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A novel chromogenic medium for isolation of *Pseudomonas aeruginosa* from the sputa of cystic fibrosis patients

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Abstract

Background: A novel chromogenic medium for isolation and identification of *Pseudomonas aeruginosa* from sputa of cystic fibrosis (CF) patients was evaluated and compared with standard laboratory methods.
Methods: One hundred sputum samples from distinct CF patients were cultured onto blood agar (BA), Pseudomonas CN selective agar (CN) and a Pseudomonas chromogenic medium (PS-ID). All Gram-negative morphological variants from each medium were subjected to antimicrobial susceptibility testing, and identification using a combination of biochemical and molecular methods.
Results: P. aeruginosa was isolated from 62 samples after 72 h incubation. Blood agar recovered *P. aeruginosa* from 56 samples (90.3%) compared with 59 samples (95.2%) using either CN or PS-ID. The positive predictive value of PS-ID (98.3%) was significantly higher than growth on CN (88.5%) for identification of *P. aeruginosa* ($P<0.05$).
Conclusions: PS-ID is a promising medium allowing for the isolation and simultaneous identification of *P. aeruginosa* from sputa of CF patients.

Keywords: Pseudomonas aeruginosa; Culture media; Enzyme substrates; Cystic fibrosis; Respiratory infection

1. Introduction

Pseudomonas aeruginosa is the most common and important species colonising the lungs of cystic fibrosis patients contributing to an accelerated decline in lung function. Colonisation rates are estimated at 80% in adults and 60% in the CF population overall [1]. Once *P. aeruginosa* is established in the lung it is extremely difficult to eradicate [2]. Early detection of *P. aeruginosa*, before its conversion to a mucoid phenotype, and aggressive early treatment with appropriate antimicrobials, has been advocated to limit the severity of infection [3].

Detection of *P. aeruginosa* colonisation is normally achieved by culture of sputum or laryngeal secretions onto artificial media. Typical isolation media for respiratory pathogens include blood agar and chocolate agar as well as selective agars such as MacConkey agar and cetrimide-based media. Despite the large amounts of *P. aeruginosa* often present in the sputa of colonised patients, isolation and identification may often be challenging to microbiology laboratories. *P. aeruginosa* from CF sputa often have an atypical appearance and present as mucoid phenotypes and small colony variants. Production of pyocyanin, a blue-green pigment typically produced by *P. aeruginosa*, is also frequently absent [4]. Specialised selective agars containing a detergent, cetrimide, have been recommended to facilitate isolation, however these have limited ability to differentiate *P. aeruginosa* from the polymicrobial flora often present in CF sputa. Other species of *Pseudomonas* as well as *Stenotrophomonas maltophilia, Achromobacter xylosoxidans* and *Ralstonia pickettii* have been shown to grow on the cetrimide-based media [5] and may be indistinguishable from non-pigmented strains of *P. aeruginosa*.

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Difficulties in recognising *P. aeruginosa* are compounded by difficulties in biochemical identification. Biochemical test kits such as API 20 NE are commonly used for identification [6], however a high rate of misidentification of oxidase positive Gram-negative rods including *P. aeruginosa* has been demonstrated using this system [7]. In addition, the test requires the use of a pure bacterial subculture and a minimum incubation time of 48 h. Hence, identification using this method requires at least 3 days.

We evaluated the potential of a novel chromogenic medium for the isolation and simultaneous identification of *P. aeruginosa*. As well as including selective agents for the inhibition of many other species, the medium contains a chromogenic substrate for ß-alanyl aminopeptidase, which is hydrolysed by *P. aeruginosa* resulting in the formation of purple colonies [8]. The aim of this study was to evaluate a novel prototype chromogenic agar medium (PS-ID), for the isolation and identification of *P. aeruginosa* from CF sputum samples and to compare its performance to routine media used in the laboratory.

2. Materials and methods

2.1. Materials

Columbia agar (CM0331) and Isosensitest agar (CM0471) were prepared from dehydrated powders in accordance with manufacturer's instructions (Oxoid, Basingstoke, UK). Molten Columbia agar at 50 °C was supplemented with 5% defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK) and plates were prepared. Pseudomonas CN selective agar (PO0185A) was obtained as pre-poured plates from Oxoid, Basingstoke, UK. The Pseudomonas chromogenic medium (PS-ID) was provided as pre-poured plates by bioMérieux, La Balme-les-Grottes, France, for research purposes only. Phenanthroline/C-390 agar containing 1,10-phenanthroline (Sigma Chemical Company, Poole, UK) and C-390 (Biosynth AG, Switzerland) was prepared as previously described [9]. Cetrimide agar and API 20 NE strips were obtained from bioMérieux, Basingstoke, UK. Amikacin, gentamicin, tobramycin, ciprofloxacin, aztreonam, piperacillin/tazobactam, ticarcillin/potassium clavulanate and ceftazidime were obtained from Sigma Chemical Company, Poole, UK as powders of known potency. Temocillin and meropenem were obtained from their respective manufacturers.

2.2. Culture of sputum samples

One hundred sputum samples were collected from distinct cystic fibrosis patients (mean age: 26 years, range 4–62 years), who visited the cystic fibrosis clinic at the Royal Victoria Infirmary, Newcastle upon Tyne or were attending the Freeman Hospital for lung transplant assessment. The sputum was homogenised with an equal amount of sputasol by vigorously shaking the sample on a vortex mixer at room temperature for 30 min. A 1/1000 dilution of the sputum was obtained by adding 10 μL of the homogenised sputum into 9.99 mL of sterile distilled water. A 10 μL aliquot of both the undiluted and diluted sputum were separately plated onto Columbia blood agar (BA), Pseudomonas CN selective agar (CN) and PS-ID.

The media were incubated aerobically at 37 °C for a total of 72 h and were read after each 24 h interval. Any presumptive Gram-negative isolate, including all morphological variants, from the three media were sub-cultured by touching a single colony with a sterile wire and inoculating a BA plate. Colonial appearance including size, shape and colour of colonies on the three primary isolation media was recorded. Sub-cultured colonies were subjected to Gram stain and storage at −20 °C in glycerol for later use.

2.3. Phenotypic identification

A 10 μL aliquot of each stored isolate along with the positive control strain *P. aeruginosa* (NCTC 10662) and the negative control *E. coli* (NCTC 10418) were sub-cultured onto BA and grown overnight. Standardized suspensions were prepared in water to a density equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 cfu/mL) using a densitometer. A 1 μL aliquot of each suspension was inoculated onto BA, Phenanthroline/C-390 agar and cetrimide agar using a multipoint inoculator. All plates were incubated at 37 °C and a duplicate set of blood agar plates was inoculated and incubated at 42 °C. All media were incubated for 48 h after which growth was recorded. Oxidase tests were performed from growth on BA at 37 °C. Isolates were confirmed to be *P. aeruginosa* if they were oxidase positive, grew on Phenanthroline/C-390 agar and cetrimide agar and also grew on BA at 42 °C (Fig. 1). Any oxidase negative isolates were identified using API 20 NE. Any oxidase positive isolates that did not confirm as *P. aeruginosa* using the above method were subjected to species specific multiplex PCR (see 2.4) and any isolates not confirmed as *P. aeruginosa* were identified using API 20 NE. All isolates of confirmed *P. aeruginosa* (from any medium) were inoculated onto PS-ID as described above.

Of the Gram positive organisms, *Staphylococcus aureus* isolates were confirmed by latex agglutination test (Slidex Staph Plus) and the other organisms were presumptively identified by Gram-stain and a catalase test. Yeasts and filamentous fungi were confirmed by microscopy and atypical mycobacteria with an acid fast stain.

2.4. Species-specific PCR

In preparation for the species-specific multiplex PCR the appropriate stored isolates were inoculated onto BA and incubated overnight. DNA extraction was carried out by preparing a 1 mL light suspension of each organism in PCR grade water and centrifuging this for 5 min at 13,000 rpm. The supernatant was decanted and the pellet was re-suspended in 100 μL of a 5%, w/v Chelex suspension [10]. The mixture was then boiled for 5 min at 100 °C and subsequently frozen at -20 °C for 5 min. This freeze thaw step was repeated once after which the mixture was centrifuged again at 13,000 rpm for 5 min. The supernatant containing the extracted DNA was transferred into a new centrifuge tube.

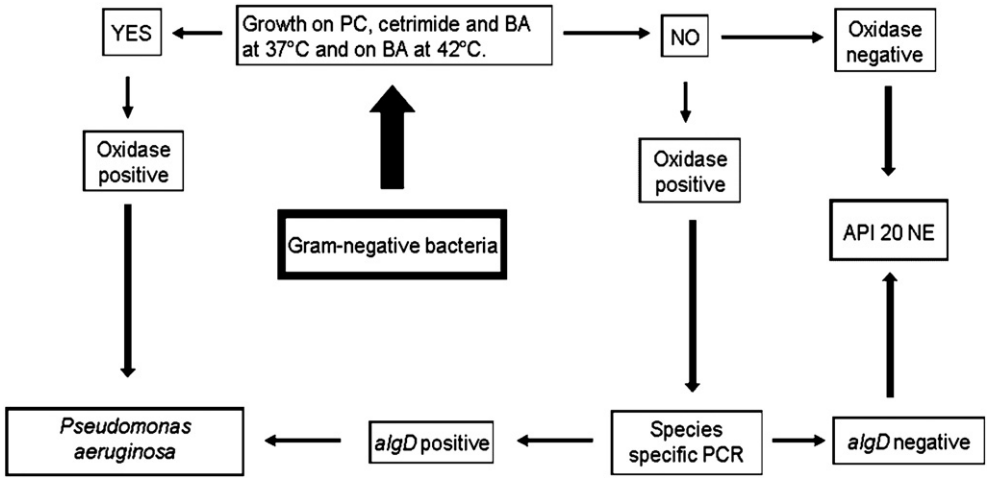

Fig. 1. Algorithm for identification of Gram-negative isolates. (PC: Phenanthroline/C-390 agar).

The species specific multiplex PCR followed a previously described method [11]. The primers used were *P. aeruginosa algD* gene specific primers VIC1 and VIC2 and universal bacterial 16 S primers 11E and 13B. Various control strains were tested in each batch of tests including *P. aeruginosa* (NCTC 10662), *Pseudomonas fluorescens* (NCTC 10688), *Pseudomonas putida* (NCTC 10475), *Pseuudomonas putida* (NCTC 10936), *S. maltophilia* (NCTC 10257) and *Burkholderia cenocepacia* (LMG 16656).

2.5. Antimicrobial susceptibility testing

Stored isolates were inoculated onto BA and incubated overnight. The ten antimicrobials listed above (see 2.1) were incorporated into Isosensitest agar at breakpoint concentrations used to define resistance (see Table 2) and inoculated with 10 000 cfu/spot of each confirmed isolate of *P. aeruginosa* using a multipoint inoculator. *P. aeruginosa* (NCTC 10662) and *Escherichia coli* (NCTC 10418) were included as controls of known susceptibility. Procedures and interpretation of antimicrobial susceptibility were performed in strict accordance with the recommendations of the British Society for Antimicrobial Chemotherapy [12]. Susceptibility to temocillin was interpreted using a breakpoint of 8 mg/L as previously recommended [13].

2.6. Statistical methods

The positive predictive value (PPV), negative predictive value (NPV) and sensitivity were calculated for the capability of each medium to isolate *P. aeruginosa*. The difference between sensitivity and specificity values for the media was assessed using the χ^2 test [14]. The difference between the mean number of antibiograms and morphological variants obtained by the different media was assessed by a two sample *t* test [14]. Differences between the number of samples with resistant *P. aeruginosa* isolates detected by the three different media were assessed using McNemar's test [14]. In all cases, a significant difference was defined as a *P* value of <0.05.

3. Results

3.1. Isolation of P. aeruginosa

Morphological 'variants' were defined as isolates that could be distinguished from other colonies on the grounds of colour, size, shape or texture. Identical variants isolated from the same sample from culture of both 'neat' and diluted sputum were counted as a single variant. Using a combination of cultures from both 'neat' and diluted sputum, 479 Gram-negative morphological variants isolated from all three media were stored for identification. Of these, 408 oxidase positive isolates were proven to be *Pseudomonas aeruginosa* using either, growth on Phenanthroline/C-390 agar and cetrimide agar and growth at 42 °C (362 isolates) or species-specific PCR (46 isolates). Using a combination of the three isolation media, *P. aeruginosa* was isolated from 62 of the 100 samples. Other Gram-negative bacteria that were recovered included *A. xylosoxidans* (from nine samples), *S. maltophilia* (from eight samples), *Burkholderia cepacia* complex (from three samples), *P. fluorescens* (from two samples) and *R. pickettii* (from one

Fig. 2. Morphological variation of *P. aeruginosa* isolates growing on PS-ID from culture of a CF sputum sample.

Table 1
Comparison of three media for the detection of *P. aeruginosa* in sputa from patients with CF

	24 h	48 h	72 h
No. of positive samples			
BA	46	55	56
CN	52	59	59
PS-ID	36	58	59
Sensitivity (%)			
BA	74.1	88.7	90.3
CN	83.9	95.2	95.2
PS-ID	58.1	93.5	95.2
PPV (%)			
BA	N/A	N/A	N/A
CN	89.7	88.5	88.5
PS-ID	100	98.3	98.3
NPV (%)			
BA	N/A	N/A	N/A
CN	76.2	90.9	90.9
PS-ID	59.4	90.2	92.5

sample). A number of specimens yielded potential Gram-positive pathogens and fungi e.g. 19 samples grew *S. aureus* of which five were methicillin resistant, 11 samples grew yeast, six grew filamentous fungi and two samples yielded mycobacteria.

P. aeruginosa typically formed purple colonies on PS-ID but some isolates generated colonies that produced shades of pink or red. Typical appearance of *P. aeruginosa* on PS-ID is shown in Fig. 2. Any pink, red or purple colonies were regarded as presumptive *P. aeruginosa*. A large majority of confirmed isolates of *P. aeruginosa* did not produce pigment on CN and therefore, for the purposes of comparison, any Gram-negative bacteria isolated on CN were also regarded as presumptive *P. aeruginosa*. Table 1 shows the performance of the three media for isolation of *P. aeruginosa*. Of 182 colonial variants of *P. aeruginosa* isolated on PS-ID, 96.7% produced purple, pink or red colonies with six isolates (3.3%) producing either orange or transparent colonies, which were regarded as 'undetected'. Mucoid colony variants had an overall inferior colour production than the non-mucoid types. For most isolates, colour production required 48 h to develop and therefore sensitivity of PS-ID was relatively poor (58.1%) after 24 h incubation. All isolates of confirmed *P. aeruginosa* from all three media showed growth when inoculated as pure cultures onto PS-ID and all but two isolates produced coloured colonies. Both of these were among the six 'undetected' isolates originally recovered on PS-ID. These two isolates were non-mucoid and produced a typical colony size and were assumed to be genuinely deficient in ß-alanyl aminopeptidase activity.

3.2. Presumptive identification on culture media

After 24 h incubation, any colony displaying purple, pink or red coloration on PS-ID was confirmed as *P. aeruginosa*. After incubation for 48–72 h, of 179 colony variants showing such coloration, 176 were confirmed as *P. aeruginosa* (PPV = 98.3%). Two isolates of *B. cepacia* complex and one isolate of *Pseudomonas fluorescens* also produced a coloration characteristic of *P. aeruginosa*. On CN, 130 colony variants were recovered of which 115 proved to be *P. aeruginosa*

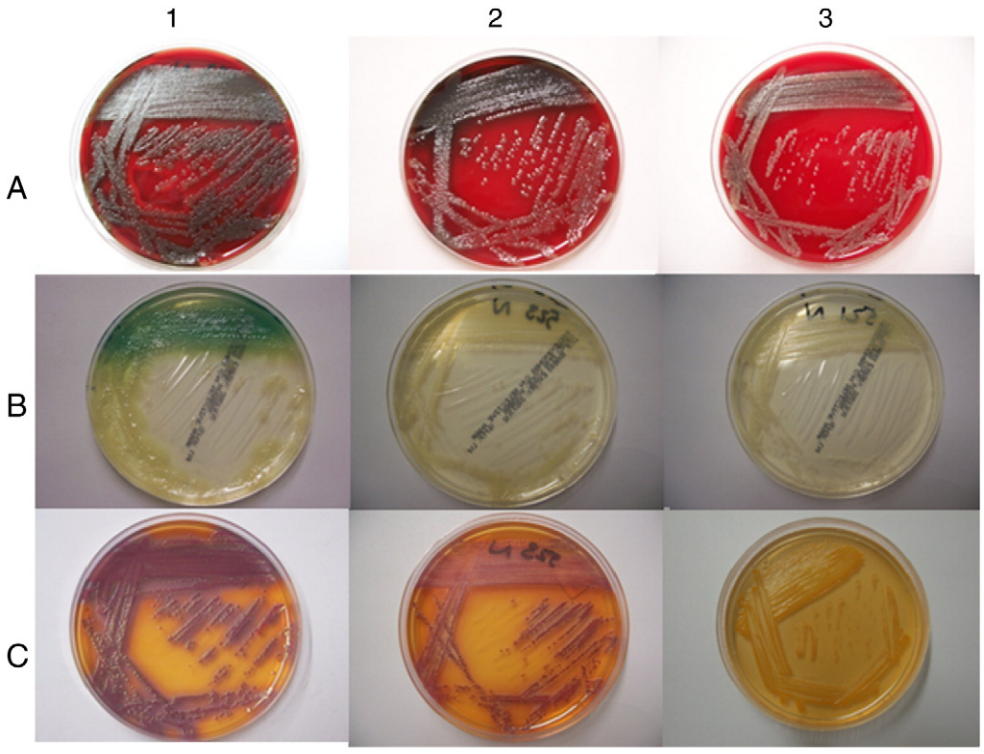

Fig. 3. Three samples of CF sputa plated onto blood agar (A), CN (B) and PS-ID (C). Sample 1 contains *P. aeruginosa* producing strong pigmentation on CN and it is easily distinguishable. In sample 2, *P. aeruginosa* is also present but pigment is weak or absent on CN thus causing it to resemble other species (e.g. *A. xylosoxidans* in sample 3). PS-ID provides distinction of both isolates of *P. aeruginosa* by their appearance as distinctive purple colonies.

(PPV=88.5%). Others were confirmed as *A. xylosoxidans* (n=11), *P. fluorescens* (n=2) and *B. cepacia* complex (n=2). No Gram-positive bacteria or yeasts were recovered on CN but occasional isolates were encountered that formed either white or blue colonies on PS-ID.

3.3. Colonial variation and antimicrobial susceptibility

A total of 111 variants of *P. aeruginosa* were detected on BA, 115 on CN and 176 on PS-ID. The 6 colourless variants isolated on PS-ID were considered as false negative and therefore not included among the variants detected on PS-ID. The number of variants per positive sample ranged from 1–3 for BA, 1–4 for CN and 1–7 for PS-ID. The differences between the mean numbers of variants per sample for both PS-ID (2.83) and BA (1.79) and PS-ID and CN (1.85) were statistically significant (P<0.001). All variants from all three media were tested concomitantly for susceptibility to each of ten antimicrobial agents on the same batch of media. A variant was regarded as having a distinct antibiogram if its susceptibility profile differed from other variants in the same sputum sample. An average of 3.87 antibiograms per sample (range 1–9) was obtained by testing variants isolated from a combination of the three media. The mean of number of antibiograms obtained per sample was 1.58 for isolates recovered on BA (range 1–3), 1.60 for CN (range 1–4) and 2.27 for PS-ID (range 1–6). The differences between the mean antibiogram values for both PS-ID and BA, and PS-ID and CN were statistically significant (P<0.001).

Table 2 shows the number of samples from which at least one antimicrobial resistant *P. aeruginosa* variant was recovered. For comparative purposes it is assumed that all antimicrobial resistant isolates were detected by a combination of the three media, although this cannot be proven. Compared to BA and CN, PS-ID showed a superior recovery of variants resistant to amikacin, gentamicin, ceftazidime, temocillin, piperacillin-tazobactam and ticarcillin-clavulanic acid. Compared with BA and PS-ID, CN recovered more variants resistant to ciprofloxacin. The same number of variants resistant to either tobramycin or aztreonam was recovered by CN and PS-ID. BA consistently recovered less resistant variants than the other two media, although it was equivalent with PS-ID for isolation of meropenem resistant isolates. These differences were not statistically significant except for the higher number of amikacin-resistant isolates recovered on PS-ID when compared with isolation on CN (P=0.045).

Table 2
Number of sputum samples containing at least one antimicrobial resistant *P. aeruginosa* isolate

	Antimicrobials* (breakpoint concentration in mg/l)									
	AMK (16)	GEN (4)	TOB (4)	ATM (8)	CAZ (8)	MEM (8)	TEM (8)	TZP (16)	TIM (64)	CIP (1)
Total (all media types)	38	53	17	36	39	36	45	37	39	42
Blood agar	26	41	8	26	30	30	30	26	28	37
%	68.4	77.4	47.1	72.2	76.9	83.3	66.7	70.3	71.8	88.1
CN	25	39	11	29	30	28	33	27	28	39
%	65.8	73.6	64.7	80.6	76.9	77.8	73.3	73	71.8	92.9
PS-ID	33	45	11	29	31	30	36	30	29	38
%	86.8	84.9	64.7	80.6	79.5	83.3	80	81.1	74.4	90.5

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

4. Discussion

P. aeruginosa is recognized as the most important pathogen affecting cystic fibrosis patients, yet media designed for the selective isolation of this important pathogen and standard biochemical kits commonly used for identification have limited utility. Pseudomonas CN agar is recommended for the isolation of *P. aeruginosa* and contains cetrimide and nalidixic acid as selective agents and potassium and magnesium ions to enhance pigment production. However, a number of reports have demonstrated the absence of pigment in strains isolated from patients with CF [4,15–17]. Other Gram-negative bacteria that can tolerate cetrimide, such as *A. xylosoxidans*, *Enterobacter* spp., *S. maltophilia* and other *Pseudomonas* spp. [5], may be indistinguishable from non-pigmented *P. aeruginosa* (see Fig. 3). This necessitates the use of biochemical tests for species identification but this can also be problematic. For example, one previous study reported 52 isolates of nontypical *P. aeruginosa* isolated from CF patients, identified by 16 S ribosomal DNA sequence typing, that were misidentified or could not be identified using API 20 NE [7].

These problems have led a number of workers to examine genotypic methods for identification of *P. aeruginosa* from CF patients and several have been described. For example, 16 S ribosomal DNA sequence typing has been reported to offer 100% sensitivity and specificity for identification of *P. aeruginosa* [18]. Real-time PCR, which can achieve identification in a matter of hours, has also been evaluated [19]. Of three gene targets targeted, the best sensitivity (98.4%) and specificity (98.9%) were found with detection of *oprI* [19]. These methods, however, were carried out on pure cultures obtained from primary culture, which means that at least 48 h had passed before the test could be carried out. Accurate identification of *P. aeruginosa* directly from sputum samples is potentially more valuable. Direct testing of CF sputa has been performed by targeting the *oprL* and ETA genes [20] and also by PCR amplification the *algD* gene [11]. However, neither of these methods obtained 100% specificity and sensitivity. Fluorescent in situ hybridisation, a rapid, specific and relatively inexpensive method, has been used to identify *P. aeruginosa* directly from CF sputa with 100% specificity but only 90% sensitivity [21].

Media containing chromogenic enzyme substrates are useful tools for clinical laboratories for the isolation and presumptive identification of a range of pathogens [22]. This report is the first to evaluate a chromogenic agar medium specifically designed for detection of *P. aeruginosa*. In this study, PS-ID had a comparable sensitivity to CN at 48–72 h incubation and afforded presumptive identification of *P. aeruginosa* with high predictive value (98.3%). Chromogenic media are typically more expensive than conventional agars, largely due to the

additional cost of enzyme substrates, however this may be offset by the requirement for fewer additional tests, thus savings should be made on reagents and workload. In contrast, genotypic methods require specialised equipment and training, which limit their suitability for routine use in many clinical laboratories [7].

An interesting observation when using PS-ID is the high number of colony variants obtained when compared to other media. Production of exopolysaccharide capsular material (alginate) is substantially reduced on PS-ID in contrast to blood agar and CN where highly mucoid isolates may obscure the presence of other morphological types. Differences in the expression levels of ß-alanyl aminopeptidase may also contribute to colour variation between colony types on the chromogenic medium. The fact that more colony variants were isolated on PS-ID and tested, inevitably led to the isolation of more isolates with varying antibiograms and consequently the detection of more antimicrobial resistance (Table 2). Foweraker et al. [23] demonstrated that identical colonies frequently showed different antibiograms, while two colonies with a different morphology could also have the same antimicrobial sensitivity pattern. It would therefore be erroneous to conclude that PS-ID has a superior ability to detect antimicrobial-resistant isolates, however this is worthy of further exploration. The mucoid aspect of a colony may be regarded as significant and, while such strains are recovered on PS-ID, the amount of capsular material is reduced. Complementary blood-based media used for the isolation of other pathogens (e.g. *Haemophilus influenzae*) may be useful alongside PS-ID for highlighting mucoid strains of *P. aeruginosa*.

In conclusion, PS-ID has shown good potential as a primary isolation medium for *P. aeruginosa* from CF sputa. The purple colonies produced by *P. aeruginosa* are easy to distinguish, and reading the cultures is quick and straightforward. Colonies can be reliably identified as *P. aeruginosa* based on colony colour alone, with a predictive value of 98.3%, without the requirement for further biochemical tests. This saves resources and allows the early confirmation of this important pathogen.

Conflict of interest statement

The Microbiology Department, Freeman Hospital receives funding from bioMérieux for the development and evaluation of culture media. C.F., D.H. and S.O. are paid employees of bioMérieux and A.J. is a paid consultant for bioMérieux.

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