ANTIMICROBIAL RESISTANCE

Mixed morphotype testing of *Pseudomonas aeruginosa* cultures from cystic fibrosis patients

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Summary. Bronchial secretions of patients with cystic fibrosis (CF) inevitably become colonised with *Pseudomonas aeruginosa*. This organism often exhibits multiple phenotypes with different antibiotic susceptibility profiles. Isolating each colonial morphotype and determining its antibiotic susceptibility profile is labour-intensive and time-consuming. Two disk diffusion methods for mixed morphotype susceptibility testing were examined; 134 morphotypes of *P. aeruginosa* from 50 respiratory specimens from CF patients were tested. Mixed cultures were prepared by (a) combining morphologically different colonies of *P. aeruginosa* directly from the sputum specimen primary culture plates from individual patients or (b) mixing colonies of individual morphotypes after isolation and subculture. Agreement with the results for testing morphotypes individually were 85.8 % and 91.6 %, respectively, for the two methods. These agreement rates rose to 95.6 % and 99.4 %, respectively, when minor errors (intermediate misclassified as susceptible or resistant or *vice versa*) were excluded. Mixed morphotype testing of *P. aeruginosa* directly from sputum specimen culture plates in chronically colonised CF patients is more efficient, reduces turn-around time and provides clinically meaningful information about antibiotic susceptibility.

Introduction

The bacteriological milieu of the cystic fibrosis (CF) lung is complex and incompletely understood. Most CF patients become chronically colonised with *Pseudomonas aeruginosa*, often carrying single strains that exhibit multiple morphological characteristics and that vary in antibiotic susceptibility. Chromosomal mutations *in vivo* may change antibiotic susceptibility without affecting colonial morphology.¹ As a result, some authors have recommended individual susceptibility testing of every isolate obtained^{2,3} and it is common practice to attempt to isolate and test the susceptibility of each colonial morphotype in any CF sputum specimen. Frequently, the laboratory combines the results and reports only the most resistant profile to the clinician. When these susceptibility profiles are used to guide antibiotic treatment during acute infective exacerbations of respiratory disease and hospitalisation, unduly pessimistic susceptibility data may lead to the use of unnecessarily toxic and expensive antibiotics. Moreover, such individual morphotype susceptibility testing is labour-intensive and time-consuming. Dunne *et al.*⁴ and Van Horn,⁵ using

disk diffusion and broth microdilution methods, respectively, found that the susceptibilities determined for mixtures of different morphotypes were equivalent to results for testing individual types. Nevertheless, both these studies required that the morphotypes of *P. aeruginosa* be identified and subcultured before mixed testing, thereby increasing the workload and delaying availability of results to the clinician. In this study mixed testing of subcultured morphotypes was compared with direct testing of multiple morphotypes from the original culture plate. If the results of testing directly from the specimen plate could be shown to be comparable to other methods it would result in considerable savings in time and materials for the laboratory, and quicker availability of results to the clinician.

Materials and methods

Isolates

Fifty sputum samples yielding more than one colonial morphotype of *P. aeruginosa* were collected from 23 patients with CF. *P. aeruginosa* was identified on MacConkey agar by colonial type, absence of lactose fermentation, and oxidase positivity.⁶ After

Table I. Percentages of susceptible, resistant and intermediate organisms based on tests with the 134 individual morphotypes

Status	Percentage of isolates tested									
	Cip	Nor	Ak	Gm	Tob	Caz	Tic	Pip	Atm	Imp
Sensitive	28	24	8	4	24	42	40	38	30	42
Resistant	52	58	90	90	76	50	60	62	48	52
Intermediate	20	18	2	6	0	8	0	0	22	6

Cip, ciprofloxacin; Nor, norfloxacin; Ak, amikacin; Gm, gentamicin; Tob, tobramycin; Caz, ceftazidime; Tic, ticarcillin; Pip, piperacillin; Atm, aztreonam; Imp, imipenem.

subculture on to Horse Blood Agar (Columbia Agar Base, Oxoid CM331, containing horse blood 5%), identification was confirmed by β -haemolysis and odour. Pigment production was also noted on Mueller-Hinton agar during disk diffusion susceptibility testing. Any isolates not fulfilling the above criteria were identified by Vitek Systems-240 (GNI card). "Mucoid" and "coliform" colony types were most commonly encountered, "rough" and "typical" types less often.⁶

Antibiotic susceptibility testing was performed by disk diffusion in accordance with the method of the National Committee for Clinical Laboratory Standards (NCCLS)⁷ with antibiotic disks (Oxoid) as follows: gentamicin 10 μ g (Gm), tobramycin 10 μ g (Tob), amikacin 10 μ g (Ak), ceftazidime 30 μ g (Caz), ciprofloxacin 5 μ g (Cip), norfloxacin 10 μ g (Nor), piperacillin 100 μ g (Pip), imipenem 10 μ g (Imp), aztreonam 30 μ g (Atm) and ticarcillin 75 μ g (Tic). Zones were graded as sensitive, moderately sensitive, intermediate or resistant according to the NCCLS criteria.⁸

Preparation of inocula for susceptibility testing

Individual colonial morphotypes were identified and subcultured on to horse blood agar. Antibiotic susceptibility tests were performed for a total of 134 morphotypes isolated from the 50 specimens. For each

combination of sputum specimen and antimicrobial agent, the morphotype with the greatest resistance was noted and the zones observed were taken as the "predicted antibiogram" to which the zones obtained in mixed morphotype testing were compared.

Mixed inocula were prepared by two methods, referred to as "specimen plate" and "pure plate" methods. Specimen plate cultures involved selection of colonial morphotypes directly from the original MacConkey agar plates for each respiratory culture. Non-lactose fermenting colonies with positive oxidase reaction and different morphological characteristics were removed with a sterile swab, mixed together in sterile normal saline 0.9% w/v and the suspension was adjusted to the opacity of a 0.5 McFarland turbidity standard. Pure plate cultures were prepared by mixing colonies from subcultures of each colonial morphotype isolated from a single sputum sample. These were mixed together in normal saline with a sterile swab and the suspension was adjusted to the turbidity of a 0.5 McFarland turbidity standard.

Data analysis

Susceptibilities for the 50 specimen plate cultures and the 50 pure plate cultures were examined and the zones of inhibition were recorded and compared with the predicted antibiogram by correlation regression analysis. Inhibition zone diameters were recorded and grouped by antibiotic class. The correlation coefficient (r) and linear regression line ($y = ax + b$) were calculated for each antibiotic class (quinolones, aminoglycosides and β -lactams). Disagreements between mixed morphotype results and predicted antibiograms were recorded as false susceptibility (FS) when mixed testing indicated as susceptible an organism that had been found to be resistant when tested in pure culture, and as false resistance (FR) when resistance was found by mixed testing but not when individual morphotypes were tested. Cases where the categorisation varied between intermediate or moderately susceptible in the predicted antibiogram and sensitive or resistant in mixed testing, or *vice versa*, were classed as minor

Table II. Agreement of specimen plate and pure plate mixed cultures with the predicted antibiograms

Number of morphotypes/ test	Number tested	Total number of tests		Minor errors (%)		FS (%)		FR (%)	
		Specimen	Pure	Specimen	Pure	Specimen	Pure	Specimen	Pure
2	26	259	260	24 (9.3)	17 (6.5)	5 (1.9)	0	6 (2.3)	1 (0.38)
3	16	160	160	15 (9.4)	14 (8.8)	4 (2.5)	0	2 (1.3)	1 (0.63)
4	7	70	70	9 (12.9)	7 (10.0)	0	0	5 (7.1)	0
5	1	10	10	1 (10.0)	1 (10.0)	0	0	0	1 (10.0)
Total	50	499	500	49 (9.8)	39 (7.8)	9 (1.8)	0	13 (2.6)	3 (0.60)

Table III. Distribution of errors in pure plate and specimen plate mixed cultures

Antibiotic	Total number of tests		Minor errors (%)		FS (%)		FR (%)	
	Specimen	Pure	Specimen	Pure	Specimen	Pure	Specimen	Pure
Caz	50	50	5 (10)	4 (8)	1 (2)	0	2 (4)	0
Cip/Nor	100	100	19 (19)	17 (17)	1 (1)	0	1 (1)	1 (1)
Pip/Tic	99	100	N/A	N/A	3 (3)	0	7 (7.1)	2 (2)
Ak/Gm/Tob	150	150	6 (4.0)	6 (4)	2 (1.3)	0	2 (1.3)	0
Atm	50	50	11 (22)	10 (20)	0	0	1 (2)	0
Imp	50	50	8 (16)	2 (4)	2 (4)	0	0	0
Total	499	500	49 (9.8)	39 (7.8)	9 (1.8)	0	13 (2.6)	3 (0.6)

N/A, not applicable as NCCLS does not have intermediate categories for these agents; other abbreviations as in table I.

Fig. 1. Scattergrams comparing predicted antibiogram inhibition zones with zones obtained by (a) specimen plate ($y = 0.89x + 1.7$; $r = 0.84$) and (b) pure plate methods ($y = 1.03x + 0.45$; $r = 0.93$). Pooled data for norfloxacin and ciprofloxacin.

Fig. 2. Scattergrams comparing predicted antibiogram inhibition zones with zones obtained by (a) specimen plate ($y = 1.01x + 0.19$; $r = 0.87$) and (b) pure plate methods ($y = 0.97x + 0.52$; $r = 0.92$). Pooled data for aminoglycosides (gentamicin, tobramycin and amikacin).

errors. Error rates between the two methods were compared by the χ^2 test.

Results

A total of 134 morphotypes was obtained from the 50 sputum cultures. These represented a wide range of antibiotic susceptibilities (table I). Of the 50 sputum

cultures, 26 yielded two morphotypes, 16 yielded three, seven yielded four and one gave five types. Correlation of the results of mixed testing with the predicted antibiogram did not appear to be influenced by the number of morphotypes mixed together (table II).

Four hundred and ninety-nine (one inoculation error) specimen plate mixed morphotype tests were performed. There were nine FS, 13 FR and 49 minor

Fig. 3. Scattergrams comparing predicted antibiogram inhibition zones with zones obtained by (a) specimen plate ($y = 0.96x + 1.43$; $r = 0.86$) and (b) pure plate methods ($y = 1.02x + 0.17$; $r = 0.95$). Pooled data for β -lactams (piperacillin, ticarcillin, ceftazidime, aztreonam and imipenem).

errors, giving 85.8% agreement between observed and predicted susceptibilities (95.6% excluding minor errors). Five hundred pure plate mixed morphotype tests were performed. There were three FR errors and 39 minor errors. Agreement between the mixed tests and the predicted antibiogram thus was 91.6% (99.4% when minor errors were excluded). These results compare favourably with the data obtained by disk diffusion mixed testing by another group.⁴ The rate of FS and FR errors was significantly higher in the specimen plate than in the pure plate method ($p < 0.01$).

Nine of the FR and three of the FS errors occurred with piperacillin and ticarcillin (table III). The NCCLS, whose criteria were used, does not recognise intermediate or moderately susceptible zone categories for these disks when used against *P. aeruginosa*, so minor variation in zone diameter meant sensitive organisms were misclassified as resistant and *vice versa*.

Figs. 1–3 illustrate correlation and linear regression between predicted inhibition zone diameters and zone diameters of specimen plate and pure plate methods for the three classes of antibiotics (β -lactams, aminoglycosides and quinolones). For all antibiotic classes,

correlation was higher between the predicted antibiogram and pure plate method than between the predicted antibiogram and the specimen plate method.

Discussion

Clinical deterioration in CF has been associated with colonisation by *P. aeruginosa*, often of a mucoid phenotype, and antibiotic therapy is largely directed at this organism. In the chronically colonised individual, clinical improvement is seldom associated with eradication of the organism.^{9,10} At the first exposure to antipseudomonal antibiotics, a significant reduction in the bacterial load of the sputum has been demonstrated, but this reduction becomes less marked on repeated therapy, although clinical improvement often still occurs.^{11,12} In many cases, clinical improvement may occur regardless of in-vitro resistance to the antimicrobial agents used.^{13,14} Nevertheless, antibiotic selection is based, at present, on laboratory susceptibility testing by conventional techniques and with reference to susceptibility standards. Laboratory time is spent isolating, subculturing and testing each morphotype. Unfortunately, the sputum sampling may be representative only of regional lung flora and overgrowth of mucoid variants on agar may obscure clinically significant non-mucoid colonial types with different susceptibility profiles. Therefore, only limited useful information can be obtained from these susceptibility results and the results cannot be regarded as an accurate picture of the in-vivo state. Nevertheless, antibiotic susceptibility testing of *P. aeruginosa* from chronically colonised patients remains the only available way of providing the clinician with guidelines for appropriate antibiotic therapy and of monitoring the emergence of antibiotic resistance during therapy. Susceptibility testing is most meaningful in the non-chronically colonised individual, where appropriate antibiotic therapy may eradicate *P. aeruginosa* and delay chronic colonisation.¹⁵

In CF patients chronically colonised with *P. aeruginosa*, it is reasonable to limit extensive susceptibility testing and adopt a simpler method that will give comparable results. Mixed morphotype testing has been shown to produce useful results when each morphotype is isolated, subcultured and mixed in equal quantities.^{4,5} This was confirmed by the present study which demonstrated that mixing pure types together in unequal quantities produced similar results (pure plate method). Furthermore, the present study also showed that mixing colonial morphotypes directly from primary cultures produced inhibition zone diameters that correlated well with predicted zone size. Although specimen plate testing correlated less well with the predicted antibiogram than did pure plate testing we believe that, in this unique CF population where susceptibility test results are of variable significance, the specimen plate testing is adequate, and has the virtue of rapidity.

FR errors were more common than FS errors for the specimen plate method and may represent contamination by resistant non-*P. aeruginosa* organisms inadvertently removed from the mixed sputum culture plate. In the clinical setting, FR and minor errors have little impact on the clinical outcome of treatment. In the non-CF patient, reporting a resistant organism as sensitive after susceptibility testing may lead to incorrect antibiotic choice and disastrous consequences. In CF patients, clinical improvement is not always related to in-vitro resistance or susceptibility, so FS errors are much less significant.

This study has compared various methods of processing sputa from CF patients in the laboratory.

Mixed susceptibility testing of flora directly removed from specimen plates without purity plating offers several advantages. For the clinician, results are available 24–28 h earlier and in the laboratory there are savings in resources and technician time. We conclude that mixed morphotype testing directly from respiratory specimen plates is adequate for testing antibiotic susceptibility of *P. aeruginosa* in chronically colonised CF patients and could be used by laboratories as a routine procedure.

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