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Evaluation of five selective media for the detection of *Pseudomonas aeruginosa* using a strain panel from clinical, environmental and industrial sources

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ABSTRACT

Isolation and correct identification of the opportunistic pathogen and industrial contaminant *Pseudomonas aeruginosa* are very important and numerous selective media are available for this purpose. A novel comparison of five selective media having positive (acetamide-based agars), negative (Pseudomonas CN selective agar [Oxoid Ltd.] and Pseudomonas Isolation agar [Sigma-Aldrich Company Ltd.]) and chromogenic (chromID® *P. aeruginosa* [bioMérieux]) selection strategies was performed using a systematically designed bacterial test panel (58 *P. aeruginosa* and 90 non-*P. aeruginosa* strains including those commonly misidentified as *P. aeruginosa* by culture-dependent techniques). Standardised inocula were added to the selective media and the results were recorded after 24 and 72 h. After 72 h of incubation at 37 °C chromID® *P. aeruginosa* displayed the highest specificity (70%) and had good sensitivity (95%), although the sensitivity was negatively impacted by the large variation in colour of *P. aeruginosa* colonies, which hampered interpretation. Both media containing inhibitory selective agents performed very similarly, both having 100% sensitivity and a specificity of approximately 30%. Raising the incubation temperature to 42 °C increased the specificity of Pseudomonas CN selective agar and Pseudomonas isolation agar (61% and 47% respectively after 72 h), but increased the number of false positives encountered with the chromogenic medium, decreasing its specificity to 68% after 72 h. Growth on the acetamide agars was weak for all strains and it was often difficult to determine whether true growth had occurred. This, compounded by the low specificity of the acetamide agars (<26%), suggested they were less suitable for application to clinical or industrial settings without further modification. Overall, the chromogenic agar was the most selective but further consideration is required to optimise interpretation of results.

1. Introduction

Pseudomonas aeruginosa is an extremely versatile microorganism with minimal growth requirements and the ability to survive in diverse habitats (Morrison and Wenzel, 1984; Stover et al., 2000). As an opportunistic pathogen, *P. aeruginosa* can cause a broad spectrum of illness including pneumonia, urinary tract infections, wound and burn infections, bacteraemia and septicaemia (Kerr and Snelling, 2009) in susceptible hosts. Notably, *P. aeruginosa* is a major pathogen associated with cystic fibrosis (CF) patients and is responsible for chronic, life-threatening pulmonary infections which are extremely difficult to eradicate (Gomez and Prince, 2007). In addition to being clinically relevant, *P. aeruginosa* also impacts on industry as a commonly encountered contaminant of industrial processes and products. Due to the health risks posed by *P. aeruginosa*, it is widely considered an 'objectionable organism' to be found in consumer products (Jimenez, 2007; Sutton and Jimenez, 2012), and represents a serious concern for manufacturers. Relatively little is known, however, about *P. aeruginosa* in industrial settings.

Correct identification of *P. aeruginosa* is paramount and the majority of routine diagnostic tests for *P. aeruginosa* are reliant on culture-dependent methods (Deschaght et al., 2011). Numerous selective agars are available for the isolation of *P. aeruginosa* including those that include inhibitory selective agents such as antibiotics and biocides (Lilly and Lowbury, 1972), those that use positive selection (Szita et al., 1998) and those that use chromogenic detection (Laine et al., 2009). Cetrimide-containing isolation media are commonly used both in clinical and industrial settings (FDA, 1998) but can lack selectivity (Kodaka et al., 2003; Laine et al., 2009). These media are often also designed to enhance pigment production as synthesis of pyocyanin can unequivocally identify *P. aeruginosa* (Reyes et al., 1981). Only 90–95% of isolates produce pyocyanin (Smirnov and Kiprianova, 1990), however, which can confound identification. Media containing acetamide as the positive selective agent have not been evaluated extensively, although have been reported to have good selectivity and may be useful for detecting low numbers of cells from 'stressed' environments (Szita et al., 2007; Szita et al., 1998). In these media acetamide represents

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the sole carbon and nitrogen source, which few species other than *P. aeruginosa* can dissociate to release ammonia and acetic acid (Szita et al., 1998). Auxotrophic *P. aeruginosa* isolates, such as those encountered in respiratory infections in cystic fibrosis patients (Thomas et al., 2000), have difficulty growing on minimal media meaning that further modification of the acetamide media may be necessary to improve sensitivity. Only one study has evaluated the recently developed chromID® *P. aeruginosa* (manufactured by bioMérieux, La Balme-les-Grottes, France) showing the chromogenic agar to have higher selectivity than the Pseudomonas CN selective agar (Oxoid, Basingstoke, UK). By virtue of an unambiguous colour change the medium has demonstrated potential for the simultaneous isolation and identification of *P. aeruginosa* (Laine et al., 2009), warranting further assessment.

Unlike previous studies, which in general have considered four or fewer selective agars with similar selection strategies (Kodaka et al., 2003), we evaluated five selective media in parallel for the detection of *P. aeruginosa*, including those using inhibitory selective agents, a positive selective agent and chromogenic detection methods (Table 1). In addition, rather than measuring the performance of these agars as part of prospective surveillance for *P. aeruginosa* in environmental or clinical samples, a bacterial test panel was systematically assembled to include genetically distinct *P. aeruginosa* strains validated by molecular identification and strains from commonly mistaken non-*P. aeruginosa* species (Spilker et al., 2004) and Gram-negative pathogens encountered in CF patients (Hauser et al., 2011). The modified Z-agar (acetamide agar) differed from the original Z-agar (Szita et al., 1998) by the addition of sufficient nutrient supplementation to facilitate growth of auxotrophic *P. aeruginosa* isolates that are commonly encountered in CF (Thomas et al., 2000). Finally, as well as including strains from clinical and environmental sources, our comparative agar study is the first to include *P. aeruginosa* strains from industry as an emerging problem area (Sutton and Jimenez, 2012). The efficacy of these isolation agars across strains, commonly mistaken species and accounting for strain source is discussed.

2. Methods

2.1. Bacterial strains and culture conditions

The strain panel comprised 148 strains (summarised in Tables 2 and 4), including 58 *P. aeruginosa* and 90 non-*P. aeruginosa* strains (full details in Supplementary Table S1). All *P. aeruginosa* isolates were confirmed to be *P. aeruginosa* by the use of a species-specific PCR (*oprL* PCR; De Vos et al., 1997). The non-*P. aeruginosa* strains belonged to other *Pseudomonas* species, other Gram-negative species and Gram-positive species. Strains originated from clinical, environmental and industrial sources. Eighty-eight strains were obtained from a collection held at Cardiff University, whilst 58 strains were supplied by industry or recovered from industrial sources, and 2 were isolated from the domestic environment. All *P. aeruginosa* strains were routinely grown on Tryptone Soya Agar (TSA; Oxoid Ltd., Basingstoke, UK) or in Tryptone Soya Broth (TSB; Oxoid Ltd.) overnight (18 h) at 37 °C. All other isolates were grown at 30 °C. Overnight cultures were prepared by inoculating 3 ml of TSB with fresh (<72 h) growth material collected using a sterile pipette tip from an area of confluent growth on a pure culture plate, and incubated on an orbital shaker (150 rpm). Bacterial strains were stored frozen in individual vials in TSB containing 8% dimethylsulphoxide (DMSO) at −80 °C.

2.2. Culture media

TSB, TSA, Pseudomonas CN selective agar (PCN; Oxoid Ltd.) and Pseudomonas isolation agar (PIA; Sigma-Aldrich Company Ltd., Dorset, UK) were prepared according to the manufacturers' instructions. Glycerol and ethanol (analytical reagent grade) were supplied by Fisher Scientific UK Ltd. (Leicestershire, UK). The chromogenic medium chromID® *P. aeruginosa* (chromID Pa) was obtained as pre-poured plates from bioMérieux UK Ltd. (Basingstoke, UK). Z-agar was prepared as described by Szita et al. (1998), and modified Z-agar had the same formula as Z-agar, but with the addition of 0.01% (w/v) Bacto™ Casamino acids (BD UK Ltd., Oxford, UK) and 0.01% (w/v) yeast extract (Oxoid Ltd.).

2.3. Comparison of selective media

Overnight cultures of strains were diluted to an optical density (OD) of 1 at 600 nm (corresponding to approximately 10^8 cfu/ml) and 200 μl of the diluted culture pipetted into separate wells of a 96 well microtitre plate. The cultures were replica plated, using a 48 pin replicator (approximately 10^5 cfu/ml transferred) from the 96 well plate onto TSA, PCN, PIA, chromID Pa, Z-agar and modified Z-agar in triplicate. All agar plates were incubated at 37 °C and 42 °C for 72 h, except the TSA plates for the non-*P. aeruginosa* panel which were incubated at 30 °C for 72 h, and the Z-agar and modified Z-agar plates which were only incubated at 37 °C. Results were recorded at 24 and 72 h. Positive results on chromID Pa were originally intended to be taken as growth accompanied by a red/pink/purple colouration as described previously (Laine et al., 2009), but this interpretation was modified to also incorporate strains that displayed a pink-brown colour. Pigment production (pyocyanin and fluorescein) by *P. aeruginosa* strains on TSA, PCN and PIA was recorded at 37 °C. Growth on any of the other media was taken as a positive result, regardless of the presence/absence of pigment production.

2.4. Evaluation criteria

The sensitivity and specificity of the agars were calculated. The sensitivity was taken as the number of *P. aeruginosa* strains identified as positive for growth on the media, divided by the total number of *P. aeruginosa* strains screened; the probability of a true positive. The specificity was taken as the number of non-*P. aeruginosa* strains negative for growth on the media, divided by the total number of non-*P. aeruginosa* strains screened; the probability of a true negative (Lalkhen and McCluskey, 2008). Positive and negative predictive values (PPV/NPV) were calculated to determine the proportions of true positives and negatives for the study population. The PPV was taken as the number of *P. aeruginosa* strains identified as positive for growth on the media, divided by the total number of strains (both *P. aeruginosa* and non-*P. aeruginosa*) positive for growth. The NPV was taken as the number of non-*P. aeruginosa* strains negative for growth on the media, divided by the total number of strains (both *P. aeruginosa* and non-*P. aeruginosa*) which were negative for growth (Lalkhen and McCluskey, 2008).

3. Results

3.1. Sensitivity of the five media

PCN, PIA and mZ-agar demonstrated 100% sensitivity as all 58 *P. aeruginosa* strains grew on these agars after 24 h of incubation at 37 °C (Table 2). The sensitivity of the chromogenic agar did not reach this level due to the variable colours produced by certain *P. aeruginosa* strains (Fig. 1). Overall, 47 out of 58 strains of *P. aeruginosa* strains were correctly identified after 24 h (81% sensitivity), with 5 strains not displaying adequate growth and 6 giving the wrong colour (yellow, orange, light brown, green and black), which may have reflected a deficiency in β-alanyl aminopeptidase activity. After 72 h the sensitivity of chromID Pa increased to 95%. Growth on the Z-agars was much weaker than that on the other agars (Fig. 2) and 1 auxotrophic *P. aeruginosa* strain LESB58 (Winstanley et al., 2009) did not grow at all on the unmodified Z-agar. The modified Z-agar had nutrient supplementation sufficient for the cultivation of the LESB58 strain although growth was still very weak. There did not appear to be any major differences in