Association between biofilm formation of *Pseudomonas aeruginosa* clinical isolates versus antibiotic resistance and genes involved with biofilm

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ABSTRACT

*Pseudomonas aeruginosa* is the most common Gram-negative bacteria and causes nosocomial infections among hospitalized patients, especially the immune compromised or patients associated with indwelling devices. These bacteria are susceptible to a wide variety of currently available antibiotics. One of the antibiotic-resistant mechanisms that the bacteria employ is the formation of biofilms, adding another degree of resistance to deleterious agents such as antibiotics, sanitizers and disinfectants. A biofilm is a community of cells attached to either a biotic or an abiotic surface and enclosed in a complex exopolymeric substance. This study’s aim was to measure biofilm production and its association with antibiotic resistance among the clinical isolates of *P. aeruginosa*. The results of biofilm production were shown for 108 isolates (79.4%). In this study, we found that antibiotic resistance was higher among biofilm producing *P. aeruginosa* than among non-producers. Only 21.4% had the *pqsA* gene, which significantly correlated with biofilm formation (P = 0.009). Our investigations suggested that the *pqsA* gene could be a candidate for screening bacteria that form biofilms and that this can be detected in clinical isolates. Bacteria-producing biofilms enhance the spread of antibiotic resistance, which can lead to an accumulation of virulence genes, further complicating treatment options. A new therapeutic strategy could be a co-treatment approach that combines traditional antibiotics with a substance that interferes with biofilms, rendering them more susceptible to treatment.

**Keywords:** biofilm, *Pseudomonas aeruginosa*, antibiotics, antibiotic resistance, *pqsA*

INTRODUCTION

*Pseudomonas aeruginosa*, a Gram-negative bacterium, is an important opportunistic bacterial pathogen that causes nosocomial infections [1, 2]. The development of antimicrobial resistance plays a crucial role in the health care system. The capability of *P. aeruginosa* to form biofilms is a key requirement for its chronic colonization of human tissues. The biofilms composed of sessile populations of microorganisms that are surrounded by a slime layer can be attached to biotic or abiotic surfaces[3]. Infections of *P. aeruginosa* associated with biofilm development are more common in immunocompromised patients and in patients with implanted medical devices in the lungs, the middle ear, as well as patients with contact lenses, catheters and other implants. The biofilm can protect all living bacteria, allowing them to achieve a high level of antibiotic resistance, stealth and invisibility. Biofilms not only provide a physical barrier to antimicrobial agents and host immune responses but also facilitate the exchange of antibiotic-resistant genetic material between organisms and may also contain antibiotic degrading enzymes such as β-lactamase[4]. Thus, biofilms render pathogenic microorganisms enormously difficult to eradicate and contribute to localized or systemic inflammation, which prolongs wound healing. Biofilms are associated with cell to cell
interactions by generating an extracellular polymeric substance (EPS) matrix. In addition, biofilms have reduced growth rates and exhibit up and down regulation of specific genes[5, 6]. The regulation of the formation and development of the biofilms is dependent on the surface area. Attachment is a complex process that is regulated by various characteristics, namely, the growth medium, substratum and the cell surface. The pathogens living inside the biofilm communicate with each other by aphenomenon called quorum-sensing. The presence of the quorum-sensing signal has been shown to be mediated by the emitting of a chemical message. These molecules display the significant signals needed for biofilm formation, bacterial motility, plasmid transfer and virulence[7]. The quorum-sensing system produces two distinct chemical classes of signal molecules:N-acyl homoserine lactones (AHLs) and 4-quinolones. Synthesis of the pseudomonas quinolone signal (PQS) depends on the pqsABCDE locus, which is responsible for generating multiple 4-quinolones[8]. The first step of the 4-quinolones synthesis pathway is the generation of the pqsA gene product, and PQS can induce virulence which includes rhamnolipids, LecA, and pyocyanin[9, 10]. Previous studies suggested an important role for the psl gene cluster in the initiation of biofilm formation in P. aeruginosa. The psl operon contains 15 genes (pslA-O) that are involved in the synthesis of the exopolysaccharide (EPS) that is important for P. aeruginosa biofilm formation[2, 3]. The sugar composition has been found to be 27% galactose and 6% mannose, referred to as the galactose-mannose rich EPS[11, 12]. The pslA gene usually the first gene of this cluster. The previous study compared a pslA mutant and a wild type strain of P. aeruginosa (PAO1) in their attachment and biofilm formation. The mutant exhibited 30% less attachment to tissue culture plates[13]. The pslHand pslic encoded for the proteins of galactosyl-transferases and mannose-transferase. The mutant strain, having lost the pslH or the pslI genes, had defective attachment, but the wild type strain rapidly attached. In addition, data supporting this conclusion showed that the pslH and psli gene mutants exhibited a decrease in biofilm formation[14]. The PslDgene exhibited a similarity of 72% to secreted proteins from the genomes of various Pseudomonas spp., and has been shown to be required for biofilm formation, presumably via its role in exopolysaccharide export[15]. Another gene, the pslB gene, is also required for exopolysaccharide synthesis and biofilm formation[16]. The production of biofilm formation by P. aeruginosarequires or involves several gene expressions. Another important gene for biofilm formation, development and maintenance is the pel gene, which is composed of seven gene operons (pelA-pelG). This gene is involved in pellicle and biofilm production, both of which are surrounded by an extracellular matrix[17, 18]. The objectives of this study are to investigate the biofilm formation ability of different P. aeruginosa clinical isolates, found to be resistant to many types of antibiotics, and to investigate which genes are associated with this biofilm formation.

EXPERIMENTAL SECTION

Bacterial strains
One hundred and thirty-six strains of clinically isolated Pseudomonas aeruginosa were collected from Rajavithi Hospital Bangkok of Thailand. The four types of specimens used were: blood (4136), urine (35136), sputum (57136) and pus (40136). Biochemical tests were performed to confirm the presence of P. aeruginosa using Gram staining, oxidase testing, carbohydrate utilization and motility. P. aeruginosa was cultured overnight on Trypticase soy agar (Oxoid) at 37°C.

Antimicrobial susceptibility testing
The disk diffusion susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[19]. Antibiotic discs used in this study included ciprofloxacin (CIP), levofloxacin (LEV), ceftazidime (CAZ), amikacin (AK), gentamicin (GN), cefepime (FEP), imipenem (IPM), meropenem (MEM), cefoperazone/sulbactam (SCF), pipercillin/tazobactam (TZP) and colistin (CL), and all were purchased from Oxoid (UK). The antibiotic discs were stored at -20°C and placed at room temperature prior to use. A single colony of P. aeruginosa was subcultured overnight on Mueller Hinton agar plates (Oxoid) at 37°C. The bacterial suspension was used as the inoculum at a McFarland no. 0.5 or 1.5 x 10⁸ colony-forming units (cfu)/mL on MHA plate. After an overnight incubation at 37°C, zones of inhibition or clear zones were measured and compared to the CLSI guidelines[19]. The results from the methods were validated using the American Type Culture Collection quality control strain P. aeruginosa ATCC 27853. Multidrug resistance (MDR) was defined as resistance to at least three of six drugs, including amikacin, gentamicin, ciprofloxacin, pipercillin, ceftazidime and imipenem, as described by the SENTRY surveillance program[20, 21].

Preparation of samples and DNA amplification of the pelA, pqsA, pslA, pslDand pslH genes
The chromosomal DNA extraction of P. aeruginosa, used as a DNA template, was prepared by heat lysis of the bacterial cell lysates at high temperatures as described previously[22]. The test strains were cultured overnight on TSA plates at 37°C and adjusted in 1.5 mL of Trypticase soy broth (TSB) at the McFarland no. 2 (6 x 10⁶ cfu/mL). The crude lysate was boiled at 90 to 100°C for 10 min and the supernatant was collected by centrifugation at 12,000 rpm for 10 min. The DNA template was precipitated by ethanol and washed 2 times with 70% ethanol. The DNA pellet was dried for 30 min and resuspended in TE buffer (pH 7.5). Polymerase chain reaction...
(PCR) assays were performed to amplify the biofilm associated genes. These reactions were carried out in 25 µL volumes containing 0.625 U of recombinant Taq DNA polymerase (Fermentus, USA), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, primers at 1 µM each, and 0.5 µL of bacterial template. The oligonucleotide primers were listed in Table 1. PCR assay (Ward medic, Singapore) conditions for all these genes were 5 min at 94°C; 35 cycles of 30 s of denaturation at 94°C, 40 s of annealing at 52°C and 50 s of extension at 72°C. The amplified products were held at 4°C until analysis.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Oligonucleotide</th>
<th>Product length (bp)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pelA</td>
<td>PelA-F</td>
<td>5'- CCTTCAGCCATCCCCTCTC-3'</td>
<td>118</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>PelA-R</td>
<td>5'- TCGGCTACGAAGTGCGATTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pqsA</td>
<td>pqsA-F</td>
<td>5'- CCCGATACCGCCGTTTATCT-3'</td>
<td>448</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pqsA-R</td>
<td>5'- ACCCGAGGGTGTATTGCAAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pslA</td>
<td>PslA-F</td>
<td>5'- TGGGTCTTCAAGTTCCGCTC-3'</td>
<td>119</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PslA-R</td>
<td>5'- ATGCTGGTCTGCGGATGAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pslD</td>
<td>PslD-F</td>
<td>5'- CTCATGAAACGCACCCTCGT-3'</td>
<td>295</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PslD-R</td>
<td>5'- TGCCACCGAYGAAGCGGATAG-3'</td>
<td></td>
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</tr>
<tr>
<td>pslH</td>
<td>PslH-F</td>
<td>5'- CAGATGCTCTGGTCCTGGAGT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PslH-R</td>
<td>5'- GGAACGAAGCCTTGCCATT-3'</td>
<td>719</td>
<td>This study</td>
</tr>
</tbody>
</table>

Microplate biofilm formation assay
The test strains were adjusted to 10⁶ cfu/mL by dilution in fresh MHB. Two hundred µL of the samples were added to each well of a 96-well microplate (Nunc, Denmark), and incubated at 37°C for 24 h. The cell suspension was removed, and then subsequently washed with tap water for a total of 3 times. Two hundred µL of 0.1% w/v crystal violet solution was added to each well and incubated at room temperature for 10 minutes. After incubation, the non-bound dye was removed by washing with tap water for a total of 3 times. Two hundred µL of absolute ethanol was added to each well and was shaken for 30 min at 150 rpm at room temperature. The absorbance of dissolved dye was measured at a wavelength of 590 nm [24]. Each assay was performed in triplicates. Biofilm measurements were calculated using the formulation SBF=(AB-CW)/G, in which SBF is the specific biofilm formation, AB is the OD₅₇₀nm of the attached and stained bacteria, CW is the OD₅₇₀nm of the attached and stained control wells containing only bacteria-free medium (to eliminate the unspecificity of the abiotic OD value), and G is the OD₆₃₀nm of cell growth in broth [25]. The level of biofilm production was classified into four categories: negative (SBF ≤ 0.35), weak (0.35 > SBF ≤ 0.79), moderate (0.79 > SBF ≤ 1.10) and strong (SBF > 1.10).

Statistical analysis of data
Pearson’s X² test was used to evaluate the statistical significance of the differences in these results. In cases where the test of homogeneity had an expected count of less than five, Fisher’s exact test was applied. A p-value of < 0.05 was considered as statistically significant. All statistical evaluations were performed with the SPSS 22 software.

RESULTS AND DISCUSSION

Antimicrobial susceptibility testing and biofilm formation
P. aeruginosa isolates from 136 clinical specimens, including blood (2.9%), urine (26.5%), pus (30.9%) and sputum (44.1%), were used for these experiments. The results of the antimicrobial susceptibility testing by the disk diffusion method showed that all isolates were susceptible to colistin, whereas other antibiotics exhibited various susceptibility rates. The antibiotics levofloxacin and ciprofloxacin showed high resistance rates of 50.7% and 50.0%, respectively. The results from ceftazidime, a third-generation cephalosporin, showed a resistance rate of 46.3%. The antibiotics combined with a β-lactam inhibitor, mainly cefoperazone/sulbactam and piperacillin/sulbactam, showed resistance rates of 40.4% and 38.2%, respectively. Similarly with the carbapenem class of antibiotics, meropenem and imipenem presented with resistance rates of 36.0% and 33.1%, respectively. The resistance rates to other antibiotics were as follows: gentamicin (27.2%), ceftazidime (23.5%) and amikacin (13.2%). Biofilms were cultivated by using static culture. The amount of biofilm was measured by crystal violet staining. P. aeruginosa from clinical isolates were found to contain 79.4% biofilm producers and 20.6% non-biofilm producers. Antibiotic resistance patterns such as amikacin, gentamicin, ceftazidime, cefepime, imipenem, meropenem, cefoperazone/sulbactam and piperacillin/sulbactam, were higher among biofilm-producing P. aeruginosa than non-producers. However, levofloxacin and ciprofloxacin were found to exhibit similar resistance in both biofilm producers and non-producers. Resistance patterns of biofilm-producing and nonproducing P. aeruginosa are shown in Figure 1.
This study classified the level of biofilm formation into 4 categories by using the SBF index. The collection of 136 *P. aeruginosa* strains was divided into strong biofilm producers (60%), negative biofilm producers (22%), moderate biofilm producers (11%) and weak biofilm producers (7%) (Figure 2).

The biofilm, produced by *P. aeruginosa* from clinical specimens, was shown in samples isolated from blood (100%), urine (88.6%), sputum (73.7%) and pus (77.5%). All antibiotic resistant strains, except to colistin, produced the biofilm at rates of more than 80% (81.1% to 86.8%) (Figure 3).
Figure 3. Percentage of antimicrobial resistance for 136 of *P. aeruginosa* clinical isolates tested with the following antibiotics: amikacin (AK), gentamicin (GN), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM), meropenem (MEM), levofloxacin (LEV), ciprofloxacin (CIP), cefoperazone/sulbactam (SCF), piperacillin/tazobactam (TZP), and colistin (CL). The black bar represents the percentage of resistant strains producing biofilms.

In the case of the biofilm-producing strains, these results found a > 50% resistance to ceftazidime (52.8%), levofloxacin (51.9%), ciprofloxacin (51.9%) and cefoperazone/Sulbactam (55.6%).

**Detection and association of the pelA, pqsA, pslA, pslD and pslH genes with biofilm formation in *P. aeruginosa* clinical isolates**

All of the five genes tested were involved in biofilm formation, which is implicated in adherence to surfaces, micro-colony formation, macro-colony formation and dispersion. This study showed a trend among the clinical isolates of *P. aeruginosa* to form a biofilm (79.4% (108/136)). The results also detected that the pelA gene is expressed heavily (97%), which is importantly associated to the polysaccharide stage of biofilm development and maintenance. Polysaccharide Synthesis Locus (psl gene) plays a significant role in the surface attachment and is normally expressed in *P. aeruginosa*. Almost all strains have been found to express pslA (94%), pslD (95%) and pslH (95%). However, the pqsA gene has been shown to be expressed in 39% of bacteria, and this is related to the development of a complex cell-to-cell communication system by the 3,4-dihydroxy-2-heptylquinoline (PQS) produced in infected cells. The non-biofilm producing strains (28/136) were similarly found to express pelA, pslA, pslD and pslH genes at 92.9%. The exception was that the pqsA gene was found only in 21.4% of bacteria; indicating that pqsA is significantly associated with biofilm formation (P = 0.009) (Table 2).

<table>
<thead>
<tr>
<th>Gene involvement</th>
<th>No (%) of <em>P. aeruginosa</em> isolates (136)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biofilm former (n=108)</td>
<td>Biofilm non-former (n=28)</td>
</tr>
<tr>
<td>pelA</td>
<td>107 (99.1%)</td>
<td>26 (92.9%)</td>
</tr>
<tr>
<td>pslA</td>
<td>103 (95.4%)</td>
<td>26 (92.9%)</td>
</tr>
<tr>
<td>pslD</td>
<td>106 (98.1%)</td>
<td>26 (92.9%)</td>
</tr>
<tr>
<td>pslH</td>
<td>106 (98.1%)</td>
<td>26 (92.9%)</td>
</tr>
<tr>
<td>pqsA</td>
<td>53 (49.1%)</td>
<td>6 (21.4%)</td>
</tr>
</tbody>
</table>

The level of biofilm production, classified by the SBF index, was present in similar numbers for all five genes associated with biofilm formation (Figure 4). Approximately 70% of the bacteria positively expressing each gene exhibited strong biofilm formation.
DISCUSSION

Previous studies have shown that the antibiotic resistance of bacteria due to biofilm formation contributes to the persistence of bacterial cells and causes problems in the complete eradication of infection [26]. The structure of biofilms is increasingly recognized as a crucial factor in the persistence of several infections. Chronic infections have been remarkably demonstrated to involve biofilm production, especially those infections associated with indwelling devices such as catheters and prostheses [27]. The ability of the biofilm to contribute to bacterial protection is widely different among microbes. Biofilms not only contribute to the resistance mechanisms against broad spectrum antibiotics but also against host immune systems. The antibiotic susceptibility of biofilm-producing bacteria is reduced because of a restricted antibiotic penetration, an adaptive response and the presence of persisting cells [28]. Therefore, this study was aimed at finding a clear association between antibiotic resistance and biofilm formation, in both phenotypic and genotypic characteristics. The formation of a biofilm is a multi-stage process that is initiated by the surface attachment of planktonic bacteria to form a monolayer, followed by aggregation leading to the formation of microcolonies, maturation to form mushroom-shaped structures and dispersal [27]. The biofilm formation is accompanied by drastic changes in gene regulation. The formation of microcolonies in *P. aeruginosa* has been attributed to many factors. These include: type IV pili, flagella, free DNA, alginate and Pel and Psl polysaccharides. Even if one of the factors is not functioning, the biofilm is still able to perform well [29]. In this study, we tested the expression of the following genes: *pelA* (97.8%), *pslA* (94.9%), *pslD* (97.1%) and *pslH* (97.1%). These genes were found in almost all clinical strains of *P. aeruginosa*, but not all of the above genes could contribute to the production of the biofilm. The presence of these genes could not predict which strains will produce a biofilm because the biofilm formation is affected by many factors. Interestingly, the *pqsA* gene was found only in 43.4% (59/136) of bacteria, and most of them were able to produce a biofilm layer. However, the non-biofilm producers carried this gene in only 21.4% (6/28) of bacteria. This was a clear difference from all the other genes. We predict that the *pqsA* gene can be used as a candidate for the screening of biofilm producers, and we have shown that the specificity was 78.57%. The conventional antibiotic susceptibility test cannot predict the bacteria involved in biofilm production. In this technique, the concentration of antibiotics is aimed at inhibiting the planktonic cell, which differs from cells in the biofilm state. The bacterial biofilm is 10-1,000 times more resistant to antimicrobial agents than the planktonic cell [30]. This can be one explanation as to why there is a higher failure rate in the eradication of biofilm-related infections. The minimum biofilm eradication concentration (MBEC), the concentration of an antimicrobial agent required to kill a bacterial biofilm, should be tested in the laboratory to select the appropriate type and concentration of antibiotics needed to eliminate bacterial biofilms. This may improve the success rate of treating infectious diseases. Moreover, the ability of bacteria to form biofilms has been enhancing the spread of antibiotic resistance and the accumulation of virulence genes. New therapeutic strategies should be aimed at a co-treatment approach that combines traditional antibiotics with a substance that interferes with biofilms, and this may render the biofilms more susceptible to treatment.

In conclusion, bacteria that have the ability to form biofilms, coupled with the emergence of multidrug resistant strains, are a significantly increasing concern in healthcare. Our results suggested that the *pqsA* gene could be used as a marker for the screening of biofilm-producing bacteria and that this could be applied to detect these...
strains from clinical isolates. We believe that this should help in the management and improvement of treatment options. The data from biofilm-producing strains, which are resistant to antibiotic or empirical treatment in individual areas, should be used to elucidate the appropriate therapies, especially in cases related to implanted devices and chronic infections.

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