<table>
<thead>
<tr>
<th>Title</th>
<th>Review Article Invasiveness of Pseudomonas aeruginosa and Its Role in Diversity of Pseudomonal Infectious Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Hirakata, Yoichi</td>
</tr>
<tr>
<td>Citation</td>
<td>Acta medica Nagasakiensia. 2002, 47(3-4), p.89-96</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2002-12-17</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/16217">http://hdl.handle.net/10069/16217</a></td>
</tr>
</tbody>
</table>

NAOSITE: Nagasaki University’s Academic Output SITE
http://naosite.lb.nagasaki-u.ac.jp
Invasiveness of *Pseudomonas aeruginosa* and Its Role in Diversity of Pseudomonal Infectious Diseases

Yoichi HIRAKATA

Department of Laboratory Medicine, Nagasaki University School of Medicine

*Pseudomonas aeruginosa* causes both invasive (bacteremic) and chronic non-invasive infections in several organs, resulting in the diversity of infectious diseases. When Madin-Darby canine kidney (MDCK) monolayers were infected with clinical isolates of *P. aeruginosa*, significantly (*P* < 0.001) more blood (30 of 32, 93.7%) than respiratory (25 of 45, 54.4%) isolates penetrated the epithelial cell monolayers by 3 h. Only eight (4 blood and 4 respiratory) isolates were cytotoxic and possessed exoU, and passed through the monolayer following epithelial cell death associated with release of lactose dehydrogenase and a marked drop in transepithelial electrical resistance. Thus, invasiveness was usually independent of cytotoxicity. The capacity to penetrate epithelial cells appears to be a critical determinant of invasiveness in susceptible hosts and may be controlled by unknown unique genes. In addition, such invasion determinant(s) are thought to be predominantly exported by *P. aeruginosa* via MexAB-OprM, which is one of multi-drug resistant (MDR) efflux systems. Hence, MDR efflux systems in *P. aeruginosa* might be critical for the efflux of virulence factors, in addition to their established role of exporting harmful substances such as antibiotics or detergents.

**Introduction**

*Pseudomonas aeruginosa* is a versatile Gram-negative bacterium that is an important pathogen in patients with compromised immunity, including those with cystic fibrosis (CF), neutropenia, thermal burns, and AIDS. *P. aeruginosa* is capable of establishing both chronic and acute infections. In patients with CF or diffuse panbronchiolitis (DPB), *P. aeruginosa* is the most predominant pathogen, causes chronic infection, and is difficult to be eliminated but usually does not invade the bloodstream. This organism, however, is invasive in other patient groups and causes bacteremia associated with high mortality rates, particularly in those with neutropenia. *P. aeruginosa* also causes several other infections in various organs. Thus, this organism develops a diversity of infectious diseases (Fig. 1).

**Figure 1.** A diversity of infectious diseases caused by *P. aeruginosa*. DPB: diffuse panbronchiolitis, CF: cystic fibrosis

Bacteria have to penetrate epithelial and endothelial barriers to invade the bloodstream. In addition to factors in the host such as immunocompromised status, virulence factors of the pathogen could be involved in different types of infections induced by *P. aeruginosa*. **
Indeed, human blood isolates of *P. aeruginosa* cause lethal endogenous bacteremia in neutropenic mice induced by cyclophosphamide but human respiratory isolates do not. The animal model used in this study (Fig. 2) reflects the important pathophysiological steps in authentic human infections, including bacterial colonization and invasion. Clinical blood isolates of *P. aeruginosa* have the ability to produce larger amounts of exoproducts, including exotoxin A and alkaline protease, in vitro, than the strains isolated from respiratory tract. The blood isolates penetrate human intestinal Caco-2 epithelial cell monolayers to a greater degree than do the respiratory isolates. These phenotypic findings suggest that blood isolates carry virulence determinants which confer the invasive phenotype. Despite evidence of putative virulence determinants in *P. aeruginosa*, none have been characterized yet. This is in contrast to what is known about other invasive bacteria such as *Yersinia*, *Listeria monocytogenes*, *Shigella*, *Salmonella*, and *Escherichia coli* (reviewed in reference 8). In this article, the author briefly reviews the recent findings in invasiveness of *P. aeruginosa*, focusing on the own data in this field.

Figure 2. Endogenous *P. aeruginosa* bacteremia model in mice. Mice were given orally *P. aeruginosa* in drinking water at the concentration of 10⁷ CFU/ml between days 1 and 4. Mice were given intraperitoneally 200 mg of ampicillin per kg between days 1 and 4, and 200 mg of cyclophosphamide per kg on days 6, 9, and 12. If the isolate inoculated is invasive, the pathogen causes endogenous gut-derived septicemia in mice.

Two phenotypes of *P. aeruginosa*: invasive phenotype and cytotoxic phenotype

Recently, it has been reported that *P. aeruginosa* strains can be differentiated into two groups: i) strains with a cytotoxic phenotype such as PA103; and ii) strains with an invasive but non-cytotoxic phenotype such as PAO1. In these studies, the invasiveness of *P. aeruginosa* has been evaluated in Madin-Darby canine kidney (MDCK) cells and corneal epithelial cells by gentamicin survival assay. Recently, it has also been reported that *P. aeruginosa* strain PA103 carries exoU, while PAO1 lacks it. *ExoU* encodes a 70-kDa protein (ExoU), which is identical to PepA. It is a cytotoxin secreted by a type III system (reviewed in reference 13) and exerts an acute cytotoxic effect on epithelial and phagocytic cells. *ExoU* is a possible candidate for the gene controlling invasiveness; however, a correlation between its presence and the clinical sources of different *P. aeruginosa* strains has not been investigated.

Establishment of MDCK cell monolayer system and penetration of representative isolates

We have developed an MDCK cell monolayer penetration assay as an in vitro screening system for invasiveness of *P. aeruginosa* (Fig. 3). When grown on permeable supports, polarized MDCK cells establish a monolayer with tight junctions and high transmonolayer electrical resistance, akin to Caco-2 cells. Monolayers of polarized MDCK cells were prepared in Transwell filter units containing 0.33-cm² porous filter membranes (3.0-μm pores) in 24-well tissue culture plates. Monolayers were infected with bacteria by adding 5 μl (ca. 3.5 x 10⁶ CFU) freshly grown bacteria cultured in L-broth overnight at 37°C.

First, *P. aeruginosa* PAO1 and PA103 were examined in this system. *Salmonella typhimurium* SL1344 was used as a positive control. Noninvasive rabbit enterotoxigenic *E. coli* strain RDEC-1, which is positive for oxidation of lactose, was used as a negative control and as an internal control of monolayer integrity, since it does not penetrate the monolayer unless the tight junctions are disrupted by Ca²⁺-free medium. Some clinical isolates of *P. aeruginosa* included five blood isolates and five respiratory isolates, which were previously evaluated in a murine model of endogenous septicemia and Caco-2 cell monolayer penetration assay, and were also evaluated. In some experiments, an equal number of *E. coli* RDEC-1 was added with *P. aeruginosa* isolates or *S. typhimurium* SL1344 at the same time. In these co-infection assay,
basolateral medium was plated on MacConkey agar (Quelab, Montreal, Quebec, Canada) to distinguish lactose-positive E. coli colonies from others.

The capacity of P. aeruginosa PAO1 and PA103 to penetrate through MDCK cell monolayers was compared with that of S. typhimurium SL1344, and E. coli RDEC-1 (Fig. 4). PAO1 was detected in the basolateral medium by 3 h after inoculation, as was S. typhimurium SL1344, whereas P. aeruginosa strain PA103 was not detected until 6 h \textsuperscript{(m)}. E. coli RDEC-1 was not detected by 12 h, and did not appear in the basolateral medium until at least 24 h after inoculation. There was no difference in growth rate between P. aeruginosa PAO1 and PA103 (data not shown).

Using the 10 clinical isolates a correlation between the data using MDCK and Caco-2 cells was examined. Times when bacteria appeared in the basolateral medium, and recovery of bacteria from the basolateral medium at 3 h and 6 h after inoculation correlated well (r = 0.911, 0.950, and 0.874, respectively) between the two cell lines (data not shown).

**Figure 4.** Bacterial penetration of P. aeruginosa PAO1 (■) and PA103 (▲), S. typhimurium SL1344 (□), and E. coli RDEC-1 through MDCK cell monolayers (reference 16). Bacteria were inoculated at 3.5 x 10^8 CFU/well to the apical surfaces of MDCK cell monolayers. The assay was performed in triplicate, and results are expressed as an average ± standard deviation. Noninvasive E. coli RDEC-1 could not be recovered from the basolateral medium up to 12 h after inoculation.

**Penetration of P. aeruginosa clinical isolates through MDCK cell monolayers.**

A total of 77 clinical isolates of P. aeruginosa, including 32 blood and 45 respiratory isolates were evaluated using the MDCK cell monolayer system described above. P. aeruginosa isolates were divided to four groups based on clinical site of isolation and their capacity to penetrate through MDCK cell monolayers (table 1). Of 32 blood isolates of P. aeruginosa, 30 (93.7%) were detected in the basolateral medium by 3 h, as was P. aeruginosa PAO1 and S. typhimurium SL1344. A total of 24 of 45 respiratory isolates of P. aeruginosa (54.4%) were detected in the basolateral medium by 3 h; this percentage was significantly lower than among blood isolates (P < 0.001). The average number of bacteria detected in the basolateral medium at 3 h in blood isolates was significantly greater than the value of respiratory isolates (3.26 ± 5.05 x 10^3 versus 1.06 ± 1.03 x 10^3 CFU, P < 0.05).

**Phenotypes of clinical isolates of P. aeruginosa.**

Serum sensitivity of P. aeruginosa strains was evaluated as previously described \textsuperscript{(22)}. P. aeruginosa strains P1 \textsuperscript{(20)} and M2 \textsuperscript{(22)} were used as serum-sensitive and -resistant controls, respectively. The motility of P. aeruginosa isolates was assessed by the diameter of colonial spreading in soft L-agar containing 0.3% agar as previously reported \textsuperscript{(1).} The presence of surface functional pili on P. aeruginosa strains was confirmed by plating a 5-μl drop of culture supernatant containing 4 x 10^7 particles of bacteriophage PO4 onto a freshly spread lawn of bacteria made on L-broth agar as previously reported \textsuperscript{(1).}

Significantly greater numbers of serum-sensitive and nonmotile phenotypes were found among respiratory isolates than blood isolates (P < 0.001 and P < 0.05, respectively), and in respiratory isolates that were not able to penetrate MDCK cell monolayer than other groups as defined in table 1 (P < 0.001 and P < 0.005, respectively) \textsuperscript{(1).} Eight respiratory isolates, which were serum-resistant and also motile could not penetrate the monolayer by 3 h. There was no difference in PO4 phage sensitivity between blood and respiratory isolates (P = 0.559), and among the four groups as defined in table 1 (P = 0.076) \textsuperscript{(1).}

Most clinical blood isolates penetrated MDCK cell monolayers by 3 h, suggesting that they possess virulence determinants capable of inducing bacteremia. It appears that serum-resistance and motility may be necessary but not sufficient, since several motile serum-resistant respiratory isolates failed to pass through the monolayer. Although pilation in P. aeruginosa is also thought to be important in the colonization of the CF airway and in the establishment of other infections \textsuperscript{(26,27)}, its significance in the penetration of isolates through MDCK cell monolayer was not clearly demonstrated in this study (table 1).
Table 1. Penetration of clinical isolates of *P. aeruginosa* through MDCK cell monolayers, their phenotypes, and possession of exoU (reference 16).

<table>
<thead>
<tr>
<th>Origin</th>
<th>Total No.</th>
<th>Capacity to penetrate MDCK cell monolayers by 3 h&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Mucoid</th>
<th>Serum-sensitive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nonmotile&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PO4-resistant</th>
<th>Cytotoxic exoU gene positive by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PCR</td>
</tr>
<tr>
<td>Blood</td>
<td>32</td>
<td>Yes</td>
<td>30</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dot blot</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory tract</td>
<td>45</td>
<td>Yes</td>
<td>24</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>21</td>
<td>3</td>
<td>11</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly greater numbers of blood than respiratory isolates penetrated MDCK cell monolayer by 3 h ($P < 0.001$).

<sup>b</sup> Significantly greater number of serum-sensitive isolates were found in respiratory isolates than blood isolates ($P < 0.001$), and in respiratory isolates which did not penetrate MDCK cell monolayer ($P < 0.001$).

<sup>c</sup> Significantly greater number of nonmotile isolates were found in respiratory isolates than blood isolates ($P < 0.05$), and in respiratory isolates which did not penetrate MDCK cell monolayer ($P < 0.005$).

Detection of exoU gene and its role in cytotoxicity.

To quantify cytotoxicity of *P. aeruginosa* isolates on the MDCK cell monolayers, the concentration of lactose dehydrogenase (LDH) released from the MDCK cells into the medium in the filter units was measured<sup>9</sup>. Triton X-100 was used as a positive control to destroy the cells and make LDH release. First, the LDH level in both the apical and basolateral medium was measured for monolayers incubated with either PAO1 or PA103 at several time points after infection (Fig. 5). Although LDH was not detected by 1 h, LDH was released into the apical medium from the MDCK monolayer inoculated with PA103 by 3 h. When the MDCK cell monolayer was infected with PAO1, LDH was not detected until at least 6 h and not in substantial levels until 10 h. LDH was not detected in the basolateral medium until 6 h and 10 h after infection with PA103 and PAO1, respectively. Cytotoxic strains were defined as those which caused statistically significant levels of LDH release from MDCK cell monolayers in apical medium by 6 h, as compared with spontaneous LDH release. Using this definition, 8 of 77 clinical *P. aeruginosa* isolates (10.4%) were cytotoxic. These 8 cytotoxic isolates included four blood isolates (B22, B24, B34, and B39) and four respiratory isolates (S33, S36, S45, and S50). There was no significant difference in cytotoxicity between blood and respiratory isolates ($P = 0.609$).

The exoU gene was detected from *P. aeruginosa* isolates by both PCR and dot blot hybridization analysis. Chromosomal DNA was purified from the isolates as previously described<sup>29</sup>. The oligonucleotide primers used for PCR detection of the exoU gene were designed from the sequence previously published (11, accession number U97065) for this study as follows; sense, 5'-TAG AAC GCC TAT TGC GCG-3'; antisense, 5' CTC GAG CTG CAG CAT TTC-3'. PCR was performed in a final volume of 25 μl containing 20 ng of *P. aeruginosa* DNA, 20 pmol each primer, 250 μM...
each deoxynucleoside triphosphate, and 1 U of Taq polymerase in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM MgCl₂, as follows: 30 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, followed by final extension at 72 °C for 10 min. A DNA probe for dot blot hybridization was prepared by amplification of a 572 bp fragment of the exoU gene from P. aeruginosa PA103 by PCR as described above and was simultaneously labelled with digoxigenin-11-uridine-5’-triphosphate (DIG-dUTP)²⁰. Approximately 1 μg of DNA in TE from each of the P. aeruginosa isolates was filtered under vacuum onto positively charged nylon membranes by using a 96-well dot-blot apparatus. The filters were hybridized with the DIG-labelled exoU gene fragment and were developed by chemiluminescence detection as previously described²⁰.

Figure 6 shows LDH levels in apical medium for the cytotoxic isolates together with isolates showed a slight but not statically significant level of LDH (S31, S32, S35, and S56). The isolates positive for exoU are also indicated in figure 6. The expected 572 bp fragment of exoU was detected by PCR from 8 isolates including blood isolates B22, B24, B34, and B39, and respiratory isolates S33, S36, S45, and S50 (table 1 and Fig. 6). All these isolates were also positive for exoU by dot blot hybridization (table 1 and Fig. 7). There were no strains that showed discrepancies in exoU gene detection between PCR and dot blot hybridization. Figure 6 also shows that typical invasive isolates, including PAO1 and blood isolates B15, B16, and B51, and non-invasive respiratory isolates such as S3 and S40 were non-cytotoxic and also were negative for exoU.

In our study, 8 of 77 clinical isolates of P. aeruginosa showed significant cytotoxicity towards MDCK cells and all of these isolates carried exoU (table 1, figure 6)²⁰. To our knowledge, this is the first report of the frequency of exoU in clinical isolates. It has been reported that P. aeruginosa isolates can be differentiated into two groups as determined by cytotoxic or invasive phenotype, as previously described⁹. In the current study, we could further differentiate clinical isolates of P. aeruginosa into four groups as follows: i) invasive and lack exoU (non-cytotoxic), ii) noninvasive and carry exoU (cytotoxic), iii) invasive and carry exoU (cytotoxic), and iv) noninvasive and lack exoU (non-cytotoxic). Most blood isolates (27 of 32, 84.5%) and fewer respiratory isolates (22 of 45, 48.9%) belonged to the first group. One blood (B34) and two respiratory (S33 and S50) isolates belonged to the second group. Blood isolates, B22, B24, and B39, and respiratory isolates S36 and S45 belonged to the third group. Invasive non-cytotoxic isolates PAO1, B15, and B16, noninvasive non-cytotoxic isolates S3 and S40 were negative for exoU. Four additional clinical isolates (B24, B34, S33, and S45) were also positive for the gene by both methods (text, table 1, and figure 6).
Influence of *P. aeruginosa* infection on epithelial cell damages.

Transmonolayer electrical resistance (TER) was measured with a Millicell-ESR apparatus at sequential timed intervals.

Figure 8 shows the mean values of TER for cytotoxic isolates, PA103, S50, and B22, and non-cytotoxic isolates, PAO1, B15, and B16, with those of *S. typhymurium* SL1344 and *E. coli* RDEC-1. When MDCK cell monolayers were inoculated with *E. coli* RDEC-1, TER decreased slowly over time. The changes in TER for non-cytotoxic isolates was quite similar to that for *S. typhymurium* SL1344. TER for cytotoxic isolates were significantly lower than those for non-cytotoxic isolates between 4 and 12 h (P < 0.05). When either *P. aeruginosa* PAO1 or B16 was added simultaneously to the apical medium with *E. coli* RDEC-1, *P. aeruginosa* alone was detected in the basolateral medium at 3 h, while *E. coli* RDEC-1 did not appear until 10 h after inoculation (data not shown). Similar results were obtained when the monolayer was infected with either *P. aeruginosa* B15 or B51 together with *E. coli* RDEC-1 (data not shown). In contrast, when cytotoxic *P. aeruginosa* strains PA103 or S50, and B22 were each inoculated together with *E. coli* RDEC-1, RDEC-1 was detected at the same early time as the *P. aeruginosa* isolates.

The co-infection study clearly revealed that cytotoxic isolates passed through MDCK cell monolayer by disrupting the monolayer, while non-cytotoxic isolates, such as PAO1, B15, B16, and B51, did not markedly affect monolayer integrity. The results in TER study and LDH assay also supported this. Consequently cytotoxic clinical isolates and PA103 are thought to pass through the monolayer following monolayer disruption due to intoxication.

Role of multidrug efflux systems in the invasiveness of *P. aeruginosa*

*P. aeruginosa* is intrinsically resistant to conventional penicillins and cephems due to its low outer membrane (OM) permeability coupled to the production of an inducible chromosomal β-lactamase, which hydrolyzes these β-lactams and can become mutationally resistant to even newly-developed anti-pseudomonal agents. In addition to the recent emergence of metallo-β-lactamase producing *P. aeruginosa*, multidrug resistant (MDR) efflux systems are becoming recognized as important antimicrobial resistance mechanisms for this organism. To date, four resistance-nodulation-division (RND) MDR efflux systems have been well characterized in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY/OprM (reviewed in reference 42). More recently, an additional system, MexJK/OprM has been reported.

Recently, certain studies have suggested a relationship between efflux and virulence in *P. aeruginosa*. For instance, it has been reported that MexAB-OprM exports homoserine lactones which are involved in quorum sensing (cell-to-cell signaling) and consequent regulation of the expression of a variety of virulence determinants. However, the role of efflux systems in specific clinical pathogenesis of *P. aeruginosa*, including invasiveness, has not been determined. To gain a better understanding the role of efflux in bacterial pathogenesis, the invasiveness of *P. aeruginosa* PAO1 and its efflux mutants was evaluated using in vitro MDCK epithelial cell monolayer penetration. Virulence was also investigated in a murine model of
endogenous P. aeruginosa bacteremia.

Both the parent WT strain K767 and strain K1521 (\(\Delta\text{mexCD-oprJ}\)) penetrated MDCK monolayers by 3 h, whereas K1119 (\(\Delta\text{mexAB-oprM}\)) was not detected in the basolateral medium until 6 h after infection (Fig. 9). Strains K767 (WT) and K1521 (\(\Delta\text{mexCD-oprJ}\)) induced lethal endogenous septicemia in mice, whereas strain K1119 (\(\Delta\text{mexAB-oprM}\)) failed to kill any mice (\(P < 0.0001\), Fig. 10). To confirm the role of \(\text{mexAB-oprM}\) in the expression of invasiveness, the \(\Delta\text{mexAB-oprM}\) deletion strain K1119 was complemented with \(\text{mexAB-oprM}\) by introducing plasmid pRSP17 carrying the genes and examined with strains K767 (WT), K1119 (\(\Delta\text{mexAB-oprM}\)), and K1119/pRK415 (plasmid control). K1119/pRSP17 (\(\text{mexAB-oprM}\) complemented strain) showed the invasiveness equivalent to WT in both in vitro (Fig. 11A) and the animal model (Fig. 11B), while plasmid control strain K1119/pRK415 had the same phenotype, which was compromised in its capacity to penetrate MDCK monolayer (Fig. 10A, \(P < 0.0001\)) and to kill leukopenic mice (Fig. 11B, \(P < 0.0001\)) as K1119 (\(\Delta\text{mexAB-oprM}\)).

These findings strongly suggest that invasion determinant(s) are predominantly exported by \(P.\ aeruginosa\) via MexAB-OprM. Hence, MDR efflux systems in \(P.\ aeruginosa\) might be critical for the efflux of virulence factors, in addition to their established role of exporting harmful substances such as antibiotics or detergents. It seems practical for bacteria to utilize efflux systems to export virulence determinants and physiological products, as a physiological process.

References


