Identification of *Pseudomonas aeruginosa* by Pyocyanin Production on Tech Agar

E. ALBERTO PICHARDO REYES, MARTHA J. BALE, WAYNE H. CANNON, and JOHN M. MATSEN

Departments of Pathology and Pediatrics, University of Utah College of Medicine, Salt Lake City, Utah 84132

*Pseudomonas aeruginosa* is the only gram-negative bacillus capable of producing the very distinctive water-soluble pigment pyocyanin. We evaluated the reliability of this characteristic as a unique test for the identification of this organism by using Tech agar (BBL Microbiology Systems, Cockeysville, Md.) medium. A retrospective and prospective analysis was performed with a total of 835 strains of *P. aeruginosa*; 818 (98%) produced pigment within 48 h of incubation, and 96% of those which produced pigment were positive after overnight incubation. Seventeen strains (2.0%) failed to produce pigment; 15 were mucoid strains from patients with cystic fibrosis. Tech agar is an effective, simple, and inexpensive medium for *P. aeruginosa* identification and may be used as a unique test for all potential *P. aeruginosa* isolates (beta hemolytic on blood agar; lactose-negative, oxidase-positive colonies). Nonpigmented mucoid strains, as well as other nonpigmented organisms, will require additional testing to ensure proper identification.

*Pseudomonas aeruginosa* is by far the most common nonfermentative gram-negative rod isolated in the clinical microbiology laboratory. In a hospital setting, this may represent as many as 10 to 15% of all gram-negative bacilli isolated.

The identification of this particular organism is carried out in most laboratories by somewhat complex biochemical test batteries (7-9) or by kit methodology (3, 6, 10). These particular diagnostic approaches are expensive and may require as long as 48 h to derive an answer.

*P. aeruginosa* is the only known organism capable of producing the very distinctive water-soluble pigment pyocyanin, other than a species of *Streptomyces* which produces cyanomycin, which is apparently identical to pyocyanin. *P. aeruginosa* also grows at 42°C, whereas some other closely related *Pseudomonas* strains will not (*P. putida, P. fluorescens*), and has a capacity for growth on cetrimide agar as well as a distinctive antimicrobial susceptibility pattern.

We evaluated Tech agar (BBL Microbiology Systems, Cockeysville, Md.) as a means of identifying *P. aeruginosa* strains in a relatively inexpensive and direct manner.

In 1954 King et al. (5) reported two media which were demonstrated to enhance the production of water-soluble pigments by *P. aeruginosa*. Tech agar is essentially the Medium A described by those authors and is specifically formulated for the enhancement of pyocyanin production. It is commercially available, inexpensive, and easy to prepare.

We report the efficacy and reliability of Tech agar as a single test for the identification of most *P. aeruginosa* strains.

**MATERIALS AND METHODS**

**Organisms.** All organisms evaluated in this study were consecutive clinical isolates of *P. aeruginosa*, recovered from a spectrum of clinical sources in the Clinical Microbiology Laboratories of the University of Utah Medical Center.

**Retrospective analysis.** Five hundred thirty-five consecutive isolates of *P. aeruginosa*, collected over approximately 6 months, were evaluated in a retrospective analysis to determine, by work sheet notations, the production of pigment on Tech agar, growth at 42°C, and growth on cetrimide agar. Organisms which did not have all three tests completed, with results annotated on the work sheet, were excluded from the study.

**Prospective study.** A prospective study was done by one of the investigators (E.A.P.R.), in which 300 consecutive strains of *P. aeruginosa* were observed for their reactions on Tech agar, lactose fermentation, and growth on cetrimide agar and at 42°C. If incubation was required for longer than 18 to 24 h, the further-incubated tests were reread by the investigator and by personnel in the routine Clinical Microbiology Laboratory. If no pigment was present after 48 h of incubation, additional tests were utilized to completely identify the isolate to assure that we were not dealing with a significantly aberrant strain.

**Identification methods.** An oxidase test (1% tetramethyl-p-phenylenediamine dihydrochloride) was performed on all non-lactose-fermenting colonies iso-
lated on MacConkey agar. All oxidase-positive colonies were subcultured to triple sugar iron agar (Difco Laboratories, Detroit, Mich.), Tech agar, Pseudosel agar (cetrimide; BBL Microbiology Systems), and Trypticase soy broth (BBL Microbiology Systems). The triple sugar iron, Tech, and cetrimide agars were incubated aerobically at 35°C. Trypticase soy agar was incubated in a 42°C water bath. All tests were read after 18 to 24 h of incubation, and if no pigment was observed on the Tech agar after 18 h, tests were reincubated for a further 18 to 24 h. If no pigment was observed after 48 h of incubation, further classic biochemical tests were conducted (oxidation-fermentation sugars including glucose, xylose, mannitol, lactose, sucrose, and maltose; nitrate; Christensen urea; gelatin liquefaction; catalase; growth on MacConkey; growth on salmonella-shigella agar; Simmons citrate; motility; and indole and H2S production). The charts from the Center for Disease Control, done by Elizabeth King and Robert Weaver (4), were utilized to identify the isolate completely.

Interpretation of pigment production. The presence of a green or bluish color was interpreted as indicating the production of pyocyanin. The presence of a yellow pigment only was interpreted as indicating the production of pyoverdin, or fluorescein. In the prospective study all slants were observed by one of the investigators, whereas in the retrospective study, strains were observed only by the technologist who was responsible for the culture evaluation on a given day.

RESULTS

A total of 835 strains of P. aeruginosa were analyzed for pigment production in this evaluation. Retrospectively, 524 strains (97.9%) of the 535 P. aeruginosa strains thus analyzed produced pigment on Tech agar; 294 (98.0%) of 300 strains evaluated in a prospective analysis produced pigment within 48 h of incubation. The majority of the strains analyzed prospectively produced pigment after 1 day of incubation (96% of those positive), and only 12 isolates (4.0%) required an additional 24-h incubation period.

There were 17 strains (2.0%) of the total 835 strains of P. aeruginosa evaluated which failed to produce pigment. Of these, 15 were mucoid strains isolated from the sputum of patients with cystic fibrosis.

All strains evaluated in this study, in both the retrospective and prospective portions, grew at 42 and 37°C and on cetrimide agar and were nonfermentative on triple sugar iron agar.

Analysis of technologist interpretation did not demonstrate any difference in terms of percent positive readings. Interestingly, in most instances where a mucoid, non-pigment-producing strain was isolated, there were also other morphological types of P. aeruginosa present which did produce pyocyanin pigment. No demonstrable susceptibility differences were detected between those mucoid strains which did not produce pigment and the large body of those strains which were pigment producers.

Those strains which were delayed and required 48 h of incubation to produce pigment were generally mucoid, thus adding a predictive factor with regard to these particular strains.

DISCUSSION

P. aeruginosa, P. fluorescens, and P. putida all have the capacity to produce water-soluble pigments including pyoverdin, but P. aeruginosa is the only organism of the pseudomonads and other glucose-nonfermenting gram-negative bacilli known to be capable of producing pyocyanin. Although a Streptomyces species may produce cyanomycin, which is said to be identical to pyocyanin, the colonial and Gram stain morphologies of the Streptomyces would be radically different from that of P. aeruginosa. P. aeruginosa is also by far the most common isolate among the Pseudomonas species as well as among the nonfermenting gram-negative rods. Pyocyanin produced by P. aeruginosa is used in many clinical microbiology laboratories as an adjunct test in the various testing approaches used for the identification of P. aeruginosa. It has been our experience that pyocyanin is a very accurate sole indicator of the presence of P. aeruginosa, and in this particular study it was evaluated as a possible single test for the identification of this organism. Discretion must be used when interpreting pigment production. Pyoverdin may appear as a yellow-green color, which could be subjectively misread as the green color indicating pyocyanin production. However, pyocyanin is soluble in chloroform, whereas the pyoverdins are not. Therefore, any questionable green pigments may be confirmed by a simple extraction with chloroform.

In our combined prospective and retrospective studies, 818 of 835 strains (97.9%) produced pyocyanin, most of them within a 24-h incubation time frame. Those that required an additional day of incubation to produce pigment were almost all mucoid strains. This high percentage of pigment producers confirms the validity of using pyocyanin production as a constant and specific characteristic of P. aeruginosa in an identification scheme, especially among nonmucoid strains. Further, the kind of accuracy demonstrated with this particular biochemical test is comparable to that achieved with kit and standard identification methodologies.

Some mucoid strains of P. aeruginosa from patients with cystic fibrosis may not be pigment producers and may be misidentified if only pigment production is used as the identification
mechanism for this special and selective group. Growth at 42°C would indicate that these mucoid strains may be P. aeruginosa and would be indicative of the necessity for further biochemical testing to ascertain species identification in the absence of pyocyanin production.

P. aeruginosa is a very common isolate in the clinical microbiology laboratory, and its identification by commercial kits or by conventional biochemical or automated means (2) may result in a somewhat expensive process of identification. Further, more than 24 h may be required to carry out conventional or kit identification.

Tech agar is a very satisfactory alternative to extensive identification schema, and the cost of the tube would vary from $0.15 (our calculation of the cost to make it in our laboratory) to approximately $0.50 if purchased commercially. The use of Tech agar would allow identification after overnight incubation.

Most P. aeruginosa strains isolated in the clinical laboratory are strains which undergo susceptibility testing, the results of which can be used to confirm the diagnosis of these organisms. This adds further validity to the process proposed in this study. P. aeruginosa is susceptible to carbenicillin and is almost always kanamycin resistant, whereas P. fluorescens and P. putida tend to have an opposite susceptibility pattern with these two antibiotics (1).

Tech agar is thus an effective, simple, and inexpensive medium for P. aeruginosa identification, through the modality of pyocyanin production, and may be used as a single test for the identification of all potential P. aeruginosa strains from primary plating medium.

LITERATURE CITED