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Comparative Evaluation of Selective Media for Isolation of *Pseudomonas cepacia* from Cystic Fibrosis Patients and Environmental Sources

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Pseudomonas cepacia **has recently emerged as an important pathogen affecting cystic fibrosis (CF) patients. We evaluated three selective media to assess their comparative potential for identification of patients colonized with *P. cepacia* and for efficacy of detection of *P. cepacia* in environmental fluids. Test organisms included *P. cepacia* isolates from CF patients (10 each from two CF centers), non-CF patients (10 isolates), and environmental sources (10 isolates). Microbiologic assays were done by the membrane filter procedure; filters were placed on *P. cepacia* medium (PCM), OFPBL, TB-T, MacConkey agar (MAC), and blood agar (BA) or Standard Methods (SM) sugar, and colonies were counted after incubation at 30 or 35°C for 72 h. Mean recovery efficiencies (MREs) (mean CFU/ml on selective media compared with CFU/ml on BA controls) for environmental and non-CF *P. cepacia* and patient isolates from one CF center showed a rank order of PCM > OFPBL > TB-T; for isolates from a second CF center, a rank order of PCM > TB-T > OFPBL was obtained. MREs for CF center isolates were generally lower than for non-CF patients or environmental isolates on *P. cepacia*-selective media. With MAC, the MREs for each group of CF isolates were extremely low (14 and 2%) compared with those for non-CF patient (47%) or environmental (84%) isolates. In laboratory and field studies, PCM and OFPBL showed good selectivity against bacteria commonly associated with CF patient respiratory secretions. These findings show that selective media should be used in clinical settings where *P. cepacia* is sought. With environmental fluids from CF centers, *P. cepacia*-selective media showed low selectivity against a variety of gram-negative water bacteria and appeared to afford little advantage over SM agar for isolating *P. cepacia* from environmental samples.**

In recent years, *Pseudomonas cepacia* has emerged as an important pathogen affecting patients with cystic fibrosis (CF) disease (3, 4, 6, 7, 10, 12). The prevalence of *P. cepacia* colonization in CF patients has been reported to be as high as 15 to 27% in some CF centers (2, 5–7, 11), and colonization has been linked to more rapid clinical deterioration (3, 6, 7, 9). Historically, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have constituted the most significant pathogens routinely isolated from the sputum samples of CF patients (5–7, 12). Because *P. cepacia* tends to grow slowly and to exhibit variable colony morphology on media commonly used in clinical laboratories for sputum culture, this organism is easily obscured by overgrowth of mucoid *P. aeruginosa* and other respiratory pathogens or overlooked because of the morphologic similarity of *P. cepacia* colony variants to *S. aureus* (1, 2, 7, 9). Similarly, efforts to define the role of the environment in the acquisition and transmission of *P. cepacia* in CF patients have been hampered by overgrowth of other gram-negative bacteria taken from environmental fluids from CF centers and plated on standard laboratory media (1, 2, 7, 10, 14).

With the development of media selective for *P. cepacia* (2, 5, 13, 14), increased recovery of *P. cepacia* from the sputum samples of CF patients and from environmental sources has been reported by a number of centers (1, 2, 5, 9, 13). However, a questionnaire survey of CF center directors showed that although 113 of 125 responding centers (90.4%) reported the use of screening for *P. cepacia*, only 11 of 113 centers (9.7%) reported the use of *P. cepacia*-selective media. In 1985, a prospective study was initiated by the Hospital Infections Program, Centers for Disease Control, in collaboration with the Cystic Fibrosis Foundation, to assess the national incidence and significance of *P. cepacia* colonization in CF patients.

The studies reported here were designed to provide data for the development of recommended standardized microbiologic procedures for screening for *P. cepacia* in CF centers participating in the national prospective study, in order to minimize bias and ensure greater accuracy in identifying cases. These studies included: (i) comparison of quantitative recovery of *P. cepacia* isolates from CF and non-CF patients and environmental sources on *P. cepacia*-selective and routine clinical laboratory media; and (ii) evaluation of the efficacy of *P. cepacia*-selective media in isolating *P. cepacia* from sputum samples from patients and from environmental fluids containing mixed microbial flora by streak-plating and membrane filtration procedures.

MATERIALS AND METHODS

Media. Selective media with bacteriostatic dyes, antibiotics, or low pH have been described for the selective isolation of *P. cepacia*, and the following were evaluated to determine their efficacy for recovery of *P. cepacia*: (i) *P. cepacia* medium (PCM) (21), containing crystal violet, polymyxin B, and ticarcillin (pH 6.2); (ii) OFPBL (13), containing polymyxin B and bacitracin (pH 6.8); and (iii) TB-T medium (C. Hagedorn, W. D. Gould, T. R. Bardinelli, and D. R. Gustavson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I53, p. 155) containing trypan blue and tetracycline (pH 5.5). Blood agar (BA) and MacConkey agar (MAC) (Baltimore Biological Laboratories, Cockeysville, Md.), which are rou-

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tinely used in CF clinical laboratories for sputum culture, were used as controls.

Growth studies. Quantitative recovery of *P. cepacia* on selective media was assessed with the following test strains: 20 *P. cepacia* sputum isolates from CF patients (10 each from two CF centers), and 20 non-CF isolates (10 from non-CF hospital patients and 10 from a variety of environmental sources). The organisms were subcultured on Standard Methods (SM) agar at 35°C for 18 h; cells were suspended in commercially obtained sterile distilled water (CDW) to approximately 10^6 CFU/ml and held at 30°C for 96 h. These stock suspensions were diluted in CDW to approximately 10^4 CFU/ml, and microbiologic assays were done by the membrane filter (MF) procedure. Aliquots of 10-fold dilutions in buffered distilled water were filtered (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and rinsed with buffered distilled water, and filters were placed on each of the five test media. Colonies were counted after incubation at 30 (OFPBL and TB-T) or 35°C (PCM, BA, and MAC) for 24, 48, and 72 h; plates showing no growth were held 7 days before discarding. Recovery efficiency was determined as the ratio of recovery (CFU/ml) on selective media to that on BA, and data are presented as the mean recovery efficiency (MRE) for each group of isolates.

The selectivity of PCM, OFPBL, and TB-T media was assessed by using the following organisms: *Acinetobacter* spp., *Corynebacterium* spp., *Diplococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella* spp., *Moraxella* spp., *Neisseria* spp., *P. aeruginosa* (mucoid and nonmucoid), *Pseudomonas maltophilia*, *S. aureus*, and streptococci. Isolates were subcultured on BA or chocolate agar (candle jar incubation) at 35°C for 18 h, and stock suspensions (1×10^3 to 1×10^4 CFU/ml) were prepared in CDW or 0.9% saline. Aliquots of 10-fold dilutions were assayed (MF procedure), and filters were placed on PCM, OFPBL, and BA or CA. In one experiment, cells from stock suspensions of each of four water-adapted *P. cepacia* strains (two from CF patients, one from a non-CF patient, and one from an environmental source) were added to each of six non-*P. cepacia* cell suspensions to obtain approximately 10^2 CFU/ml of each isolate. The mixed suspensions ($n = 24$) were assayed by the MF method with PCM, OFPBL, and TB-T media. Recovery efficiency for each *P. cepacia* and non-*P. cepacia* isolate was determined after incubation at 30 or 35°C for 72 h.

Field studies. These were designed to compare streak-plating and MF procedures for isolating *P. cepacia* from fresh sputum samples of CF patients and to assess the effect of cold storage (24-h refrigeration, ca. 4°C) of sputum specimens on subsequent recovery of *P. cepacia*. Fresh sputum samples from CF patients were streaked onto BA, MAC, OFPBL, and PCM within 15 min of collection at a CF center, and plates were incubated at 30 or 35°C. The sputum samples were placed in refrigerated containers and transported to the Centers for Disease Control, and at 24 h, the sputum samples were assayed both by streak-plating and MF procedures.

To assess the efficacy of selective media for detection of environmental *P. cepacia*, we assayed (MF procedure) environmental fluids obtained from one CF center with SM, PCM, OFPBL, and TB-T media and from a second CF center with SM, PCM, and OFPBL media.

Identification of isolates. All *P. cepacia* isolates from CF patient sputum and environmental samples were presumptively identified with a screening set of six conventional biochemical tests (oxidation of glucose, lactose, and mannitol in OF media, presence of lysine decarboxylase, and absence of arginine dihydrolase, and beta-galactosidase activity). *P. cepacia* and non-*P. cepacia* isolates were confirmed by using rapid diagnostic test systems: N/F system (Flow Laboratories, Inc., McLean, Va.), API Rapid NFT system (API Analytab Products, Plainview, N.Y.), and Minitek (BBL Microbiology Systems, Cockeysville, Md.).

TABLE 1. Comparative recovery of *P. cepacia* isolates from CF patients and non-CF sources on various selective media

Type of medium	% Recovery[a]			
	CF patient isolates		Non-CF isolates	
	Center A ($n = 10$)	Center B ($n = 10$)	Patient strains ($n = 10$)	Environmental strains ($n = 10$)
PCM	93 ± 20	87 ± 17	95 ± 15	100 ± 7
OFPBL	75 ± 29	64 ± 39	90 ± 20	99 ± 4
TB-T	44 ± 27	81 ± 31	55 ± 50	66 ± 47

[a] % recovery was calculated as follows: [(CFU/ml on selective media)/(CFU/ml on blood agar control)] × 100. Each value shown is the mean ± standard deviation.

RESULTS

Growth studies. Overall, PCM appeared to be most effective in recovering pure cultures of *P. cepacia* strains, irrespective of the source of isolates (Table 1). Comparison of MRE (mean CFU/ml) on selective media compared with CFU/ml on BA controls showed higher recovery on PCM than on TB-T for isolates from patients from center A ($P < 0.001$, Student's t test), non-CF patient isolates ($P = 0.02$), and environmental isolates ($P = 0.03$). MREs on OFPBL were greater than on TB-T for center A isolates ($P = 0.02$) and for environmental isolates ($P = 0.03$); for non-CF patient isolates, the difference was not significant ($P = 0.05$). With center B isolates, differences in MREs among the three selective media were not statistically significant. In comparing recovery of all CF patient isolates ($n = 20$) with non-CF isolates ($n = 20$) on each of the three selective media, OFPBL showed significantly lower recovery for CF isolates ($P < 0.01$); differences with PCM and TB-T were not significant. *P. cepacia* isolates from CF and non-CF patients showed poor recovery on MAC, a medium routinely used in clinical laboratories for primary isolation (Table 2). MREs for each group of CF isolates were extremely low (14 and 2%) compared with non-CF patient (47%) or environmental (84%) isolates.

Tests to assess the ability of *P. cepacia*-selective media to inhibit growth of non-*P. cepacia* organisms commonly found in patient respiratory secretions showed that with inocula of 1×10^3 to 1×10^4 CFU/ml, none of 17 strains examined was able to grow on PCM (Table 3). On OFPBL, *P. maltophilia* grew readily, and viable counts of *P. aeruginosa* were reduced by a factor of 10^2 when compared with that for BA controls. Further studies with *P. maltophilia* and *P. aeruginosa* showed that at high levels of inoculum (1×10^6 to 1×10^7 CFU/ml), *P. maltophilia* could be recovered on PCM (10^3 CFU/ml), whereas *P. aeruginosa* continued to show no growth.

A comparison of MREs for *P. cepacia* strains from mixed microbial suspensions (Table 4) showed considerable variation from results obtained with pure *P. cepacia* suspensions. MREs for isolates from CF patients (center A), non-CF patients, and environmental isolates were in the following

TABLE 2. Comparative recovery of *P. cepacia* on BA and MAC

Organisms	MRE[a] (log_{10} CFU/ml)		*P* value[b]
	BA	MAC	
CF isolates			
Center A (n = 10)	3.60 ± 0.49	1.79 ± 1.23	$P<0.001$
Center B (n = 10)	4.04 ± 0.30	0.59 ± 0.77	$P<0.001$
Non-CF isolates			
Patient (n = 10)	4.19 ± 0.20	3.82 ± 0.30	$P<0.01$
Environmental (n = 10)	3.82 ± 0.78	3.73 ± 0.82	NS[c]

[a] Mean ± standard deviation.
[b] Student's *t* test.
[c] NS, Not significant.

descending order: OFPBL > TB-T > PCM. Center B isolates, which had shown the highest MRE on TB-T (81%) of the four test groups, now showed an extremely low MRE for TB-T (6%), and an overall recovery order of PCM > OFPBL > TB-T. *P. cepacia*-selective media were generally effective in inhibiting non-*P. cepacia* organisms in the mixed suspensions; none of the strains tested was able to grow on TB-T, and *P. aeruginosa* was recovered only on OFPBL; although *Klebsiella* spp. grew on PCM or OFPBL, mean viable counts were reduced by a factor of 10^2.

Clinical specimens. Overnight refrigeration (ca. 4°C) of sputum samples appeared to affect recovery of *P. cepacia* by the streak-plating method. *P. cepacia* was recovered from three fresh sputum specimens streaked on BA, PCM, or OFPBL (Table 5) but from only two of three refrigerated specimens streaked on BA or PCM and from one of three streaked on OFPBL medium. On the other hand, refrigeration did not affect recovery of *P. cepacia* by MF procedures; assays using BA, PCM, and OFPBL demonstrated that *P. cepacia* was in fact still present in high numbers (1×10^6 to 1×10^7 CFU/ml). With MAC, *P. cepacia* was recovered from only one of three fresh sputum samples, and no samples were positive after overnight refrigeration.

Environmental samples. To assess the efficacy of selective media for detecting *P. cepacia* in environmental fluids, samples were collected from the water treatment and distribution system of a CF center (e.g., particulate and carbon filters, deionizer, holding tanks, and water taps in the pharmacy and CF clinic [n = 8]), and from pulmonary function-testing machines (n = 2). Results of microbiologic assays showed total viable counts ranging from no growth (3 of 10) to 3.3×10^4 CFU/ml on PCM, from no growth (3 of 10) to 4.2×10^4 CFU/ml on OFPBL, and from no growth (5 of 10) to 1.6×10^4 CFU/ml on TB-T. Total viable counts on SM agar controls ranged from 1.9×10^1 to 5.0×10^6 CFU/ml. *P. cepacia* was isolated from a sample that had been through a carbon filter (7.3×10^1 CFU/ml on PCM and 7.7×10^1 CFU/ml on SM agar) and from deionization tank effluent (3.3×10^4 CFU/ml on TB-T and 3.5×10^4 CFU/ml on SM agar); *P. cepacia* was not detected on OFPBL for any of the water samples tested. Gram-negative non-*P. cepacia* isolates included *Acinetobacter*, *Alcaligenes* spp., *Achromobacter* spp., *Moraxella*, *Pseudomonas mesophilica*, *Pseudomonas paucimobilis*, *Pseudomonas pickettii*, *Pseudomonas putida*, and *P. stutzeri*.

At a second CF center, samples of tap water that were used to rinse nebulizers between use in the rooms of patients and water samples from the common tub, shower, and drinking fountain in the CF patient wing (n = 8), showed total viable counts on SM agar ranging from 4.0×10^{-2} to 2.3×10^2 CFU/ml. No growth was observed on PCM medium (50-ml sample portions) for any of the samples. On OFPBL, viable counts of 5.1×10^0 and 1.4×10^0 CFU/ml were obtained, respectively, in shower and drinking fountain samples. *P. cepacia* was not identified in any of these samples. Sterile rinses (CDW) of tubing used in pulmonary function-testing machines before and after use showed no detectable organisms. Nebulizer fluids (n = 2) remaining at the end of treatment of the patient showed viable counts of 2.0×10^{-1} and 3.5×10^1 CFU/ml on SM agar. In this latter sample, *P. cepacia* was detected on PCM (9.3×10^0 CFU/ml), OFPBL (3.0×10^0 CFU/ml), and SM agar (1.0×10^1 CFU/ml).

DISCUSSION

With the recent increase in the incidence of respiratory infections and continued reports of accelerated clinical deterioration and poorer prognosis in CF patients colonized with *P. cepacia* (3, 4, 6, 10, 11), the question of how *P. cepacia* is acquired has become a major area of concern. *P. cepacia* is ubiquitous, being found in a wide variety of natural aquatic and soil environments, as well as in hostile environments, such as aerosol antibiotics, topical anaesthetics, and disinfectants (4, 5). Although studies have sug-

TABLE 3. Efficacy of *P. cepacia* selective media in inhibition of growth of bacteria commonly found in CF respiratory secretions

Organism	Growth (log_{10} CFU/ml)		
	Blood-chocolate agar control	Selective media	
		PCM	OFPBL
Neisseria meningitidis	3.69	NG[a]	NG
N. flavescens	3.64	NG	NG
N. subflava	3.74	NG	NG
D. pneumoniae	3.39	NG	NG
Alpha-streptococcus	3.55	NG	NG
Beta-streptococcus	3.52	NG	NG
P. aeruginosa	3.72	NG	1.72
Corynebacterium diphtheriae (biotype mitis)	3.71	NG	NG
C. pseudodiphtheriticum	3.57	NG	NG
C. minutissimum	4.19	NG	NG
P. maltophilia			
Pm-1	4.06	NG	3.83
Pm-2	4.25	NG	4.16
Pm-3	4.20	NG	4.20
Pm-4	4.31	NG	4.20
Pm-5	4.10	NG	4.10
Pm-6	3.93	NG	3.49

[a] NG, No growth.

TABLE 4. Selectivity of various media for isolation of *P. cepacia* from mixed cultures containing non-*P. cepacia* organisms

Organisms	No. of tests	% Recovery[a]					
		PCM		OFPBL		TBT	
		Range	Mean	Range	Mean	Range	Mean
P. cepacia							
CF patient isolates							
Center A	6[b]	56–100	81	84–100	97	90–100	98
Center B	6	46–100	86	45–100	6	<1–10	6
Non-CF isolates							
Patient	6	47–100	81	83–100	95	65–100	92
Environmental	6	19–76	40	71–100	91	52–84	67
Non-*P. cepacia*							
P. aeruginosa	4[c]	NG[d]		46–100	80	NG	
Moraxella spp.	4	NG		NG		NG	
Acinetobacter spp.	4	NG		NG		NG	
E. coli	4	NG		NG		NG	
Klebsiella spp.	4	4–13	9	3.28	11	NG	
S. aureus	4	NG		NG		NG	

[a] Calculated as [(CFU/ml on selective media)/(CFU/ml on blood agar control)] × 100.
[b] Six test suspensions, each containing a *P. cepacia* strain and one of six non-*P. cepacia* strains.
[c] Four test suspensions, each containing a non-*P. cepacia* strain and one of four *P. cepacia* strains.
[d] NG, No growth.

gested a possible role for patient contact with other *P. cepacia*-colonized patients or siblings as a risk factor for acquiring *P. cepacia* (5, 10, 11), this has not been demonstrated unequivocally. Similarly, the role of the inanimate environment during hospitalization (i.e., pulmonary function equipment, aerosol solutions, stethoscopes, sinks, showers, etc.) in the acquisition and transmission of *P. cepacia* among CF patients remains undetermined (5, 6, 10–12).

To elucidate epidemiologic factors for *P. cepacia* in CF patients, methods of detecting *P. cepacia* must be sensitive and reproducible. This requires selective media for *P. cepacia* that have a high efficiency for recovery of *P. cepacia* and the ability to inhibit background contaminants in the mixed microbial populations typically found in the sputum samples of CF patients and in environmental fluids (2, 7, 14). It also requires that such media be easily prepared or readily available commercially, if they are to be routinely used by clinical laboratories. One of the media reported to be highly selective for *P. cepacia*, contained a chemical selective agent (C390) that was not readily available for routine laboratory procedures, and this medium was not included in the study (14).

In tests to compare the sensitivity of the three selective media for recovering known numbers of *P. cepacia*, differences in recovery efficiency were observed between two groups of isolates from two CF centers, as well as between CF-patient, non-CF patient, and environmental isolates. This may reflect differences in genetic makeup and physiologic characteristics of *P. cepacia* strains or in the type or frequency of usage of prescribed antibiotics so that predominant subtypes become established in different CF centers. This may further reflect differences in the adaptive mechanisms that allow *P. cepacia* to colonize CF patients, in contrast to mechanisms that allow *P. cepacia* to grow in natural fluid environments and to survive in such hostile environments as dyes, preservative solutions, antiseptics, and disinfectants (4).

PCM showed good inhibition of pure cultures of bacterial strains most commonly found in the respiratory secretions of CF patients and allowed good recovery of *P. cepacia* when mixed suspensions of organisms were tested. TB-T similarly showed good inhibition, whereas OFPBL only minimally inhibited *P. aeruginosa*, *P. maltophilia*, and *Klebsiella* spp. Recovery rates of *P. cepacia* isolates (center B) from mixed suspensions were extremely low both on OFPBL and TB-T. PCM was previously shown to have a good recovery efficiency (75.8%) in quantitative studies using six *P. cepacia* isolates from the sputum samples of CF patients (2). In another study (1), comparable recovery of *P. cepacia* taken from the sputum samples of CF patients and plated on PCM and OFPBL was reported; however, no quantitative data from studies with known numbers of *P. cepacia* or other organisms were presented.

PCM, OFPBL, and TB-T may not have similar selectivity for *P. cepacia* in environmental fluids containing high levels of naturally occurring gram-negative water bacteria. Isolates from environmental fluids that grew on these media (up to 10^4 CFU/ml) included *Alcaligenes* and *Achromobacter* spp.,

TABLE 5. Effect of refrigeration (24 h) on recovery of *P. cepacia* on nonselective and selective media by streak-plating and MF procedures

Patient	Type of medium	Recovery of *P. cepacia* from:		
		Fresh sputum samples by streak plating	Refrigerated sputum samples	
			Streak plating	MF
1	BA	+	+	+
	MAC	−	−	ND[a]
	PCM	+	+	+
	OFPBL	+	+	+
2	BA	+	−	+
	MAC	−	−	ND
	PCM	+	+	+
	OFPBL	+	−	+
3	BA	+	+	+
	MAC	+	−	ND
	PCM	+	−	+
	OFPBL	+	−	+

[a] ND, Not done.

P. paucimobilis, *P. stutzeri*, *P. mesophilica*, *P. pickettii*, *P. putida*, *P. maltophilia*, *P. aeruginosa*, *Flavobacterium* spp., molds, and yeasts, These findings are consistent with those reported by other investigators (1, 7, 13). In addition, when *P. cepacia* was the predominant organism present, SM agar appeared to be as effective as PCM or TB-T in recovering *P. cepacia* from environmental fluid samples.

It is interesting to note that MAC, a commonly used primary isolation medium, did not support good growth of laboratory strains of *P. cepacia* isolated from CF patients and was not effective in isolating *P. cepacia* from fresh sputum samples of *P. cepacia*-colonized CF patients; these results are consistent with those of other studies reporting its low sensitivity and selectivity (2, 9, 13).

Standardized procedures in processing sputum specimens and environmental fluids are needed, because the techniques now used vary from simple streak-plating of various amounts of raw sputum (e.g., calibrated loop, swab, and pipette [9]), to homogenizing and diluting sputum before streak plating. In addition, environmental fluids are frequently treated as clinical specimens; volumes as low as 0.01 or 0.1 ml are cultured by spread plating. Not infrequently, sputum may be refrigerated for an unspecified length of time before microbiologic assays; this can affect either the quantity or type of organisms recovered (8). We found that overnight refrigeration of CF patient sputum resulted in reduced recovery of *P. cepacia* on PCM and OFPBL media by streak-plating methods. Membrane filter procedures were found to be efficient for the microbiologic assay of either sputum samples or environmental fluids.

In summary, these studies confirm that the use of selective media, such as PCM or OFPBL, which were developed for use in clinical settings for the isolation of *P. cepacia* provide greater sensitivity and reproducibility in detecting *P. cepacia* in the sputum specimens of CF patients. The findings also suggest that there is probably a need for an improved medium for detecting *P. cepacia* in environmental specimens.

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