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Isolation Medium for the Recovery of *Pseudomonas cepacia* from Respiratory Secretions of Patients with Cystic Fibrosis

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A new medium for the isolation of *Pseudomonas cepacia* from respiratory tract secretions of patients with cystic fibrosis (CF) is described. This medium consists of inorganic salts, 0.5% pyruvate, and 0.1% proteose peptone as nutritive components and 0.0001% crystal violet, 0.15% bile salts, 100 μg of ticarcillin per ml, and 300 U of polymyxin B per ml as selective agents. The medium, designated PC medium, supported superior growth of 38 of 50 stock isolates of *P. cepacia* after 48 h of incubation when compared with MacConkey agar (0 of 50). The medium completely inhibited the growth of 112 of 124 stock isolates of organisms commonly found in respiratory secretions of CF patients. Cultures were made on PC medium with respiratory secretions of 169 CF patients. *P. cepacia* was recovered from 35 patients with isolates on PC medium but from only 21 patients with isolates on MacConkey agar. Of 221 other potentially pathogenic isolates found in these specimens, only six (two *Pseudomonas aeruginosa* isolates, two molds, one yeast, and one *Serratia marcescens* isolate) grew on PC medium. PC medium should facilitate the recovery of *P. cepacia* from CF patients.

Recent studies have suggested that *Pseudomonas cepacia* may be emerging as an important pulmonary pathogen in patients with cystic fibrosis (CF) (6, 8, 12). In a case-controlled study, CF patients colonized with *P. cepacia* were more frequently hospitalized and died earlier than control CF patients (O. C. Tablan, T. L. Chorba, D. V. Schidlow, K. A. Hardy, P. H. Gilligan, J. H. White, W. M. Morgan, L. A. Carson, W. J. Martone, J. M. Jason, and W. R. Jarvis, J. Pediatr., in press). Clinical experience suggests that the emergence of this organism in the respiratory tree of older CF patients can be associated with rapid deterioration in pulmonary status and death (6). Despite these data, the role of *P. cepacia* in pulmonary disease of CF patients remains unclear. One of the major problems in determining the actual incidence of colonization by *P. cepacia* in CF patients with severe pulmonary disease has been difficulty in isolating this organism. Although *P. cepacia* can survive and multiply in hostile environments such as distilled water and disinfectants (2–4), it has been our experience that it often grows more slowly on commonly used laboratory media such as blood and MacConkey agar than do other organisms, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* commonly found in the respiratory tree of CF patients. We have also observed that *P. cepacia* can be overgrown by these organisms on media commonly used for its isolation. We designed an isolation medium for the recovery of *P. cepacia*, designated PC medium, to enable investigators to isolate *P. cepacia* more reliably from CF patients. PC medium is based in part on a holding medium designed by one of us (B.T.D.) for long-term maintenance of *P. cepacia*, other pseudomonads, and *Enterobacteriaceae*.

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MATERIALS AND METHODS

PC medium formulation. DeCicco holding medium contained, per liter, $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $Fe(NH_4SO_4)_2 \cdot 6H_2O$, 0.01 g; phenol red, 0.02 g; and agar, 15 g, dissolved in 950 ml of 0.04 M potassium phosphate buffer (pH 6.2). After being autoclaved at 121°C for 15 min and cooled to 50°C, 50 ml of 10% pyruvic acid sodium salt was added to the basal medium. The following additions to DeCicco holding medium were necessary to ensure that PC medium would support rapid growth of all strains of *P. cepacia* while remaining sufficiently selective to inhibit the growth of other organisms commonly found in the respiratory tree of CF patients. Before the medium was autoclaved, 0.1 ml of 1% crystal violet, 1.0 g of proteose peptone no. 3, and 1.5 g of bile salts were added per liter of medium. After being autoclaved, sterile solutions of ticarcillin sodium salt (100 mg) and polymyxin B sulfate (300,000 U) were added per liter of medium. All inorganic chemicals used were reagent grade. Proteose peptone no. 3, bile salts (Oxgall), and agar were purchased from Difco Laboratories, Detroit, Mich. Polymyxin B sulfate and pyruvic acid sodium salt were purchased from Sigma Chemical Co., St. Louis, Mo. Ticarcillin was a gift from Beecham Laboratories, Bristol, Tenn.

Isolates used in growth studies. Fifty strains of *P. cepacia* isolated from CF patients at St. Christopher's Hospital for Children (SCHC), Philadelphia, Pa., were used in growth studies. The identity of all 50 strains as *P. cepacia* was confirmed by the Hospital Infection Program, Centers for Disease Control, Atlanta, Ga. The *P. aeruginosa* and *S. aureus* strains used in these studies were also isolated from CF patients at SCHC. All other organisms (see Table 2) used

TABLE 1. Comparison of growth of 50 isolates of *P. cepacia* on PC medium, MacConkey agar, and 5% sheep blood agar after 48 h of incubation

Growth condition and results	No. of isolates
PC medium vs MacConkey agar	
Superior on PC medium	38
Superior on MacConkey agar	0
Similar	12
PC Medium vs 5% sheep blood agar	
Superior on PC medium	0
Superior on blood agar	8
Similar	42

in growth studies were fresh clinical isolates, identified by standard laboratory techniques, from patients at SCHC or North Carolina Memorial Hospital, Chapel Hill, N.C.

Growth studies. Growth studies were performed by inoculating the test organisms into 3 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubating them overnight at 35°C. These cultures were then adjusted to a turbidity equal to a McFarland standard of 0.5, and each isolate was inoculated with a sterile cotton swab onto a quadrant of a PC medium plate, a MacConkey agar plate, and, when *S. aureus* was tested, a 5% sheep blood agar plate. The plates were incubated at 35°C and examined daily for the presence or absence of growth. When growth of *P. cepacia* was being compared on PC medium, MacConkey agar, and 5% sheep blood agar, the relative abundance of growth was recorded for each medium on each of 3 days.

Six *P. cepacia* strains were chosen at random for the comparison of quantitative recovery of this organism on PC medium, MacConkey agar, and 5% sheep blood agar. These strains were grown overnight in Trypticase soy broth and diluted 1:10,000 in saline to make a stock suspension. This stock suspension was diluted 1:10, 1:100, and 1:1,000 in saline, and 0.1 ml of the stock suspension and each dilution was plated onto duplicate plates of each medium. After 48 h of incubation at 35°C, each plate was counted, and the number of organisms for each strain recovered on each type of medium was recorded.

Culture of clinical specimens. Respiratory secretions, primarily sputa, deep pharyngeal swabs, and bronchial washings, were cultured from patients identified by physicians or by patient registry at two CF centers as having CF. At SCHC, the cultures were made with PC medium, 5% sheep blood agar, chocolate agar, mannitol salt agar (Difco), and MacConkey agar. Cultures at North Carolina Memorial Hospital were made on the same media as at SCHC, except that 5% horse blood agar and colistin-nalixidic acid agar were substituted for chocolate agar and sheep blood agar. All plates were incubated at 35°C for 72 h and examined daily for the presence of *P. cepacia* and other potential respiratory pathogens. *P. cepacia* was identified by the following criteria: colonial morphology, disk diffusion susceptibility testing, and (at SCHC) the Uni-N/F Tek (Flow Laboratories, Inc., McLean, Va.) or (at North Carolina Memorial Hospital) conventional biochemical testing (5). All other potential pathogens were identified by standard techniques (9).

RESULTS

Preliminary studies. Studies were done to modify the holding medium formulation of DeCicco to develop an isolation medium for the recovery of *P. cepacia*. Certain additions to the medium were found to be necessary. An amino acid source (0.1% proteose peptone no. 3) was required for the growth of some of the *P. cepacia* isolates. Experiments were also done to examine different selective agents. The addition of 0.15% bile salts and either 100 or 300 U of polymyxin B per ml allowed similar growth of 10 *P. cepacia* isolates when compared with media without these components. Because of this observation, 10 clinical specimens from CF patients were cultured on the holding medium containing 0.1% proteose peptone no. 3, 0.15% bile salts, and 300 U of polymyxin B per ml to assess its selectivity. These experiments showed that *S. aureus*, mucoid and rough strains of *P. aeruginosa*, *Proteus mirabilis*, yeast, and mold, as well as *P. cepacia*, grew on this medium. Because of these findings, 100 μg of ticarcillin per ml and 0.0001% crystal violet were added to the medium, the former to inhibit gram-negative organisms and the latter to inhibit gram-positive organisms.

Growth and selectivity studies. Experiments were performed which compared the growth of 50 clinical isolates of *P. cepacia* on MacConkey and 5% sheep blood agar and on PC medium, which contained 300 U of polymyxin B per ml, 100 μg of ticarcillin per ml, 0.15% bile salts, and 0.0001% crystal violet. Growth on PC medium was equal or superior to growth on MacConkey agar after 48 h of incubation (Table 1). Comparative growth on 5% sheep blood agar and PC medium showed that most isolates (84%) grew equally well on the two media. In addition, all 50 *P. cepacia* isolates grew on PC medium within 48 h.

Quantitative studies to compare the recovery of six *P. cepacia* strains on these three media were performed by quantitatively culturing a 1:10,000 dilution of an overnight broth culture of these strains. Sheep blood was the most sensitive, with an average recovery for these six strains of 1.2×10^4 CFU/ml. PC medium was nearly as sensitive, with an average recovery of 9.1×10^3 CFU/ml, and MacConkey agar was the least sensitive, with an average recovery of 6.1×10^3 CFU/ml.

To assess the selective nature of this medium, 120 isolates of various gram-negative bacilli and 4 isolates of *S. aureus* were examined for their ability to grow on PC medium (Table 2). The choice of gram-negative bacilli for study was based on the likelihood of recovery from sputa of CF patients (1). None of the four strains of *S. aureus* grew on this medium. Of 120 gram-negative bacillus isolates, 12 grew on PC medium, and all grew on MacConkey agar. Of the organisms tested, strains of *Klebsiella pneumoniae* were able to grow most frequently on PC medium. All 12 non-*P. cepacia* isolates which grew on PC medium did poorly when compared with their growth on MacConkey agar. Because of

TABLE 2. Comparison of growth of gram-negative rods on PC medium and MacConkey agar after 48 h of incubation

Organism (no. of isolates)	Growth detected on:	
	PC medium	MacConkey agar
Achromobacter sp. (10)	2	10
Acinetobacter sp. (10)	0	10
Enterobacter sp. (4)	0	4
E. coli (6)	0	6
Klebsiella sp. (13)	6	13
Proteus sp. (10)	2	10
P. aeruginosa (43)	1	43
Pseudomonas maltophilia (10)	1	10
Serratia sp. (4)	0	4

TABLE 3. Comparative recovery of *S. aureus*, *P. aeruginosa*, and *P. cepacia* from 169 CF patients at 2 CF centers

Organism	No. positive/total at[a]:	
	Center 1	Center 2
S. aureus	61/126	20/43
P. aeruginosa	73/126	31/43
P. cepacia	34/126	1/43

[a] Number of patients positive for specific organism/number of patients cultured.

the ability of PC medium to support the growth of all *P. cepacia* isolates tested and its ability to inhibit the growth of over 90% of isolates likely to be encountered in respiratory secretions of CF patients, PC medium was examined to determine whether it could be used as a primary isolation medium to enhance the recovery of *P. cepacia* from respiratory cultures from CF patients.

Clinical studies. Respiratory secretions from 169 CF patients at two CF centers were examined for the presence of potential respiratory tract pathogens, with special emphasis on the detection of *P. cepacia*. *P. cepacia* was present in CF patients at both centers (Table 3). The recovery rate at center 1 (27%) was significantly higher than that at center 2 (2%). The recovery of *S. aureus* (48 and 47%, respectively) and *P. aeruginosa* (58 and 72%, respectively) at the two centers, on the other hand, was similar.

When recovery of *P. cepacia* on PC medium was compared with that on MacConkey agar, PC medium was clearly superior (Table 4). Of the 35 isolates of *P. cepacia* recovered, 14 were found only on PC medium. This was, in part, because in cultures from seven patients there was confluent growth of highly mucoid strains of *P. aeruginosa* on MacConkey agar which probably obscured or inhibited the growth of *P. cepacia*. In isolates from 18 patients, the growth of *P. cepacia* was superior on PC medium; three isolates showed similar growth on the two media. No isolates were found only on MacConkey agar, nor did any isolate grow better on MacConkey agar.

The ability of PC medium to inhibit the growth of other potential pathogens was also examined. Of 221 potential pathogens, only 6 grew on PC medium (Table 5). These included two ticarcillin-resistant *P. aeruginosa* isolates, two mold isolates, an isolate of *Serratia* sp., and one yeast. No organisms which would be considered normal respiratory flora, such as diphtheroids, saprophytic *Neisseria* sp., coagulase-negative staphylococci, and oral streptococci, grew on PC medium (data not shown).

Description of the growth of *P. cepacia* on PC medium. PC medium is dull yellow when uninoculated. After 24 h of grown on PC medium, *P. cepacia* colonies are usually pinpoint, and in areas of heavy growth, the medium may begin to turn pink. After 48 h of growth, isolated colonies are 1 to 2 mm in diameter. In areas of heavy growth, the agar is usually hot pink. As the colonies age, they may become slightly larger and have a purplish hue or remain grayish white. The hot-pink color reaction is due to the alkaline end products produced as a result of pyruvate metabolism by *P. cepacia*, with phenol red being the pH indicator present in the medium. Other organisms which grow on this medium may also turn the medium pink; PC medium therefore cannot be considered a truly differential medium.

TABLE 4. Comparative growth of the 35 isolates of *P. cepacia* recovered from clinical specimens on PC medium and MacConkey agar

Growth condition	No. of isolates
PC medium only	14
Superior on PC medium	18
MacConkey agar only	0
Superior on MacConkey agar	0
Similar	3

TABLE 5. Recovery of other potential pathogens on PC medium compared with recovery on all other media used for 169 CF patients

Organism	No. of isolates recovered on:	
	PC medium	All other media
Achromobacter sp.	0	2
Acinetobacter sp.	0	2
Beta-hemolytic streptococci	0	3
Citrobacter sp.	0	1
Enterobacter sp.	0	5
E. coli	0	5
Haemophilus influenzae	0	5
K. pneumoniae	0	1
P. aeruginosa	2	104
P. maltophilia	0	1
S. marcescens	1	2
S. aureus	0	81
Mold	2	7
Yeast	1	2

DISCUSSION

Chronic respiratory disease is the primary contributing factor in the premature death of patients with CF (13). *S. aureus* and *P. aeruginosa*, especially mucoid colonial variants, have been well recognized as important pulmonary pathogens in this process. With the advent of relatively effective antimicrobial therapy to control these two pathogens, life expectancy in this patient population has risen dramatically (13). Recently, *P. cepacia* has emerged as a potentially important respiratory pathogen in at least three large CF centers (6, 8, 12). One characteristic of *P. cepacia* which has made its emergence particularly distressing has been its resistance, in vitro and in vivo, to both conventional and experimental antimicrobial therapy (7).

The role of *P. cepacia* in pulmonary disease remains controversial. In cases in which it is recognized, the spectrum of disease can range from long-term colonization to rapidly progressive (1 to 3 weeks) fatal illness. Unlike *P. aeruginosa*, *P. cepacia* is relatively avirulent in animal models (4, 11). Although isolates from CF patients have both proteolytic and lipolytic activities, they produce neither elastase nor toxins similar to *P. aeruginosa* toxin A, both of which are postulated to be important virulence factors in pulmonary disease in CF patients (10; P. H. Gilligan, W. H. Yeung, D. Yucht, and D. Schidlow, Cystic Fibrosis Club **25**:113, 1984).

To assess the role of *P. cepacia* in pulmonary disease of CF patients, an isolation medium specific for it appears to be essential. *P. cepacia* has been reported to be present in as few as 1% to as many as 27% of CF patients, depending on the reporting center (1). These may represent real differ-

ences in the centers, as was shown in this study (with a selective medium, 27% of the patients were positive for *P. cepacia* in one center and 2% in the other), or differences in the ability of different laboratories to recognize *P. cepacia* among flora growing in isolates from the respiratory tract of CF patients. A major concern when clinical specimens from CF patients are examined for *P. cepacia* is its slow growth on commonly used laboratory media when compared with other organisms present in these specimens. Mucoid strains of *P. aeruginosa* are of particular concern, as shown in this study: 20% of the *P. cepacia* isolates were not recovered on MacConkey agar in the presence of heavy growth of mucoid strains of *P. aeruginosa*. These isolates would not have been detected by means other than PC medium. Overall, 40% of the *P. cepacia* strains isolated in this study were found only on PC medium. These data suggest that a selective medium specific for *P. cepacia* isolation, such as PC medium, should be used to obtain more accurate epidemiological information concerning the role that *P. cepacia* plays in the pulmonary deterioration seen in CF patients.

Two other selective media for the isolation of *P. cepacia* have recently been described. One medium (M. J. Muszynski, D. A. Pickett, and D. F. Welch, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 179, 1984), in which bacitracin and polymyxin B are used as selective agents, and production of acid from lactose is used as a differential test, has been shown to support the growth of stock cultures of *P. cepacia*, which has been recovered on this medium from sputa seeded with it. With this medium, *P. cepacia* was not recovered from sputum specimens obtained from 25 CF patients (D. Welch, personal communication).

The other medium that was recently described (14) was designed to recover *P. cepacia* from environmental sources. With 9-chlora-9-(4-diethylaminophenyl)-10-phenylacridan and polymyxin B as selective agents, it effectively inhibits the growth of most organisms. However, *Serratia marcescens* and *P. cepacia* grow equally well on this medium. In addition, the efficacy of recovery of *P. cepacia* is judged by using only environmental water specimens. It would be difficult to extrapolate the utility of this medium for isolation of *P. cepacia* from clinical specimens based on studies with environmental water specimens.

The use of PC medium should enhance the recovery of *P. cepacia* from respiratory secretions of CF patients. The more reliable recovery of *P. cepacia* from CF patients should aid in the evaluation of the role of this organism in pulmonary disease in CF patients.

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