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Differential Primary Plating Medium for Enhancement of Pigment Production by *Pseudomonas aeruginosa*

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A cost-effective and more rapid means of detection of *Pseudomonas aeruginosa* in cultures from clinical specimens would be very advantageous. We have developed a modified MacConkey agar (MMA), which enhances pigment production of *P. aeruginosa* and which, if pyocyanin pigment is present, provides a relatively rapid and very cost-effective identification. The MMA medium inhibits the gram-positive organisms, while lactose- and non-lactose-fermenting gram-negative rods are easily distinguishable from pigment-producing pseudomonads. Organisms that produce pyocyanin, pyoverdin, or pyorubin, or both pyocyanin and pyoverdin, are easily recognized on the medium. Pyocyanin production is clearly distinguishable from other *Pseudomonas* pigments on MMA. In a comparative study, MMA identified 97% of the *P. aeruginosa* strains 24 h earlier than routine laboratory biochemical methods. Highly mucoid strains which did not produce detectable pigments on standard biochemicals produced detectable pigments on the MMA within 48 h. This medium can provide a very practical, reliable, and cost-effective means for early characterization of *P. aeruginosa*.

Nonfermenting gram-negative rods account for ca. 15% of all organisms isolated in the clinical microbiology laboratory. *Pseudomonas aeruginosa*, an important agent of human disease, is the most commonly isolated of these organisms (1), and the rapid isolation and identification of *P. aeruginosa* is an important consideration for the laboratory. Most strains of *P. aeruginosa* produce pyocyanin or pyoverdin or both, as well as pyorubin, pyomelanin, or various combinations of these pigments. They are the only gram-negative rods, however, that produce pyocyanin, and further biochemical tests on these organisms only increase material and labor costs without improving the accuracy of the organism identification. This report describes a primary plating medium for the detection of *Pseudomonas* pigments.

The development of the primary plating medium described in this paper was empirically determined. To avail the clinical microbiology laboratory of a rapid, practical, cost-effective means of *P. aeruginosa* identification, it was felt that the medium must not only elicit easily detectable pigment production but must also differentiate lactose-fermenting organisms from non-lactose-fermenting organisms. The work of King et al. (8) and others (3–6, 9) provided guidelines for the inclusion of inorganic constituents in the medium. Lactose and neutral red were added to allow for the determination of lactose fermentation, and crystal violet dye was added to inhibit the growth of the gram-positive organisms.

MATERIALS AND METHODS

Organisms. All organisms in this evaluation were consecutive clinical isolates of *P. aeruginosa* recovered primarily from urine, sputum, and burn patient specimens submitted to the clinical microbiology laboratories of the University of Utah Medical Center.

Media. The modified MacConkey agar (MMA) was compared with standard laboratory media. MMA contains per liter: 10 g of Difco Proteose Peptone no. 3 (Difco Laboratories, Detroit, Mich.), 10 g of Difco Peptone, 9 g of potassium sulfate, 2 g of magnesium chloride, 1.5 g of magnesium sulfate, 1.5 g of potassium phosphate, 15 g of Bacto-Agar (Difco), and 10 g of sodium citrate. In addition, per liter, 10 g of lactose, 30 μg of neutral red, and 1.5 g of bile salts no. 2 (Difco) were added for the differentiation of the lactose-fermenting organisms. Crystal violet (1 μg/liter) was included to inhibit the gram-positive organisms.

The medium was autoclaved for 15 min at 15 lb (ca. 6.6 kg) of pressure (121°C) and poured into sterile petri dishes (15 by 100 mm). The surface pH of the medium was 7.2.

Inoculation and incubation of media. Urine specimens were inoculated onto a MacConkey agar plate (BBL Microbiology Systems, Cockeysville, Md.), a Columbia sheep blood agar plate (BBL), and an MMA plate with a 0.001-ml calibrated loop. Sputum specimens were inoculated onto a MacConkey agar plate, a selective horse blood agar plate (BBL), a Columbia sheep blood agar plate, and an MMA plate. Burn patient specimens were received in the laboratory on Rodac plates (Micro Bio Products, Phoenix, Ariz.). All media were incubated at 36°C in 5% CO_2 for 18 to 24 h.

Interpretation of pigment production. Plates were examined after overnight incubation. Non-lactose-fermenting colonies growing on MacConkey agar were tested for indophenol oxidase production (Kovacs methodology) with a platinum loop. If the oxidase test was negative, grey, flat, beta-hemolytic colonies typical of *P. aeruginosa* were tested from the sheep blood agar plate to verify the oxidase reaction. Oxidase-positive organisms were subcultured from the MacConkey agar plate to a triple-sugar iron agar slant and a Tech agar slant (BBL) and incubated overnight. Tech agar enhances pyocyanin production. The MMA was examined for the presence of pigment, and the various pigment colors and their relative intensity were recorded. No further biochemical tests were performed on the isolates recovered from the MMA plates. If pigment was not apparent on the medium after overnight incubation, the plates were incubated for an additional 18 to 24 h. Green or blue-green pigment was interpreted as pyocyanin. The yellow-green pigments indicated the production of pyoverdin. A few isolates pro-

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duced a red-brown pigment that was recorded as pyorubin. Several strains of *P. aeruginosa* produced various combinations of the pigments. The *Pseudomonas* isolates that were subcultured to Tech agar also produced the same apparent variety of pigments. The results of pigment production on Tech agar were recorded as positive or negative, and the pigments were noted. No attempt was made to define the pigments elaborated on the Tech agar.

RESULTS

A total of 862 clinical specimens were plated in duplicate onto the routine primary plating media and onto MMA. Of these specimens, 135 resulted in growth of *P. aeruginosa*. Ninety-seven percent of these isolates elaborated detectable pigments on MMA within 24 h. Several strains of *P. aeruginosa* (83%) produced pigment on MacConkey agar and sheep blood agar as well, but most required 48 h of incubation. However, the pigments demonstrated on MMA suggested both increased pigment production and intensity.

Twenty-one mucoid strains of *P. aeruginosa* recovered from sputum cultures of cystic fibrosis patients failed to produce pigment on Tech agar after 48 h and required additional biochemical testing for confirmation of identity. All but three of these strains produced detectable pigment on MMA medium within 24 h. Additional incubation (up to 48 h) resulted in enhanced pigment production in all of these isolates. Ninety-five percent of the isolates that were subcultured to triple-sugar iron and Tech agars produced pigments within 24 h. However, the subculture of these organisms to the biochemicals resulted in a time loss of 18 to 24 h.

An additional capability of the MMA provides for the easy differentiation of lactose-fermenting and non-lactose-fermenting non-*Pseudomonas* organisms. The differentiation on MMA is very similar to that noted on regular MacConkey agar plates. The gram-positive organisms are inhibited on MMA.

DISCUSSION

P. aeruginosa has become an important cause of infection. In a survey of community hospitals (2), this organism was found to have caused 343 infections per 100,000 discharged patients, or ca. 12% of all reported infections. *P. aeruginosa* reportedly caused 10% of urinary tract infections, 9% of surgical wound infections, 17% of lower respiratory tract infections, and 11% of bacteremias, as revealed by the hospital surveillance program of the Centers for Disease Control (1). In addition, this organism has become an important pathogen among debilitated, burned, and immunocompromised individuals (2). Because of the ubiquitous nature of this pathogen, and since *Pseudomonas* infection typically progresses rapidly, a diagnostic tool which allows for more timely identification would be of value. Because the demonstration of pyocyanin pigment is virtually diagnostic in *P. aeruginosa* identification, a primary differential medium which enhances the pigment production of this organism may allow for a relatively rapid, inexpensive identification.

P. aeruginosa produces several extracellular pigments that are excreted into the culture medium surrounding the colonies; however, enhancement of pigment production is dependent on the composition of the medium. Pigment formation and elaboration rely on a dynamic metabolic equilibrium provided by medium constituents such as peptones, minerals, and various ions. Inasmuch as this medium was empirically derived, magnesium, potassium, phosphate, and sulfates were incorporated according to guidelines established by the work of King et al. (8) and others (3–6, 9). The precise function of these components is not known. However, systematic adjustments in concentrations of these compounds had either a stimulatory or an inhibitory effect on the production of pigment. Pyocyanin production was enhanced by the addition of 1% (wt/vol) sodium citrate, whereas the incorporation of Protease Peptone no. 3 (Difco), a peptone with a phosphorus content of nearly 0.5%, stimulated the production of pyoverdin.

MMA can provide the laboratory with a viable alternative to MacConkey agar in that it enhances the pigment production of *P. aeruginosa*, as well as maintaining the differentiating capability between the lactose-fermenting and non-lactose-fermenting gram-negative rods. The use of primary plating medium as an aid in *P. aeruginosa* identification has the potential for saving 24 h of time. Although not all of the *P. aeruginosa* isolates produced pigment on MMA, the degree of accuracy demonstrated in this study is closely comparable to most tube biochemicals and kit methodologies routinely utilized in the clinical laboratory. Furthermore, nonpigmented isolates can be analyzed by other means. Therefore, the actual accuracy of diagnosis is very high indeed. In this study, 97% of the *Pseudomonas* isolates elaborated pigment on the experimental medium. It is possible that occasionally the production of pyoverdin by *Pseudomonas fluorescens* may lead to an erroneous identification, although those isolates are uncommon, and ancillary tests such as the susceptibility profile of the organism will aid in the correct identification.

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