long chain signal molecules, 29 isolates produced short chain signal molecules. The elastase and alkaline protease values of the strains that produced short chain signal molecules were significantly higher than those that did not produce them or produced them in small amounts. The pyocyanin values were also higher, but this was not statistically significant.

Since the QS system enables bacteria to determine the density of the population of the bacteria of the same species around them and consequently

leads to the expression of the genes that code the virulence factors, it plays an important role in the virulence and pathogenicity of the pathogens that are capable of invasion[21]. The regulative role of the QS system and the production of AHL should be considered important as these may play a multiple role in the pathogenesis of P. *aeruginosa*. Although the QS system has not been resolved yet for many pathogens, the therapeutic agents targeting the QS system may be an alternative solution in the future in the treatment and prophylaxis of infections.

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