Recovery of antimicrobial-resistant *Pseudomonas aeruginosa* from sputa of cystic fibrosis patients by culture on selective media

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Objectives: To assess the utility of direct plating of whole sputa onto selective media as a means of identifying antimicrobial resistance in strains of *Pseudomonas aeruginosa* from the sputa of patients with cystic fibrosis (CF).

Methods: A total of 45 sputum samples from CF patients were cultured onto conventional culture media for isolation of *P. aeruginosa* and were also cultured directly onto Iso-Sensitest agar plates containing each of 10 antimicrobials incorporated at a 'breakpoint' concentration. A representative of each colonial type (morphotype) recovered from both routine media and selective media was tested for its susceptibility to 10 antimicrobials using a standard agar dilution MIC technique.

Results: Of the samples shown to contain resistant strains, the proportion (%) detected using routine media and selective media, respectively, was: 42 and 100 for amikacin, 57 and 100 for gentamicin, 54 and 100 for tobramycin, 88 and 77 for aztreonam, 62 and 90 for ceftazidime, 70 and 97 for meropenem, 61 and 100 for piperacillin/tazobactam, 80 and 90 for ticarcillin/clavulanic acid, 90 and 86 for temocillin, 66 and 100 for ticarcillin/clavulanic acid, and 80 and 90 for ciprofloxacin resistance. The increased rates of isolation on selective media were statistically significant \((P < 0.05)\) for amikacin, gentamicin, tobramycin, meropenem, piperacillin/tazobactam and ticarcillin/clavulanic acid.

Conclusions: For most antimicrobials, selection of colonies from conventional media for antimicrobial susceptibility testing provided a considerable underestimation of resistance in *P. aeruginosa*. The use of selective media for the culture of whole sputum was effective for the detection of resistant isolates of *P. aeruginosa*.

Keywords: susceptibility testing, minimum inhibitory concentration, antimicrobial resistance

Introduction

Chronic lung colonization with *Pseudomonas aeruginosa* contributes significantly to the morbidity and mortality of patients with cystic fibrosis (CF), and appropriate antimicrobial chemotherapy remains important in the management of acute episodes of infection. Conventional susceptibility testing involves the selection of individual colony variants or 'morphotypes', or the testing of a mixture of colony variants ('mixed morphotype testing'), typically followed by disc susceptibility testing.1–3

In a recent report, Foweraker et al.1 questioned the value of conventional antimicrobial susceptibility testing of *P. aeruginosa* once it becomes established in the CF lung. The authors examined the susceptibility of *P. aeruginosa* colonies recovered from CF sputa and found that, from colonies belonging to a single morphotype, an average of three antibiograms could be demonstrated. The authors also demonstrated that when a mixture of morphotypes was prepared as a single inoculum for susceptibility testing, the results correlated very poorly with those obtained by testing each morphotype individually. The aim of this study was to evaluate direct susceptibility testing of CF sputa and compare with conventional susceptibility testing for detection of resistance in *P. aeruginosa*.

Materials and methods

Materials

Unless otherwise stated, all media were prepared in-house from dehydrated powders supplied by Oxoid Ltd, Basingstoke, UK,
according to the manufacturer’s instructions. *Pseudomonas CN* selective agar (44171) was supplied as pre-poured plates from bioMérieux, Basingstoke, UK. API 20 NE strips were also obtained from bioMérieux. Horse blood was obtained from TCS Biosciences Ltd, Buckingham, UK. 9-Chloro-9-[4-(diethylamino)phenyl]-9,10-dihydro-10-phenylacridine hydrochloride (C-390) was obtained as a powder from Biosynth AG, Switzerland, and 1,10-phenanthroline dihydro-10-phenylacridine hydrochloride (C-390) was obtained from Sigma Chemical Company. Other antimicrobials were obtained from their respective manufacturers. *P. aeruginosa* (NCTC 10662) and *Escherichia coli* (NCTC 10418) were obtained from the National Collection of Type Cultures, Colindale, London, UK.

**Patient samples**

All sputum samples were from distinct patients with CF (mean age: 25 years; range 5–53 years). Samples were pre-selected as being likely to contain *P. aeruginosa* on the basis of laboratory results of sputum culture obtained within the previous 3 months.

**Routine culture**

Each of the 45 sputa was treated with an equal volume of sputasol (SR0233A; Oxoid Ltd) until visibly homogeneous. Typically, this required 30 min at room temperature with regular vigorous agitation using a vortex mixer. A 10 µL sample of homogenized sputum was then inoculated onto ‘routine media’, which comprised Columbia blood agar supplemented with 5% horse blood, chocolate agar supplemented with 70 µg/mL bacitracin, cystine lactose electrolyte deficient agar, *Pseudomonas CN* selective agar and Iso-Sensitest agar as a control. Sputum samples were then further diluted 1000-fold by addition of 10 µL of homogenized sputum to 9.99 mL sterile distilled water. After thorough mixing, this was then cultured onto the same range of media using 10 µL aliquots. All routine media were incubated for 72 h at 37°C and examined after each 24 h incubation period. At each stage, a description of each morphotype on each individual medium was recorded and an ongoing assessment was made of the number of distinct colonial variants present on the routine media. Subculture of distinct morphotypes involved touching a single colony and inoculating a Columbia blood agar plate that was incubated at 37°C for 24–72 h as required. These subcultures were used for MIC testing and also for immediate storage in 10% glycerol at −80°C. Only one subculture was permitted prior to MIC testing.

**Culture on selective media**

A 10 mg sample of ‘active’ antimicrobial was weighed out and dissolved in 10 mL of sterile distilled water to give a stock solution of 1000 mg/mL. This was further diluted in Iso-Sensitest agar at 45°C to produce 100 mL agar containing a breakpoint concentration of antimicrobial. For example, 1.6 mL of 1000 mg/mL amikacin was added to 98.4 mL of Iso-Sensitest agar and well mixed. Plates were poured in 20 mL volumes containing a final concentration of 16 mg/L. Breakpoint concentrations that defined resistance or intermediate resistance were incorporated into agar so that any colonies growing on such agar plates should generally be resistant. Where available, the choice of breakpoint was guided by recommended breakpoints for *P. aeruginosa* published by the BSAC. A breakpoint of 16 mg/L was selected for temocillin as recommended by Fuchs et al. The following concentrations of antimicrobials were incorporated into Iso-Sensitest agar: amikacin (16 mg/L), gentamicin (4 mg/L), tobramycin (4 mg/L), aztreonam (8 mg/L), cefazidime (8 mg/L), meropenem (4 mg/L), piperacillin/tazobactam (16 mg/L), temocillin (16 mg/L), ticarcillin/clavulanic acid (32 mg/L) and ciprofloxacin (1 mg/L).

A 10 µL aliquot of undiluted homogenized sputum was inoculated onto a single plate for each test antimicrobial and the inoculum was spread to obtain single colonies (10 selective plates in total per sputum sample). All plates were incubated and interpreted as described above for routine culture plates. All distinct morphotypes from all selective media were referred for MIC testing following a single subculture on Columbia blood agar.

**MIC determination**

For any particular sputum sample, all morphotypes from both routine culture media and selective media were tested concomitantly in the same batch of MIC tests. MICs were performed using a standard agar dilution methodology. Brieﬂy, antimicrobials were incorporated into Iso-Sensitest agar and poured in 20 mL volumes into Petri dishes to produce agar plates containing the following concentration ranges: amikacin (64-2 mg/L), gentamicin (16-0.5 mg/L), tobramycin (16-0.5 mg/L), aztreonam (32-1 mg/L), cefazidime (32-1 mg/L), meropenem (16-0.5 mg/L), piperacillin/tazobactam (64-2 mg/L), temocillin (64-2 mg/L), ticarcillin/clavulanic acid (128-4 mg/L) and ciprofloxacin (4-0.125 mg/L). Each isolate was suspended in sterile distilled water and adjusted to a density equivalent to that of a 0.5 McFarland standard (~1.5 × 10^8 cfu/mL) using a densitometer. Each suspension was then diluted 1/10 in sterile distilled water and 1 µL was inoculated onto plates containing antimicrobial, using a multipoint inoculator (final inoculum ~1.5 × 10^4 cfu). Iso-Sensitest agar without antimicrobial was also inoculated and all plates were inoculated with *P. aeruginosa* (NCTC 10662) and *E. coli* (NCTC 10418) as control strains of known MIC. All plates were incubated for 48 h at 37°C in air and the MIC for each isolate was recorded after 24 and 48 h of incubation. All strains showed growth on Iso-Sensitest agar within 48 h of incubation.

**Identification**

Using a multipoint inoculator, all strains of Gram-negative bacteria were inoculated onto PC agar that has been reported to have 100% specificity for identification of *P. aeruginosa*. All strains were also inoculated onto cetrimide agar (Oxoid) and blood agar. PC agar and cetrimide agar were incubated for 48 h at 37°C, and blood agar was incubated for 48 h at 42°C. Any strain showing growth on all three media was regarded as *P. aeruginosa*. All other strains were identiﬁed using API 20 NE strips.

**Statistical analysis**

Differences between the two methodologies were statistically assessed using McNemar’s test with continuity correction applied. Statistical signiﬁcance was deemed as P < 0.05. The positive predictive value (PPV) expresses the number of proven resistant strains isolated on selective agar as a percentage of those presumed to be resistant by virtue of growth on selective agar. It was calculated by dividing the number of true positives by the number of true positives plus the number of false positives. The higher the PPV, the more accurate the method was for predicting resistance. The negative predictive value (NPV) expresses the number of specimens proven to contain only susceptible strains as a percentage of those...
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presumed to contain only susceptible strains by virtue of no growth on selective agar. It is calculated by dividing the number of true negatives by the number of true negatives plus the number of false negatives. The higher the NPV, the more accurate the method was for predicting the absence of resistant strains in a specimen.

Results

Using a combination of all media, 705 isolates of non-fermentative Gram-negative bacteria were investigated from the 45 samples. These were identified as *P. aeruginosa* (*n* = 645), *Achromobacter xylosoxidans* (*n* = 27), *Pseudomonas fluorescens* (*n* = 22), *Stenotrophomonas maltophilia* (*n* = 8), *Burkholderia cepacia* complex (*n* = 1), *Moraxella* species (*n* = 1) and *Sphingobacterium spiritivorum* (*n* = 1).

One sample yielded *B. cepacia* complex only and a second sample yielded *A. xylosoxidans* only. *P. aeruginosa* was isolated from the other 43 samples. Due to the presence of a heavy growth of *S. maltophilia*, *P. aeruginosa* was not recovered from one of these 43 samples on routine media and was only recovered using selective media. An average of three colony variants or morphotypes was isolated from each of the 43 samples using routine media (range 1–5 variants).

Detection of resistance in *P. aeruginosa*

Table 1 shows the number of specimens identified as harbouring at least one resistant isolate of *P. aeruginosa* using both routine and selective media. For comparative purposes only, it is assumed that all resistant strains were detected using a combination of the methods, although this cannot be proven. For temocillin and aztreonam, resistant strains were more readily detected using isolation on non-selective media followed by susceptibility testing of distinct morphotypes, although the difference between this methodology and isolation on selective agar was not statistically significant for either agent. For the other eight agents, a higher proportion of samples was found to contain at least one resistant isolate of *P. aeruginosa* using culture on selective media, and this difference was statistically significant (*P* < 0.05) in relation to meropenem, piperacillin/tazobactam, ticarcillin/clavulanic acid and all three aminoglycosides. Selective media showed the greatest advantage for recovery of aminoglycoside-resistant strains. For example, of 31 samples proven to contain amikacin-resistant *P. aeruginosa* using selective media, only 13 samples (42%) were shown to contain an amikacin-resistant strain using routine media and methods.

Colonies growing on selective agar plates were expected to be resistant to the antibiotic that had been incorporated and this was generally true but varied for different agents. Table 1 shows that the predictive value of *P. aeruginosa* growth on plates containing aztreonam was 100% (i.e. all isolates from plates containing aztreonam proved to be resistant when tested using a reference MIC method). However, the predictive value of growth on plates containing aminoglycosides was lower (88% to 92%). Strains producing anomalous results typically showed borderline MIC values, for example, 12% of isolates recovered by culturing sputum on selective isolation media containing 4 mg/L gentamicin (presumptively resistant), had an MIC of 4 mg/L gentamicin (susceptible) when tested using the reference MIC method. This discrepancy may have been attributable to the presence of the specimen or other flora or a higher inoculum of *P. aeruginosa* on the selective medium when compared with that used in standardized MIC tests.

When strains of *P. aeruginosa*, which were proven to be antibiotic-resistant, were recovered on selective media, growth was detected within 48 h in at least 94% of cases (Table 1). Using routine media, incubation for at least 48 h was typically necessary before distinction of morphotypes could be made and susceptibility testing required at least a further 24 h. Hence, the use of selective media typically allowed for detection of antimicrobial resistance 24 h earlier than the use of routine methods.

Detection of other Gram-negative bacteria

Selective media assisted in the occasional isolation of other potentially pathogenic Gram-negative bacteria. For example, using selective media, four additional samples of sputum were found to contain *P. fluorescens*, three contained *A. xylosoxidans*, three contained *S. maltophilia* and one contained *S. spiritivorum*. No additional Gram-negative species were isolated using routine media only.

Discussion

This study adds further weight to the hypothesis that the results of conventional susceptibility testing grossly underestimate the amount of antimicrobial resistance in *P. aeruginosa* isolated from CF sputa. Differences in MIC, obtained by testing colonies isolated by the two different methods, were typically more than one dilution apart and use of selective media frequently resulted in a dramatically different outcome in the assessment of susceptibility. For example, the following MIC results (mg/L) were obtained for the most resistant morphotype of *P. aeruginosa* isolated from specimen 23 using the two different methods (MIC results listed second in parenthesis): amikacin (4, 64), gentamicin (1, 16), tobramycin (≤0.5, 16), aztreonam (≤1, 32), ceftazidime (≤1, 32), meropenem (2, 16), piperacillin/tazobactam (≤2, >64), ticarcillin (4, >64), ticarcillin/clavulanic acid (≤4, >128) and ciprofloxacin (0.5, 2). This sample, from a 14-year-old patient awaiting lung transplantation, yielded three morphotypes of *P. aeruginosa* on routine media that were all fully susceptible as determined by a reference MIC method. Using selective media, *P. aeruginosa* could be isolated showing resistance, often at a high level, to all 10 antimicrobials.

It has been recognized for many years that *P. aeruginosa* from CF sputa shows great phenotypic variability and that selection of different colonies may generate a range of antibiograms. After 5 years of colonization, strains frequently become hypermutable leading to even more variability and an even greater propensity to acquire antibiotic resistance.8,9

In the context of processing CF sputa, the first priority of susceptibility testing should arguably be to reliably detect antimicrobial resistance so that inappropriate therapy may be avoided. Direct susceptibility testing of whole sputum using selective media offers a means of achieving this aim. Using this approach, resistant phenotypes are actively selected for, whereas there is accumulating evidence that conventional susceptibility testing,
Table 1. Number of specimens containing strains of P. aeruginosa resistant to antimicrobials

<table>
<thead>
<tr>
<th>AMK</th>
<th>GEN</th>
<th>TOB</th>
<th>ATM</th>
<th>CAZ</th>
<th>MEM</th>
<th>TZP</th>
<th>TEM</th>
<th>TIM</th>
<th>CIP</th>
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<tr>
<td>Total (any method)</td>
<td>31</td>
<td>35</td>
<td>13</td>
<td>26</td>
<td>29</td>
<td>30</td>
<td>33</td>
<td>29</td>
<td>35</td>
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<tr>
<td>Routine method no. detected</td>
<td>13</td>
<td>20</td>
<td>7</td>
<td>23</td>
<td>18</td>
<td>21</td>
<td>20</td>
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<td>62</td>
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<td>61</td>
<td>90</td>
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<tr>
<td>Selective method no. detected</td>
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<td>13</td>
<td>20</td>
<td>26</td>
<td>29</td>
<td>33</td>
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<td>93</td>
<td>93</td>
<td>96</td>
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<td>NPV (%)b</td>
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<td>100</td>
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<td>Detection within 48 h (%)</td>
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<td>95</td>
<td>100</td>
<td>97</td>
<td>100</td>
<td>96</td>
<td>100</td>
</tr>
</tbody>
</table>

AMK, amikacin; GEN, gentamicin; TOB, tobramycin; ATM, aztreonam; CAZ, ceftazidime; MEM, meropenem; TZP, piperacillin/tazobactam; TEM, temocilin; TIM, ticarcillin/clavulanic acid; CIP, ciprofloxacin.

aPPV (%) indicates the positive predictive value of growth on selective agars to predict antimicrobial resistance as confirmed by MIC testing.

bNPV (%) indicates the negative predictive value of the absence of P. aeruginosa on selective media to predict susceptibility as confirmed by MIC testing.

involving selection of colonies, is akin to a lottery and may detect resistance only by chance. This perception is reinforced by the data from this study.

In previous studies, Maduri-Traczewski et al. employed three selective media containing tobramycin, azlocillin and ticarcillin for direct culture of sputum samples and compared these with non-selective media for recovery of resistant P. aeruginosa from 30 CF sputa. The use of selective media enabled detection of an increased number of resistant isolates and reduced the time required for detection of resistance by 24 h. Van Dalsen et al. compared batches of MacConkey agar with and without 25 mg/L tobramycin for culture of 240 CF sputa. They found that 98% of tobramycin-resistant isolates could be detected using selective media compared with only 47% using non-selective media (P = 0.001). In our study, 100% of samples containing tobramycin-resistant P. aeruginosa were detected using selective media compared with 54% using routine media (P < 0.05).

In other studies, Zebouh et al. examined the use of the Etest for direct susceptibility testing of 310 CF sputa with six antimicrobials and reported a sensitivity of at least 89% for detection of resistance when compared with conventional methods. Serisier et al. reported the direct susceptibility testing of whole sputum using Etests to detect synergistic interactions between antimicrobials. The results were used to guide therapy and were linked to an improved clinical outcome; however, the study comprised a case report for a single patient. Some authors have suggested that the presence of sputum on culture media may provide an environment closer to the situation in the lung, where biofilm mode of growth and quorum-sensing effects are important factors, although given the complexity of the micro-environment of the CF lung, this seems optimistic.

It is widely reported that there is a poor correlation between the results of antimicrobial susceptibility tests and treatment outcomes for Pseudomonas infections in CF patients. Indeed, it is unsurprising that in vitro susceptibility tests on synthetic media do not accurately predict therapeutic outcomes in the vastly contrasting environment of the CF lung for reasons concisely summarized by Govan in a recent editorial. However, the inability of conventional susceptibility tests to reliably detect resistance, as highlighted in this study and elsewhere, may also contribute to this poor correlation.

In conclusion, we have shown that direct culture of CF sputa on selective agars containing breakpoint concentrations of antimicrobials leads to a significantly improved detection of resistance over conventional testing. Growth on selective agars reliably predicts resistance in most cases (PPV: 88% to 100%) although MIC tests may be necessary to confirm antimicrobial resistance with certainty.

Transparency declarations

None to declare.

References

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