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Belfast Agar–a simple laboratory medium to separate *Pseudomonas aeruginosa* from pan-resistant *Burkholderia cenocepacia* isolated from the sputum of patients with cystic fibrosis (CF)

S Caskey[a], JE Moore[a,b], J McCaughan[a,c] and JC Rendall[a]

[a]Regional Adult Cystic Fibrosis Centre, Belfast City Hospital, Belfast, UK; [b]Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast, UK; [c]Department of Medical Microbiology, The Royal Group of Hospitals, Belfast, UK

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Cystic fibrosis (CF) is the most commonly inherited life-limiting disease in persons originating from a white and European background and has a genetic carriage rate of approximately 1 in 20 persons and an incidence of 1 in 2500 live births [1]. It is an autosomal recessive condition whereby two alleles carrying a polymorphism in the CF transmembrane conductance regulator (CFTR) gene phenotypically manifest the disease state through a variety of multiorgan problems, associated with a pharmacological dysfunction to regulate chloride ion secretion across cell membranes. The most common complication of CF is the recurrence of chronic chest infections usually caused by bacterial pathogens [2]. CF patients continue to suffer from recurrent and chronic respiratory tract infections and most of their morbidity and mortality is due to such infections throughout their life [3]. These infections are usually dominated by Gram-negative organisms, especially by the pseudomonads, including *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia cepacia* complex (BCC) organisms, particularly *Burkholderia cenocepacia* (*B. cenocepacia*) and *Stenotrophomonas maltophilia* [3].

The clinical microbiology of CF is complicated by high levels of antibiotic resistance frequently observed in such Gram-negative organisms, originating particularly from the sputum of adult CF patients, combined with few phenotypic/colonial differences, when grown on basal agar, such as Columbia Blood Agar (CBA). Generally, CF selective microbiology isolation methods may exploit differences in antibiotic susceptibility to select for a target pathogen, for example, employment of *Pseudomonas* Isolation Agar (PIA) containing cetrimide (200 mg/L) and sodium nalidixate (15 mg/L), or *Burkholderia cepacia* Selective Agar (BCSM) containing polymixin B (150,000 IU/L), gentamicin (5 mg/L) and ticarcillin (100 mg/L), which specifically select for *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, respectively. Given the multi- and pan-resistance characteristics of these species, they can be difficult to separate, when co-infecting the lungs of CF patients. This, therefore, presents a diagnostic dilemma to the successful separation from sputum and reliable reporting of the more antibiotic susceptible species, namely *P. aeruginosa,* in the presence of the more antibiotic-resistant *B. cenocepacia*.

B. cenocepacia has an unusual characteristic, recently reported by our group [4], in that it is susceptible to the aminocoumarin antibiotic, novobiocin, whereas *P. aeruginosa* is resistant [4]. Indeed, this trait may be *B. cenocepacia*'s 'Achilles heel', as it generally tends to be resistant to all available antibiotics, hence its dominance in the pathophysiology of CF disease, where it is present, due to a clinical inability to successfully treat and eradicate this organism. This trait also leads to its dominance on selective culture agar, as it usually outcompetes all other flora, including *P. aeruginosa*. Until now, no report has exploited this recently described universal susceptibility of *B. cenocepacia* to novobiocin. We now describe a simple laboratory method, which exploits this characteristic, to aid in the laboratory separation of *P. aeruginosa* from pan-resistant *Burkholderia cenocepacia* isolated from sputum in patients with CF.

Belfast agar was prepared by combining a presterilised selective supplement of the antibiotic agent, novobiocin (Oxoid SR0181), to a sterile and precooled basal agar base, consisting of gelatin peptone (16 g/L), casein hydrolysate (10 g/L), potassium sulphate (10 g/L), magnesium chloride (1.4 g/L), agar (11 g/L) and distilled water, 1000 ml, (pH 7.1 ± 0.2 @ 25 °C), followed by pouring into sterile petri dishes (Sterilin Ltd., UK). The basal agar was dissolved by thorough mixing and was sterilised by autoclaving at 121 °C for 15 min. The antibiotic,

CONTACT JE Moore jemoore@niphl.dnet.co.uk

novobiocin, was prepared by dissolving 1 vial in sterile distilled water, followed by filter sterilisation through a 0.22 µm filter (Whatman Inc., USA). Sterilised selective supplement was added to cooled basal agar (45 °C) to give a final concentration of novobiocin of 100 µg/ml in the final agar. After thorough mixing, the final agar was poured into sterile Petri dishes and allowed to cool and set under sterile laminar air-flow. After drying of the petri dishes for 2 h at room temperature in sterile air, prepared medium was wrapped in plastic, shrink-wrapped and stored in the dark at 4 °C until used. Quality control of the medium involved the employment of two well-characterised control CF clinical organisms, namely *Pseudomonas aeruginosa* (positive control) and *B. cenocepacia*, whereby the former bacterium grows on the medium, whilst the latter bacterium is unable to grow under appropriate incubation conditions.

An experiment was therefore designed to evaluate the effectiveness of this agar to separate CF clinical isolates of *P. aeruginosa* from *B. cenocepacia*. Ten clinical isolates including *P. aeruginosa* [PA] ($n = 4$) and *B. cenocepacia* [BC] ($n = 6$) [ET12 lineage] were obtained from the Northern Ireland Health & Social Care Microbiology Repository (HSC MicroARK) (www. microark.com). These isolates had been previously isolated from the sputum of patients with a confirmed diagnosis of CF. All isolates were taken from storage on blood at −80 °C and were recovered aerobically on CBA (Oxoid CM0331, Basingstoke, UK) for 24 h at 37 °C, followed by a further two passages on CBA to allow full recovery from storage, as above, prior to use in this experiment. Fresh inocula of each organism was prepared to a 0.5 McFarland standard (approx 1 x 10^8 colony forming units (cfu)/ml) and inoculated onto freshly prepared Belfast Agar and incubated aerobically up to 48 h at 37 °C.

All PA and BC organisms were plated individually onto the following agar media: CBA (Oxoid CM0331) supplemented with 5% sterile defibrinated blood; BCSM (Oxoid CM0995 + SR0189); PIA (Oxoid CM0559 + SR0102); *Pseudomonas* Agar Base (Oxoid CM0559); and Belfast Agar. Plates were incubated aerobically at 37 °C for 48 h and growth assessed at the end of this incubation period. Simulated sputum was prepared employing Peptone Saline (0.1% w/v) solution (Oxoid CM0733) to which various *P. aeruginosa*/*B. cenocepacia* organism combinations were added, as detailed in Table 1. Following this, re-isolation of both *P. aeruginosa* and *B. cenocepacia* was attempted in freshly prepared Belfast Agar, as described above.

Results of the ability of each PA and BC organism and the PA/BC combinations to grow on the various agar media are shown in Table 1. Most significantly, we were able to recover all the PA isolates from each of the simulated sputa combinations on Belfast Agar, containing a single mixture of one isolate of PA + six BC organisms at high bacterial cell densities (ca. 6 x 10^8 cfu), employing MALDI-TOF (bioMérieux) technology, whilst simultaneously not being able to recover any BC organisms from the simulated sputa combinations on this agar. Additionally, we were unable to grow any BC organism on Belfast Agar, either individually or in combination from simulated sputa.

Previous work from our group in Belfast demonstrated that CF sputum contains large numbers of pathogens, in the range 10^6–10^9 cfu/g sputum [3]. Therefore, we performed our experiments in simulated sputum in this range, in an attempt to emulate the numbers naturally found in CF sputum, employing a range of clinical PA ($n = 4$) and BC ($n = 6$), which had previously been isolated from CF sputum.

When developing this method, we placed great emphasis on how such a method could be most conveniently rolled out into biomedical practice in busy NHS Clinical Microbiology laboratories. Most importantly,

Table 1. Growth of clinical isolates of *Pseudomonas aeruginosa* and *Burkholderia ceneocepacia* on Belfast Agar and commercially available selective agars.

Isolate	Growth on CBA	Growth on BCSA	Growth on PIA	Growth on PAB	Growth on Belfast Agar
Pseudomonas aeruginosa (PA)1	+	−	+	+	+
Pseudomonas aeruginosa 2	+	−	+	+	+
Pseudomonas aeruginosa 3	+	−	+	+	+
Pseudomonas aeruginosa 4	+	−	+	+	+
Burkholderia cenocepacia (BC) 1	+	+	+	+	−
Burkholderia cenocepacia 2	+	+	+	+	−
Burkholderia cenocepacia 3	+	+	+	+	−
Burkholderia cenocepacia 4	+	+	+	+	−
Burkholderia cenocepacia 5	+	+	+	+	−
Burkholderia cenocepacia 6	+	+	+	+	−
Isolate combinations in simulated sputum**					
PA1 + [BC1+BC2+BC3+BC4+BC5+BC6]	+	+	+	+	+*
PA2 + [BC1+BC2+BC3+BC4+BC5+BC6]	+	+	+	+	+*
PA3 + [BC1+BC2+BC3+BC4+BC5+BC6]	+	+	+	+	+*
PA4 + [BC1+BC2+BC3+BC4+BC5+BC6]	+	+	+	+	+*

Abbreviations: CBA = Columbia Blood Agar (Oxoid CM0331) supplemented with 5% sterile defibrinated blood ; BCSA = *Burkholderia cepacia* Selective Agar (Oxoid CM0995 + SR0189); PIA = *Pseudomonas* Isolation Agar (Oxoid CM0559 + SR0102); PBA = *Pseudomonas* Agar Base (Oxoid CM0559).
+=growth; - = no growth.
*Only growth of *Pseudomonas aeruginosa* detected. None of the *Burkholderia cenocepacia* organisms were detected from simulated sputa combinations on Belfast Agar; **bacterial density = approx. 10^8 colony forming units (cfu) *Pseudomonas aeruginosa* + 10^8 cfu of each of the individual isolates of BC1 –BC6.

the ability of any resulting method to be pragmatic and adoptable into the workflow of current NHS clinical microbiology laboratory practice was of primary importance. The scientific literature describes many excellent innovations into the detection of bacterial pathogens in CF patients, including molecular (PCR)-based methods, as well as serological methods. Whilst these new assays are effective, most NHS service laboratories have not adopted such methods as part of their SOPs, for several reasons, including lack of molecular experience, training and cost. Therefore, it is important to consider aspects of test acceptance and adoptability into any NHS laboratory with a desire to employ this method. To support this, our first consideration was choice of diagnostic platform to use. In this instance, we opted for simple bacteriological culture and not molecular or serological methods, for the reasons stated above. Secondly, our next decision was what basal agar base to use in Belfast Agar, for growing *Pseudomonas aeruginosa*. Given that a predefined agar base, namely PIA, has already been described and formulated for the specific nutritional requirements of *Pseudomonas* spp., coupled with its commercial availability from major UK laboratory suppliers, made this our choice for the agar base. Previously, when we originally described the antibacterial effect of novobiocin against clinical *B. cenocepacia* from CF patients, we further quantified this activity, in terms of minimum inhibitory concentration (MIC) [4].In this previous study, all *B. cenocepacia* isolates examined showed sensitivity *in vitro* to this antibiotic, with a mean zone size of 21 mm and a MIC range from 3.25 to 26 µg/mL, with MIC_{50} and MIC_{90} values (MICs that inhibit 50 and 90% of the isolates, respectively) of 8.0 and 18.0 µg/mL, respectively. We therefore chose a concentration of 100 µg/mL, as the its concentration in the final agar medium, as this concentration exceeded the MIC approximately fourfold with the least susceptible isolates tested, as well as being relatively easy to re-constitute in a busy NHS laboratory from commercially available pre-weighed vials, without the need for complicated dilution and re-working. Presently, novobiocin is available commercially from Oxoid (SR0181), in 10 mg vials, that can be easily purchased and reconstituted as detailed above.

Overall, we therefore commend employment of this method, when trying to isolate and separate *P. aeruginosa* from *B. cenocepacia* organisms from the sputum of patients with CF.

This work represents an advance in biomedical science because it describes a novel and simple method to separate two phenotypically close species, with ease and in a cost-effective manner in NHS clinical microbiology laboratories servicing patients in CF centres.

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No potential conflict of interest was reported by the authors.

ORCID

JC Rendall http://orcid.org/0000-0001-5836-1966

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