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Accuracy and Cost of Antibiotic Susceptibility Testing of Mixed Morphotypes of *Pseudomonas aeruginosa*

GREGORY L. MORLIN,[1]* DONNA L. HEDGES,[2] ARNOLD L. SMITH,[1] AND JANE L. BURNS[1]

Division of Infectious Disease, Department of Pediatrics, University of Washington School of Medicine,[1] and Division of Pulmonary Medicine, Children's Hospital and Medical Center,[2] Seattle, Washington 98105

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Chronic *Pseudomonas aeruginosa* colonization of the lower respiratory tract of patients with cystic fibrosis frequently results in pulmonary exacerbations requiring treatment with antimicrobial agents. Multiple morphotypes with different antibiotic susceptibilities are often isolated from a single sputum sample. Determination of MICs of antibiotics for each sputum morphotype is used to guide therapy but is time-consuming and expensive. We explored an alternative assay for determining MICs for all *P. aeruginosa* morphotypes cultured from a homogenized sputum sample. We sought correlations of those MICs with the MIC for the most resistant morphotypes tested separately. The MICs determined for a mixture of morphotypes correctly predicted the highest MICs (± one dilution) determined for isolated morphotypes 73.5% of the time. The MIC for the mixed morphotypes correctly predicted susceptibility in 90.4% of samples. In contrast, determination of the MIC for the mixture of morphotypes correctly predicted resistance in only 57.0%. For sputa containing susceptible isolates, testing the mixed culture may provide adequate susceptibility data with significant laboratory time and cost savings. However, for sputa with resistant strains, the traditional method of testing isolated morphotypes should still be used.

Pseudomonas aeruginosa is an important endobronchial pathogen associated with morbidity and mortality in patients with cystic fibrosis (CF). Chronic *P. aeruginosa* colonization and subsequent pulmonary exacerbations may require therapy with antimicrobial agents (5). Because of the frequent administration of antibiotics in CF and the high incidence of multiple antibiotic resistance in *P. aeruginosa*, it is necessary to determine the antibiotic susceptibility of all isolates recovered from CF sputum. However, multiple morphotypes with differing susceptibilities are often isolated from a single sputum specimen (1). Currently, susceptibility testing is based on isolating individual *P. aeruginosa* morphotypes from sputum and determining the antibiotic susceptibility profile for each by disk diffusion or quantitative susceptibility testing. Although antibiotic susceptibility testing of *P. aeruginosa* is useful in choosing antimicrobial agents, determination of MICs for multiple *P. aeruginosa* morphotypes prolongs the interval until potentially active antibiotic therapy can be instituted.

Multiple *P. aeruginosa* morphotypes, distinguishable by color, size, and texture, are often identified in a sputum sample from a single CF patient. Phenotypically distinct colony types may exhibit different antibiotic resistance patterns over time (8). Consequently, antibiotic susceptibility testing is usually performed on all *P. aeruginosa* morphotypes isolated from a sputum sample. Because susceptibility may change in serial isolates, several studies (4, 6, 9) have proposed circumventing tedious isolation procedures by testing a mixed culture of *P. aeruginosa* rather than each individual morphotype. Maybury et al. (6) found that a mixed inoculum of morphologically different *P. aeruginosa* had susceptibilities which fell within one dilution of the MIC for the most resistant morphotype when each had been tested separately. Other authors have demonstrated a correlation between the susceptibility of mixed and isolated morphotypes tested by disk diffusion (4) and broth microdilution (9) assays. On the basis of these data, we questioned whether determination of MICs for *P. aeruginosa* from homogenized sputum growing on selective media would reflect the MIC for the most resistant *P. aeruginosa* morphotype. We compared the MIC determined for the mixture of *P. aeruginosa* from sputum directly plated on cetrimide agar with the MICs determined for the most resistant morphotype.

MATERIALS AND METHODS

Subjects. Ninety-four sputum cultures from patients with CF who were seen and treated at the CF center at the Children's Hospital and Medical Center, Seattle, Wash., between June 1988 and July 1992 were processed as described previously (11). An average of two morphotypes were isolated from each culture, with 34, 50, 17, and 2% of the cultures harboring one, two, three, or four morphotypes, respectively. Patient data were excluded if they were incomplete because of low bacterial density ($<10^3$ CFU/g) or if *P. aeruginosa* was not isolated. Carbenicillin was analyzed for only 86 specimens; susceptibility testing with this antibiotic was discontinued in October 1991.

Laboratory procedures. Sputum specimens (~0.5 g) were treated in a similar manner for both mixed- and isolated-*P. aeruginosa*-morphotype MIC methods. The samples were diluted 2:1 in 6.5 mM dithiothreitol (Sputolysin; Calbiochem) and vortexed for 5 min to ensure homogenization. In the procedure for mixed *P. aeruginosa* morphotypes, a 0.1-ml aliquot of sputum homogenate was spread on a *P. aeruginosa*-selective cetrimide agar plate (Difco, Detroit, Mich.), which was subsequently incubated at 37°C. After 24 h of incubation, the lawn of mixed *P. aeruginosa* morphotypes was harvested and used as the inoculum for semiautomated antibiotic susceptibility testing.

For isolated-morphotype MICs, three serial 100-fold dilutions of the original sputum homogenate (to a final dilution of 10^{-6}) were made in phosphate-buffered saline, pH 7.4, and a 0.1-ml aliquot of each dilution was spread on cetrimide agar. The plates were incubated at 37°C for 48 h. Distinct bacterial

* Corresponding author. Mailing address: 4800 Sand Point Way N.E., Seattle, WA 98105. Phone: (206) 526-2116.

morphotypes were identified by size, color, and texture. Each morphotype was subcultured to ensure purity. After 48 h of incubation, the MIC of each morphotype was determined. Dilutions of each sputum homogenate were also spread on MacConkey agar (Difco) and oxidation-fermentation base supplemented with agar, lactose, and two antimicrobial agents (10) to enable the identification of other gram-negative organisms which might grow on cetrimide agar. Eleven sputum specimens contained non-*P. aeruginosa* gram-negative bacteria, including *Pseudomonas cepacia* and *Xanthomonas maltophilia*. These isolates were streaked onto cetrimide agar, and none grew.

MIC determination. MICs were determined with the Sensititre system (Radiometer, Westlake, Ohio) for both mixed and isolated *P. aeruginosa* morphotypes. A loopful of bacterial growth was dispersed in 6 ml of sterile water. The optical density was adjusted to an equivalent of 1×10^8 CFU/ml (A_{625} = 0.08 to 0.1). A 1:1,000 dilution was made in Mueller-Hinton broth, resulting in approximately 1×10^5 CFU/ml. Aliquots of 50 μl were dispensed into commercially prepared (Sensititre) microdilution wells. This assay used a 96-well plate containing twofold serial dilutions of the following antimicrobial agents: amikacin, 0.5 to 64 μg/ml; carbenicillin, 2.0 to 512 μg/ml; ceftazidime, 1.0 to 2,048 μg/ml; ciprofloxacin, 0.5 to 4.0 μg/ml; gentamicin, 0.25 to 512 μg/ml; ticarcillin, 2 to 4,096 μg/ml; and tobramycin, 0.25 to 512 μg/ml. The tray was incubated at 37°C for 18 to 24 h. After incubation, the microtiter plates were examined on a Sensititre light box and the MIC was determined as the lowest concentration at which the antibiotic inhibited visible bacterial growth. A single determination was made for each sample.

Data analysis. All comparisons of susceptibility data for mixed and isolated *P. aeruginosa* morphotypes used the results for isolated, morphologically distinct colonies as the reference standard. Interassay comparisons were made by calculating the discrepancies (number of twofold dilutions or single-well differences) between the MIC determined for an individual morphotype and the MIC determined for mixed morphotypes. Resistance levels were defined according to National Committee for Clinical Laboratory Standards guidelines (6): MICs of amikacin (≥64 μg/ml), carbenicillin (≥512 μg/ml), ceftazidime (≥32 μg/ml), ciprofloxacin (≥4 μg/ml), gentamicin (≥8 μg/ml), ticarcillin (≥128 μg/ml), and tobramycin (≥8 μg/ml) defined resistance.

Because selection of the optimum antibiotic therapy is the goal of susceptibility testing, two interpretations of the data were considered clinically important: (i) whether the direct sputum MIC was predictive of susceptibility or resistance as determined by the isolated MICs and (ii) how frequently the direct sputum MIC falsely identified the isolated organisms as susceptible. The first of these measures we defined as predictive of susceptibility or predictive of resistance. The calculation of these two values is similar to the calculation of specificity and sensitivity (3): predictive of susceptibility = true susceptibles/(true susceptibles + false resistants); predictive of resistance = true resistants/(true resistants + false susceptibles). The percentage of samples in which the mixed-morphotype method falsely predicted that the individual isolates were susceptible was also calculated for each sputum.

Cost calculations. The cost and the length of time required for completion of susceptibility testing for each protocol were compared. Time considerations were based on the assumption of an 18- to 24-h incubation period for *P. aeruginosa* growth. Expenses were compared as the costs for repeated or additional procedures required to determine the MIC for an isolated morphotype in comparison with testing a mixture of morphotypes. Cost estimates were based on the expenditures necessary for assay kits and reagents, culture media, and technician salary time.

TABLE 1. Differences in MICs when comparing mixed morphotypes with isolated colonies

Antibiotic	No. of strains[a] within range									Correlation coefficient
	More susceptible			No difference			More resistant			
	>−4[b]	−3	−2	−1	0	1	2	3	4	
Amikacin	2	4	1	20	24	24	10	6	3	0.70
Carbenicillin	6	1	5	12	36	12	5	1	0	0.65
Ceftazidime	7	2	7	14	33	14	8	6	3	0.48
Ciprofloxacin	0	3	4	8	58	12	6	3	0	0.61
Gentamicin	2	1	2	16	45	14	7	4	3	0.78
Ticarcillin	7	5	4	11	39	11	2	6	9	0.64
Tobramycin	3	1	3	10	47	18	7	3	2	0.75

[a] n = 94, except for the carbenicillin data (n = 86).
[b] Fold difference. Fold difference denotes the number of twofold-dilution differences between mixed and isolated *P. aeruginosa* morphotype MICs. A positive fold dilution difference suggests a more resistant mixed-morphotype MIC. A negative fold dilution suggests a more susceptible mixed-morphotype MIC.

RESULTS

MIC correlation. The correlations of MICs determined by the direct sputum method with the MICs determined for isolated morphotypes are listed in Table 1. A positive fold dilution difference suggested that the mixed culture was more resistant. A negative fold dilution difference suggested that the mixed culture was more susceptible. The accepted variability in MIC determination is ±1 twofold dilution (2). The mixed culture method correctly predicted the actual MIC ±1 dilution 73.5% of the time.

Accuracy of identification of susceptibility. Table 2 depicts the ability of the MIC determined for mixed morphotypes to accurately predict whether isolated morphotypes will be susceptible or resistant to a given antibiotic. The method had a greater ability to predict susceptibility (overall mean = 90.4%) than to predict resistance (overall mean = 57.0%). The percentage of specimens falsely predicted to be susceptible ranged from 5 to 17%.

Cost. The expense and time differences between MICs determined for individual morphotypes compared with the mixed-morphotype method are listed in Table 3. Because the average number of morphotypes isolated from each sputum specimen in this study was two, the example was calculated for two isolates. Determination of the MICs for individual morphotypes requires the additional steps of isolating colonies by

TABLE 2. Predictability of MIC determination for a mixture of morphotypes

Antibiotic	% Predictive of susceptibility	% Predictive of resistance	% Falsely identified as susceptible
Amikacin	89.3	70.0	8
Carbenicillin	94.7	36.4	14
Ceftazidime	88.6	46.7	17
Ciprofloxacin	87.0	41.7	7
Gentamicin	93.9	83.0	5
Ticarcillin	82.9	50.0	17
Tobramycin	96.6	71.4	7

TABLE 3. Cost and time comparisons for the mixed-morphotype MIC determination versus the isolated-morphotype MIC determination for two morphotypes

Procedure	Cost		Time (h)	
	Isolated	Mixed	Isolated	Mixed
Sample handling and incubation	$100	$33	24	24
Incubation	NE[a]	NE	24	NT[b]
Isolation and purification	$33	NE	24	NT
MICs	$56	$28	24	24
Total	$189	$61	4 days	2 days

[a] NE, no extra expense necessary.
[b] NT, no extra time necessary.

dilution and overnight incubation on selective media and subculturing those colonies prior to MIC determination. Thus, the mixed-morphotype method can be performed for $128 less and be available two days earlier than determination of the MICs for two distinct strains. The cost, but not time, would be increased for increased numbers of morphotypes.

DISCUSSION

Selection of appropriate antibiotic therapy for the treatment of CF patients colonized with *P. aeruginosa* may be difficult when susceptibility data are unavailable. Current methods of isolating and testing individual colony types from a sputum sample are expensive and time-consuming. This study explores an alternative technique for determining susceptibility based on MICs determined with a mixture of *P. aeruginosa* morphotypes from homogenized sputum samples. Other studies (4, 6, 9) have shown a correlation between the MICs for isolated *P. aeruginosa* colonies and the MICs for a mixture of those colonies. In contrast to previous work, our method correlates the MIC for the most resistant isolated *P. aeruginosa* morphotype from a single sputum culture with the MIC for a mixture of *P. aeruginosa* morphotypes cultured directly from a sputum homogenate plated onto selective media.

Similar studies comparing the MICs for mixed forms with isolated colonies have used both disk diffusion (4) and broth microdilution (6, 9) assays. A correlation of greater than 90% was achieved with approximately 50 specimens in each of the studies mentioned above (4, 6, 9). Among those specimens with discordance, 65% of all discrepancies were MIC results falsely indicating susceptibility. In contrast, using 94 specimens our mixed method of determining MICs for all strains in sputum homogenates correlated with the MIC for an isolated organism 74% of the time, within a twofold dilution. Discrepant results had a greater likelihood of overestimating the MIC for the most resistant *P. aeruginosa* isolate.

Discrepancies between the MICs determined for isolated colonies versus those for mixed morphotypes need to be questioned. Although the standard method using isolated colonies is considered the gold standard, it is possible that the sputum homogenate technique is more representative of what is going on in the lung. Possible variables which might affect MIC results (inoculum size, phase of growth, test medium, and drug stability) were constant for all samples. However, MICs for a mixture of *P. aeruginosa* morphotypes discrepant with that of the reference standard may have been caused by non-*P. aeruginosa* pathogens or endogenous factors present in sputum homogenates. Although cetrimide agar is selective for *P. aeruginosa*, after incubation the cetrimide plate may continue to harbor nonreplicating organisms. Other gram-negative organisms, such as *P. cepacia* and *X. maltophilia*, may be carried over in inoculating the MIC broth from the cetrimide plate. When 11 of the 94 sputums which contained *P. cepacia* and *X. maltophilia* were eliminated, the adjusted predictive values of susceptibility and resistance to all antibiotics were 91 and 69%, respectively. Without samples containing these bacteria, which are intrinsically resistant to β-lactams and aminoglycosides, the likelihood of correctly identifying a sputum containing a resistant morphotype increased by 12%. Because synergism and antagonism by other sputum components may alter susceptibility in vivo as well as in vitro, it is possible that the MIC for the mixture of morphotypes freshly plated from a sputum homogenate best represents the relative quantity and phenotype of organisms causing pulmonary infection. Clinical correlation should be performed to determine whether the MIC for mixed morphotypes is a more accurate estimate of in vivo susceptibility within the lung.

In addition to the potential benefit of more accurately predicting bacterial susceptibility within the milieu of the CF lung, the technique of determining *P. aeruginosa* MICs directly from selectively plated sputum homogenates is easier, less expensive, and more rapid than the standard method of determining the MIC for each individual morphotype. The accurate prediction of susceptibility is more clinically relevant than the accurate prediction of resistance. The ability of the mixed-morphotype method to predict susceptibility and resistance, as defined, does not change with the prevalence of antibiotic resistance at a given CF center. However, the method will be most cost-effective when used at centers, such as ours, where the incidence of resistance is low. For susceptible *P. aeruginosa*, the mixed-morphotype method will provide adequate susceptibility data with significant cost and time savings. For resistant isolates, the traditional method should still be used.

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