Citywide emergence of *Pseudomonas aeruginosa* strains with reduced susceptibility to polymyxin B

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**Objectives:** To determine the prevalence of *Pseudomonas aeruginosa* isolates with reduced susceptibility to polymyxin B, and to assess the *in vitro* activity of antibiotic combinations.

**Methods:** All unique patient isolates of *P. aeruginosa* were collected from 11 Brooklyn, NY hospitals during a three month period in 2003. Isolates with reduced susceptibility to polymyxin B (MIC > 2 mg/L) underwent ribotyping. The activity of polymyxin B combined with rifampicin, azithromycin and/or imipenem was tested by the chequerboard and time–kill methods against a subset of isolates.

**Results:** Of 527 isolates, only 61% were susceptible to imipenem. Twenty-five isolates (5%), from 8/11 hospitals, had reduced susceptibility to polymyxin B (MICs 4–8 mg/L), compared with 0/691 isolates collected in 2001. Ten of 25 were resistant to multiple other antibiotic classes. Ribotyping of the isolates revealed 19 unique types. Chequerboard testing of the 10 multiresistant isolates demonstrated synergy for the combinations of polymyxin B with azithromycin, imipenem and rifampicin in 6, 2, and 1 isolates, respectively. Time–kill studies revealed bactericidal activity for the following antibiotics when combined with polymyxin B: imipenem plus rifampicin against all 10 isolates, rifampicin in 9/10 isolates, imipenem in 8/10 isolates and azithromycin in 4/10 isolates. MICs of bacteria surviving incubation in polymyxin B alone rose for 4/9 isolates (MIC range 12–48 mg/L).

**Conclusions:** *P. aeruginosa* with reduced susceptibility to polymyxin B have emerged in multiple strains in Brooklyn, NY. Combinations of polymyxin B with rifampicin and/or imipenem are bactericidal. The clinical utility of these combinations remains to be determined.

Keywords: antibiotic resistance, imipenem, rifampicin, azithromycin

**Introduction**

*Pseudomonas aeruginosa* is an important cause of nosocomial infections, including pneumonia, bacteraemia and urinary tract infection. The relative impermeability of the outer membrane of *P. aeruginosa* produces intrinsic resistance to many antibiotics. The presence of acquired mechanisms of resistance, with multiple mechanisms often present in single strains, has made *P. aeruginosa* an even more formidable pathogen. Resistance to β-lactam antibiotics, including carbapenems, has been increasing among strains of *P. aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* in some regions. Because of this trend, there has been renewed interest in the use of polymyxins against multidrug-resistant strains. As polymyxin usage increases, the emergence of resistance to this agent of last resort becomes an obvious concern. A citywide surveillance study in Brooklyn, NY revealed no resistance to polymyxin B among nearly 700 unique patient isolates of *P. aeruginosa* in 2001. This report documents the development of reduced susceptibility to polymyxin among more recent isolates of *P. aeruginosa* and evaluates the *in vitro* activity of polymyxin combined with other agents.

**Materials and methods**

During a three month period in 2003, all unique patient isolates of *P. aeruginosa* were collected from the Microbiology laboratories at 11 Brooklyn, NY hospitals. Isolates were identified by standard techniques in the individual laboratories. MICs were performed by the agar-dilution method according to NCCLS standards.
The following antibiotics were tested: ciprofloxacin, cefepime, ceftazidime, imipenem, meropenem, piperacillin plus tazobactam (4 mg/L), amikacin and polymyxin B. ATCC strains of *P. aeruginosa* 27853 and *Escherichia coli* 35218 were included as controls. Susceptibility breakpoints were as recommended by the NCCLS for all agents except polymyxin B. While no breakpoint has been established for polymyxin B, a breakpoint of $\leq 2\text{ mg/L}$ has been suggested. Isolates with a polymyxin MIC $> 2 \text{ mg/L}$ were considered to have reduced susceptibility. The reduced susceptibility of these isolates was confirmed by repeat testing by agar dilution, as well as testing by the broth microdilution and Etest methods. Susceptibility rates were compared with those found in a prior surveillance study by $\chi^2$ analysis. Isolates with reduced polymyxin B susceptibility were ribotyped with the Riboprinter Microbial Characterization System (Qualicon, Wilmington, DE, USA) using the restriction endonuclease *Pvu* II.

A subset of isolates with reduced polymyxin B susceptibility was selected for *in vitro* synergy studies. The isolates were selected based on their concomitant resistance to imipenem and at least one additional antibiotic class. Chequerboard studies were performed as previously described. Polymyxin B was tested in combination with azithromycin, rifampicin and imipenem. These combinations were tested based on their activity against multidrug-resistant strains of *A. baumannii*. FIC indices of $\leq 0.5$, $> 0.5$–4, and $> 4$ were interpreted as synergy, no interaction and antagonism, respectively. Time–kill studies were performed on the same isolates as previously described. The concentrations tested were: polymyxin B 2 and 4 mg/L, rifampicin 1 mg/L, imipenem 4 mg/L and azithromycin 4 mg/L. These concentrations were chosen to reflect clinically relevant levels. Antibiotic carryover was eliminated by diluting the final cultures 200-fold in pour plates. Bactericidal activity was defined as a decrease of $\geq 3$ log cfu/mL in 24 h. Bacterial regrowth was defined as an increase of $\geq 2$ log cfu/mL at 24 h following an initial decline at 4 h. Bacteria surviving incubation in the presence of polymyxin B underwent repeat Etest MIC testing after subculture on fresh agar.

**Results**

A total of 527 unique patient isolates of *P. aeruginosa* were collected during the surveillance period. Results of susceptibility testing are shown in Table 1. Compared with a similar surveillance study conducted in 2001, there was an increase in the percentage of isolates non-susceptible to imipenem (30 to 39%, $P < 0.01$), meropenem (23 to 29%, $P < 0.05$), cefepime (29 to 39%, $P < 0.001$), piperacillin/tazobactam (17 to 21%, $P < 0.05$) and amikacin (5 to 14%, $P < 0.001$). In 2001, 100% of nearly 700 isolates were inhibited by 2 mg/L or less of polymyxin B. In contrast, 25 isolates (5%) from the present surveillance had polymyxin B MICs of 4–8 mg/L by the agar dilution and Etest methods ($P < 0.001$), including 13 with MICs of 6–8 mg/L by Etest. The polymyxin B MICs of the isolates were two-fold lower when tested by the broth microdilution method.

Isolates with reduced susceptibility to polymyxin B were present in 8/11 hospitals. Ribotyping of the 25 isolates revealed 19 distinct types (Figure 1); two strains were isolated at more than one hospital. The susceptibility of the 25 isolates to the other anti-pseudomonal agents was similar to the rest of the isolates. Ten of 25 (40%) were resistant to multiple classes of antibiotics (including carbapenems), whereas 12 of 25 (48%) were susceptible to all agents except polymyxin B.

The 10 multidrug-resistant isolates, comprising seven unique ribotypes, were selected for *in vitro* synergy studies. The MICs of azithromycin and rifampicin for these isolates were all $> 256\text{ mg/L}$ and $> 32\text{ mg/L}$, respectively. Chequerboard studies revealed synergy of polymyxin B combined with azithromycin for 6/10, with imipenem for 2/10 and with rifampicin for 1/10. For the remainder of isolates, no interaction was observed.

The results of time–kill studies are summarized in Table 2. Polymyxin B produced an initial decline in cfu/mL followed by regrowth for many isolates at 24 h; regrowth was observed in 9/10 and 5/10 isolates following exposure to polymyxin B 2 and 4 mg/L, respectively. In separate experiments using a well-diffusion bioassay, the concentration of polymyxin B was unchanged after 24 h of incubation (data not shown). Bacteria surviving 24 h of incubation in polymyxin 2 or 4 mg/L underwent repeat MIC testing. The polymyxin B MIC increased three- to eight-fold for four of the isolates, with resulting MICs of 12–48 mg/L. The combination of polymyxin B with azithromycin had variable activity; this combination was bactericidal against four strains and bacteriostatic against five. Regrowth following an initial decline in colony counts occurred for three strains with this combination. The combination of polymyxin B with either rifampicin or imipenem was bactericidal against most of the strains, and the three-drug combination against all strains. The three-drug combination was most rapidly bactericidal; there were no surviving bacteria at 4 h for six, seven and nine of 10 strains for polymyxin B combined with rifampicin, imipenem and both drugs, respectively.

Information regarding usage of polymyxin B was available from the hospital with the greatest number of *P. aeruginosa* isolates with reduced polymyxin susceptibility (nine of the 25 isolates). For the five year period from 1999–2003, the total

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**Table 1. Susceptibility results for 527 isolates of *P. aeruginosa* from 11 Brooklyn, NY hospitals**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
<th>MIC$_{50}$ (mg/L)</th>
<th>MIC$_{90}$ (mg/L)</th>
<th>Range (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>53%</td>
<td>3%</td>
<td>44%</td>
<td>1</td>
<td>$&gt; 4$</td>
<td>$&lt; 0.125$–$&gt; 4$</td>
</tr>
<tr>
<td>Cefepime</td>
<td>61%</td>
<td>15%</td>
<td>24%</td>
<td>8</td>
<td>32</td>
<td>$&lt; 0.5$–$&gt; 32$</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>72%</td>
<td>6%</td>
<td>22%</td>
<td>4</td>
<td>$&gt; 32$</td>
<td>$&lt; 0.5$–$&gt; 32$</td>
</tr>
<tr>
<td>Imipenem</td>
<td>61%</td>
<td>3%</td>
<td>36%</td>
<td>4</td>
<td>32</td>
<td>$&lt; 0.5$–$&gt; 32$</td>
</tr>
<tr>
<td>Meropenem</td>
<td>71%</td>
<td>13%</td>
<td>16%</td>
<td>1</td>
<td>16</td>
<td>$&lt; 0.125$–$&gt; 32$</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>79%</td>
<td>21%</td>
<td>21%</td>
<td>8</td>
<td>$&gt; 128$</td>
<td>$&lt; 0.5$–$&gt; 128$</td>
</tr>
<tr>
<td>Amikacin</td>
<td>86%</td>
<td>14%</td>
<td>14%</td>
<td>8</td>
<td>32</td>
<td>$1$–$&gt; 64$</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>95%</td>
<td>5%</td>
<td>5%</td>
<td>2</td>
<td>2</td>
<td>0.5–8</td>
</tr>
</tbody>
</table>
increased nearly six-fold between 2001 and 2003 at one of these hospitals. Therefore, P. aeruginosa isolates may have been affected by exposure to polymyxin B used in the treatment of resistant A. baumannii infections. This possibility is supported by the fact that most of the polymyxin B-resistant P. aeruginosa isolates were susceptible to other agents and were, therefore, unlikely to trigger polymyxin therapy. As the problem of multidrug-resistant Gram-negative infections continues to spread, clinicians may need to consider combining polymyxins with other agents as a strategy to prevent the emergence of polymyxin resistance.

The clinical significance of small elevations in polymyxin B MIC is unclear. A susceptibility breakpoint has not been established for polymyxin B, although a breakpoint of 2 mg/L has been suggested based on serum concentrations. In a previous report, polymyxin B demonstrated concentration-dependent activity against P. aeruginosa strains with MICs of 1 or 2 mg/L, and was uniformly bactericidal at a concentration of 4 mg/L. Polymyxin B 4 mg/L was less active against strains with MICs of 4–8 mg/L in the present study. Moreover, bacterial regrowth occurred in many strains exposed to 2 or 4 mg/L, concentrations that will not be exceeded for a portion of the dosing interval. The significant rise in MIC in some bacteria surviving exposure to polymyxin B demonstrates the potential for higher level resistance to emerge. The use of higher doses of polymyxin B to maintain serum and tissue levels above 4 mg/L might prove to be more effective, but would probably result in greater toxicity. Taken together, these data suggest that reduced polymyxin susceptibility may be clinically relevant and could compromise the use of polymyxin B monotherapy against these strains.

The use of polymyxins combined with other agents has not been well studied. In vitro studies have demonstrated synergy between polymyxin B and azithromycin, rifampicin, and/or imipenem against strains of A. baumannii. In vitro synergy has also been demonstrated between polymyxin B and azithromycin and between colistin and rifampicin against polymyxin-susceptible strains of P. aeruginosa. In the latter report, anecdotal success was noted in the treatment of four patients with colistin plus rifampicin. The efficacy of polymyxin agents alone, or in combination, against strains with reduced polymyxin susceptibility is unknown. This study suggests that combining polymyxin B with rifampicin and/or imipenem, and possibly azithromycin, may prove to be useful and might allow the use of lower doses of polymyxin. The clinical use of these combinations deserves further study.

Discussion

Multidrug-resistant Gram-negative bacilli have become increasingly problematic in certain regions. In particular, the spread of multiresistant A. baumannii and P. aeruginosa has seriously limited therapeutic options. The recent emergence of carbapenemase-producing multidrug-resistant K. pneumoniae only adds to the problem. For many of these pathogens, polymyxins are the only remaining agents with reliable antimicrobial activity. While P. aeruginosa are typically susceptible to polymyxins, resistance has been known to occur. In 2003, at least nine isolates were reported non-susceptible to colistin in Greece. Recent studies in the USA have demonstrated uniform susceptibility of P. aeruginosa to polymyxin B. Nevertheless, increased usage of polymyxins might be expected to result in the development of polymyxin resistance in some strains. This report documents the emergence of reduced polymyxin B susceptibility among multiple strains of P. aeruginosa in Brooklyn, NY.

P. aeruginosa isolates with reduced polymyxin susceptibility were detected at most hospitals in the study. However, the majority of isolates (20 of 25) came from three hospitals. These three hospitals were also among the four hospitals with the greatest number of carbapenem-resistant A. baumannii isolates during the study period (data not shown). The greater prevalence of multiresistant A. baumannii at these hospitals might have resulted in greater usage of polymyxins. In fact, the usage of polymyxin B increased nearly six-fold between 2001 and 2003 at one of these hospitals. Therefore, P. aeruginosa isolates may have been affected by exposure to polymyxin B used in the treatment of resistant A. baumannii infections. This possibility is supported by the fact that most of the polymyxin B-resistant P. aeruginosa isolates were susceptible to other agents and were, therefore, unlikely to trigger polymyxin therapy. As the problem of multidrug-resistant Gram-negative infections continues to spread, clinicians may need to consider combining polymyxins with other agents as a strategy to prevent the emergence of polymyxin resistance.

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Table 2. Results of time–kill studies against 10 multidrug-resistant isolates of P. aeruginosa with reduced susceptibility to polymyxin B

<table>
<thead>
<tr>
<th>Antibiotic(s)</th>
<th>Change in log cfu/mL (±SD)</th>
<th>No. bactericidal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>None</td>
<td>+1.5 ± 0.4</td>
<td>+2.7 ± 0.6</td>
</tr>
<tr>
<td>Azithromycin 4 mg/L</td>
<td>+1.1 ± 0.3</td>
<td>+1.7 ± 0.4</td>
</tr>
<tr>
<td>Imipenem 4 mg/L</td>
<td>−0.2 ± 1.5</td>
<td>+2.0 ± 0.8</td>
</tr>
<tr>
<td>Rifampicin 1 mg/L</td>
<td>+1.0 ± 0.4</td>
<td>+2.5 ± 0.4</td>
</tr>
<tr>
<td>Polymyxin 2 mg/L</td>
<td>−3.5 ± 1.0</td>
<td>−0.4 ± 2.0</td>
</tr>
<tr>
<td>Polymyxin 4 mg/L</td>
<td>−3.9 ± 0.9</td>
<td>−2.2 ± 3.2</td>
</tr>
<tr>
<td>Polymyxin 2 mg/L + azithromycin</td>
<td>−3.6 ± 1.1</td>
<td>−2.1 ± 2.4</td>
</tr>
<tr>
<td>Polymyxin 2 mg/L + imipenem</td>
<td>−4.3 ± 0.9</td>
<td>−4.5 ± 2.1</td>
</tr>
<tr>
<td>Polymyxin 2 mg/L + rifampicin</td>
<td>−4.0 ± 0.8</td>
<td>−4.8 ± 1.1</td>
</tr>
<tr>
<td>Polymyxin 2 mg/L + imipenem + rifampicin</td>
<td>−4.3 ± 1.1</td>
<td>−5.4 ± 0.4</td>
</tr>
</tbody>
</table>
Multidrug-resistant *P. aeruginosa* with reduced susceptibility to polymyxin B are now present in most hospitals in Brooklyn, NY. Microbiology laboratories in regions affected by multiresistant Gram-negative bacteria should consider routine susceptibility testing of polymyxins. Further studies are needed to assess the strategy of combining polymyxins with other agents to prevent the emergence of further resistance.

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**References**


