Antibiotic Susceptibility of Multiply Resistant Pseudomonas aeruginosa Isolated from Patients with Cystic Fibrosis, Including Candidates for Transplantation

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Chronic lung disease caused by antibiotic-resistant Pseudomonas aeruginosa in patients with cystic fibrosis (CF) is difficult to treat, especially in those who are lung transplantation candidates. Analysis of antibiotic susceptibility and synergy studies of 1,296 isolates revealed that 172 (13.3%) were multiply resistant (i.e., resistant to two or more classes of anti-Pseudomonas agents). β-Lactam agents (including imipenem and aztreonam) or aminoglycosides inhibited only 11% of the multiply resistant strains, while ciprofloxacin inhibited 34%. High concentrations of tobramycin and gentamicin (200 μg/mL), achievable by aerosol administration, inhibited 95% of isolates and overwhelmed permeability-resistance mechanisms. Antimicrobial pairs tested in checkerboard dilutions of clinically achievable drug concentrations inhibited 75% of the multiply resistant strains. On average, three additive and 2.4 synergistic pairs of antimicrobial agents had activity per strain. Transplantation candidates were older than nontransplantation candidates (P = .034), and isolates from transplantation candidates were less likely to be inhibited by antibiotic combinations (P < .001). Administration of aerosolized aminoglycosides and synergy testing of antimicrobial combinations may represent viable therapeutic options for patients with CF.

Continuing advances in the diagnosis and treatment of cystic fibrosis (CF) during the past 2 decades have led to markedly increased longevity for patients with CF, and many now live into adulthood [1, 2]. Increased longevity has important consequences for the management of chronic lung disease associated with CF. Most patients are initially infected with susceptible strains, but Pseudomonas aeruginosa is almost never eradicated despite aggressive antimicrobial therapy [3]. Large numbers of organisms (10^9–10^10 cfu per gram of sputum) may be present, and after years of symptomatic lung disease and consequent exposure to numerous antibiotics, strains resistant to multiple antibiotics emerge. Molecular typing methods demonstrated that most patients who have CF are infected with the same clone of P. aeruginosa that acquires multiple mutations and becomes progressively resistant to antibiotics [4].

A substantial number of patients with CF are candidates for lung transplantation, despite the risk of infectious disease-related complications [5, 6]. Such patients require pretransplantation antimicrobial therapy in an attempt to decrease chronic infection in contaminated airways, and they need post-transplantation therapy to prevent potentially fatal pseudomonas septicemia during maximal immunosuppression. While infection with multiply resistant P. aeruginosa is not an absolute contraindication for lung transplantation, many centers are understandably concerned about transplantations in patients infected with these organisms.

We have systematically studied the activity of 10 antimicrobial agents against P. aeruginosa isolates from patients with CF who were not responding to conventional antibiotic regimens, to establish the spectrum of resistance in these clinical isolates. The activity of pairs of antibiotics against highly resistant strains was tested to determine potential synergy. We also tested the ability of higher concentrations of tobramycin (achievable by the aerosol route) to inhibit multiply resistant strains, and we consider the potential role of this specialized susceptibility and synergy testing for managing CF patients.

Materials and Methods

Source of Clinical Isolates

From August 1991 through July 1994, physicians caring for patients with CF referred multiply resistant strains of P. aeruginosa to Columbia University (New York) for susceptibility testing. Information about the availability of this testing was disseminated through the CF newsletter sent regularly to all CF center directors. A brief questionnaire was sent to physicians, inquiring about the age, sex, and transplantation status of the patients whose isolates were included in this study.

Sixty-seven centers in 31 states sent 1,296 strains, and 234 (18%) were multiply resistant P. aeruginosa isolates, according to the definition below. Sixty-two of these 234 strains (26.5%) were multiply resistant P. aeruginosa from patients with CF referred multiply resistant strains of P. aeruginosa to Columbia University (New York) for susceptibility testing. Information about the availability of this testing was disseminated through the CF newsletter sent regularly to all CF center directors. A brief questionnaire was sent to physicians, inquiring about the age, sex, and transplantation status of the patients whose isolates were included in this study. Only the most resistant strain from an individual patient was selected for this study. Thus, 172 multiply resistant P. aerugi-
nosa strains from 172 patients were used in this study. Statistical analysis was performed with the use of Excel software (Microsoft, Seattle).

**Identification of Species of Isolates**

The species of the multiply resistant isolates was confirmed at Columbia University with use of either the API 20E system (Analytab Products, Plainview, NY; used through 1993) or the Microscan (Baxter Diagnostics, West Sacramento, CA) and oxidative-fermentative biochemical panel (BBL/Becton Dickinson, Cockeysville, MD), which were used in 1994.

**Susceptibility Testing**

Conventional anti-Pseudomonas antimicrobial agents. MICs of 10 anti-Pseudomonas antimicrobial agents were determined for all 1,296 strains by the microdilution method, with use of commercially prepared trays (Microtech Medical Systems, Aurora, CO). The trays contained twofold serial dilutions of the following: ticarcillin (4–128 μg/mL), ticarcillin + clavulanate (4–128 μg/mL), piperacillin (4–128 μg/mL), cefazidime (1–128 μg/mL), imipenem (0.5–16 μg/mL), aztreonam (1–32 μg/mL), tobramycin (1–16 μg/mL), gentamicin (1–16 μg/mL), amikacin (1–64 μg/mL), and ciprofloxacin (0.25–8 μg/mL). The trays were stored at ~70°C and then thawed at room temperature immediately before use.

Overnight cultures grown in Mueller-Hinton broth at 37°C were diluted 1:20 in such broth and held for 3–4 hours at 37°C. These cultures were then diluted with distilled water to a 0.5 McFarland standard, according to the specifications of the National Committee for Clinical Laboratory Standards (NCCLS) [7]. Inocula of 5 × 10^8 cfu/mL were distributed with a multipoint inoculator into the microdilution trays and incubated at 37°C. The MIC was the lowest antibiotic concentration at which there was no visible turbidity at 24 hours (or at 48 hours, if slow growth occurred). Susceptibility of the 10 antimicrobial agents was established according to the following NCCLS breakpoint standards for P. aeruginosa: ticarcillin, ticarcillin + clavulanate, and piperacillin, ≤64 μg/mL; cefazidime, ≤8 μg/mL; imipenem, ≤4 μg/mL; aztreonam, ≤8 μg/mL; tobramycin and gentamicin, ≤4 μg/mL; amikacin, ≤16 μg/mL, and ciprofloxacin, ≤2 μg/mL.

High-level aminoglycosides. Susceptibility to high concentrations of aminoglycosides was tested by inhibition of growth in Mueller-Hinton broth containing tobramycin or gentamicin (100 or 200 μg/mL).

**Mechanisms of Resistance to Aminoglycosides**

Two methods determined the mechanisms of resistance to aminoglycosides in 70 strains. Specific aminoglycoside-modifying enzymes were detected by patterns of resistance to aminoglycoside compounds, and their presence was confirmed by DNA hybridization.

Identification of aminoglycoside-modifying enzymes. Isolates resistant to gentamicin and/or tobramycin per the NCCLS breakpoint standards were screened for the presence of aminoglycoside-modifying enzymes with use of a panel of aminoglycoside-impregnated disks containing gentamicin, tobramycin, amikacin, netilmicin, isepamicin, 2'-N-ethyl-netilmicin, 6'-N-ethyl-netilmicin, apramycin, 5-epi-sisomicin, or fortimicin (Schering-Plough Research Institute, Kenilworth, NJ). These 10 aminoglycosides have functional groups that prevent modification of the molecule by specific aminoglycoside-modifying enzymes. Classification of the enzymatic mechanism was determined on the basis of the previously established patterns of resistance [8].

DNA hybridization. DNA probes encoding portions of the structural genes of nine aminoglycoside-modifying enzymes were used for dot-blot hybridizations [9]. In brief, 10 μL of an overnight culture in Mueller-Hinton broth was spotted on Gene Screen paper (NEB Research Products, Boston) and air-dried. The filters were treated with 0.5 M NaOH and 1.0 M Tris (pH, 7.0) twice, air-dried, and prehybridized for 4 hours at 42°C; then they were hybridized with 32P-labelling probe (1 × 10^6 cpm per 5 mL of hybridization solution) overnight at 42°C. The filters were washed as previously described at 55°C and exposed to roentgenographic film for 48 hours [9].

**Synergy Testing**

Microtiter plates containing nine pairs of anti-Pseudomonas antimicrobial agents in clinically achievable concentrations (Microtech Medical Systems) were used to test for synergistic combinations. Four or five serial twofold dilutions of the antibiotic pairs were prepared in checkerboard fashion. Pairs of drugs included agents from different antibiotic classes. Inocula and growth conditions were the same as described for the MIC determinations.

Potentially effective combinations were determined by calculation of the fractional inhibitory concentration (FIC) index for each pair of antibiotics [10, 11]:

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FIC = \frac{MIC_{A \text{ combination}}}{MIC_{A \text{alone}}} + \frac{MIC_{B \text{ combination}}}{MIC_{B \text{alone}}}
\]

Interpretations of the calculated FICs were as follows: <0.5, synergism; ≥0.5 to <1, addition; 1–2, indifference; and >2, antagonism. Thus, a synergistic combination of antibiotics demonstrated a >4-fold reduction in the MIC of either drug tested alone, as compared with the MIC obtained when both drugs were combined.

**Definition of Multiple Resistance**

The definition of multiple resistance for these studies was resistance to all of the agents in two or more of the following...
antimicrobial categories: β-lactam antibiotics, including the carbapenem imipenem and the monobactam aztreonam; aminoglycosides; and/or the fluoroquinolone ciprofloxacin. This definition was developed at the Microbiology and Infectious Diseases Consensus Conference sponsored by the Cystic Fibrosis Foundation in 1994.

Results

Comparison of the Susceptibility of 1,296 Strains vs. 172 Multiply Resistant Strains

All 1,296 isolates received at the referral center demonstrated high levels of resistance, and by definition, the 172 multiply resistant strains included in this analysis were substantially more resistant to all classes of agents (Figure 1). Notably, the β-lactam antibiotics maintained activity against 18%–25% of the 1,296 isolates. However, the 172 multiply resistant strains were resistant to ticarcillin, ticarcillin + clavulanate, and piperacillin, as well as the antimicrobial agents more recently introduced into clinical practice, imipenem and aztreonam.

Among the aminoglycosides, tobramycin demonstrated the most activity against the 1,296 strains, while no differences in the activity of gentamicin and tobramycin against the 172 highly resistant strains were detected. Ciprofloxacin had the best activity, as only 21% of the 1,296 strains were resistant to it, and among the 172 multiply resistant strains, 66% (113) were resistant to ciprofloxacin (MIC, >2 μg/mL).

Among the multiply resistant strains, more than one-half (55%) were resistant to all the antibiotics tested. While 59 strains (34%) were inhibited by ciprofloxacin, only 18 of 113 ciprofloxacin-resistant strains (11%) were inhibited by either a β-lactam agent or an aminoglycoside. Thus, ciprofloxacin resistance generally correlated with resistance to all classes of agents and thereby indicated the highest degree of resistance.

Susceptibility to High Levels of Aminoglycosides

Almost none of the strains were inhibited by conventional levels of aminoglycosides (MIC, ≤4 μg/mL), but the majority could be inhibited by high levels of tobramycin or gentamicin (100 or 200 μg/mL) achievable by aerosol administration. Ninety percent of strains were inhibited by a 100 μg/mL concentration of tobramycin, and an additional 5% by 200 μg/mL. Similar levels of inhibition were achieved with gentamicin. Thus, resistance to aminoglycosides could be categorized as low (MIC, ≤200 μg/mL) or high (MIC, >200 μg/mL).

Mechanisms of Resistance to Aminoglycosides

Specific mechanisms of resistance to aminoglycosides appeared to correlate with low vs. high levels of resistance.

Permeability mutants. Isolates with suspected permeability mutations have resistance to fortimicin and apramycin, as well as markedly diminished zones of inhibition to the other eight agents. Permeability mutation by itself was the most common mechanism of resistance observed in 56 of 70 isolates. The
majority of strains with permeability mutations could be inhibited by high concentrations of tobramycin (figure 2).

Aminoglycoside-modifying enzymes. The second most common mechanism of resistance was the acquisition of aminoglycoside-modifying enzymes. The enzyme AAC(6')-II—which confers resistance to gentamicin, tobramycin, netilmicin, 2'-N-ethyl-netilmicin, and 5-epi-sisomicin—has been shown to occur frequently in P. aeruginosa [9]. In this study, 11 strains had homology to the aac(6')-II probe [12]. These strains demonstrated high-level resistance, characterized by growth in the presence of a 200-μg/mL concentration of tobramycin. In six strains AAC(6')-II was the only mechanism detected, and in five strains AAC(6')-II was expressed along with permeability changes (figure 2).

Three additional aminoglycoside-modifying enzymes were detected in single isolates: AAC(3)-I, ANT(2')-I, and AAC(6')-I. All three of these mechanisms have been found previously in aminoglycoside-resistant P. aeruginosa [8, 9]. The isolates with ANT(2')-I and AAC(6')-I had high-level resistance to tobramycin, whereas the isolate expressing AAC(3)-I was gentamicin-resistant but tobramycin-susceptible.

Synergy Studies of Multiply Resistant Strains

More than one-half of these 172 highly resistant strains could be inhibited by drug combinations: 129 of 172 isolates (75%) were inhibited by clinically achievable levels of at least one pair of antibiotics (figure 3). On average, there were three additive and 2.4 synergistic combinations per strain. The combinations including tobramycin were generally most active, while imipenem-containing regimens did not inhibit these strains as well. No inhibitory combinations of drugs were active at biologically relevant concentrations for 25% (43) of the 172 strains. Truly antagonistic combinations were noted for only 3% of isolates.

Characteristics of Patients, Including Transplantation Status

Overall in this selected sample of 172 patients, 58% (99) were females and 42% (73) were males (table 1; P < .001, Z-test of proportions). Female patients tended to be younger; their average age was 21.9 years (mean, 20 years; range, 6–78 years). The average age for male patients was 24.6 years (mean, 24 years; range, 8–48 years) (P = .083, ANOVA). Thirty-one percent of patients (53 of 172) were transplantation candidates, comprising equal numbers of male (27) and female (26) patients (table 1). The transplantation candidates were older than the nontransplantation patients, as the average ages were 26 and 22 years, respectively (P = .034).

The organisms harbored by the transplantation candidates were not more resistant than those found in the nontransplantation patients (table 1). A comparable proportion of strains from these two groups of patients were susceptible to β-lactam agents, ciprofloxacin, and high-dose aminoglycosides. Conversely, potentially active combinations were found for significantly fewer transplantation candidates (P < .001). However, the isolates for which synergistic combinations were not found could be inhibited by high-dose aminoglycosides.

Discussion

The treatment of chronic pulmonary disease in patients with CF can be a great challenge, as prolonged exposure to subinhibitory levels of antibiotics leads to the selection of highly resistant strains of P. aeruginosa. While 81% of American patients with CF are infected with P. aeruginosa by their mid-twenties, the incidence and prevalence of multiply resistant organisms in the CF population are unknown [2]. In addition, the impact of multiply resistant organisms on the clinical course of CF has not been systematically studied.

To date, there is not a universally accepted definition of "multiply antibiotic resistant" CF strains. Most clinicians use two anti-Pseudomonas agents to treat a pulmonary exacerbation in the hopes of providing synergy and slowing the development of resistance. The definition of multiple resistance used in this study reflects the clinical dilemma if only one or no antimicrobial agent has in vitro activity against an isolate. It is important to develop a coherent approach for both the diagnosis and treatment of infection with multiply resistant P. aeruginosa.

The referral center centralizes and standardizes susceptibility and synergy studies of multiresistant P. aeruginosa. The widespread use of the referral center indicates that these organisms are detected throughout the United States. There are obviously limitations to such synergy studies. Microtiter dilution testing rather than disk-diffusion methodology was used to determine
susceptibility and generate FIC indexes. At present the most accurate susceptibility testing for CF strains is under investigation, as conventional automated susceptibility testing is most likely inadequate for slower-growing, multiply resistant *P. aeruginosa* (L. Saiman, unpublished data).

Clinically achievable antibiotic levels tested in vitro were not correlated with clinical efficacy in these studies and probably do not reflect endobronchial levels of drugs. In addition, analysis of a single isolate does not represent different populations of organisms within the CF-affected lung, as patients typically harbor multiple phenotypes with potentially different antibiograms. Finally, there may be limited clinical correlation with in vitro susceptibility studies for some patients.

The patterns of resistance to specific agents in these studies may reflect the duration of use of each antibiotic class in the general population. These strains had significant resistance to the β-lactam drugs, which have been available for 3 decades, as well as extensive resistance to the β-lactamase stable antimicrobial agents imipenem and aztreonam. This most likely is secondary to cross-resistance between these compounds and other β-lactam antibiotics.

These patterns of resistance may be increasingly relevant to non-CF-related isolates of *P. aeruginosa*. A recent study of 587 *P. aeruginosa* isolates from five American medical centers demonstrated MIC90 values of piperacillin + tazobactam (Zosyn; Lederle, Pearl River, New York), ticarcillin + clavulanate, ceftazidime, and imipenem of 8, 32, 2, and 1 μg/mL, respectively, with markedly higher MIC90 values of 64, 128, >16, and >8 μg/mL, respectively.

Resistance to the aminoglycosides could be classified as low-level or high-level resistance. While the vast majority of strains were resistant to conventional levels of tobramycin and gentamicin (MICs, >4 μg/mL), most were inhibited by high concentrations of these drugs achievable by the aerosol route (100 and 200 μg/mL), especially those strains with permeability mutations. Ramsey and colleagues demonstrated the efficacy of aerosolized tobramycin as prophylaxis for susceptible *P. aeruginosa* strains in patients with CF whose pulmonary status was stable [13]. The renewed interest in aerosolized tobramycin as treatment for pulmonary exacerbations is further supported by this report, but this role must be studied further in clinical trials.

A surprising finding was that ciprofloxacin was active against one-third of the multiply resistant isolates. Although *P. aeruginosa* often develops resistance to fluoroquinolones during therapy, reversion to a susceptible phenotype is observed when therapy is stopped [14]. Ideally, multiple, prolonged courses of ciprofloxacin should be avoided to optimize its usefulness.
Patients with CF have increased morbidity and perhaps mortality following lung transplantation, in part because of infectious complications [5]. Some centers have considered chronic infection with multiply resistant *P. aeruginosa* to be a contraindication to transplantation. Unlike patients with CF and chronic endobronchial disease, transplantation patients can acquire bacteremia with *P. aeruginosa*. As synergy testing reflects antibiotic levels achievable in serum, the efficacy of the active antibiotic combinations may be greater in transplant recipients and should be studied.

This is an era in which widespread antibiotic resistance is acknowledged as a growing crisis. In the coming decades, the number of patients with CF who are infected with multiply resistant organisms will most likely increase. Thus, it is critical to monitor these trends of resistance, to delay the emergence of resistance if possible, and to find effective means to treat such pathogens. At present there are limited anti-*Pseudomonas* agents under development, and these studies suggest that aerosolized aminoglycosides and synergy testing of antibiotic combinations may represent viable therapeutic options.

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**References**