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Comparison of Isolation Media for Recovery of *Burkholderia cepacia* Complex from Respiratory Secretions of Patients with Cystic Fibrosis

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***Burkholderia cepacia* selective agar (BCSA) has previously been devised for isolation of *B. cepacia* from respiratory secretions of patients with cystic fibrosis and tested under research laboratory conditions. Here we describe a study in which BCSA, oxidation-fermentation polymyxin bacitracin lactose agar (OFPBL), and *Pseudomonas cepacia* agar (PCA) were compared in routine culture procedures for the ability to grow *B. cepacia* and inhibit other organisms. Three hundred twenty-eight specimens from 209 patients at two pediatric centers and 328 specimens from 109 adults were tested. Plates were inoculated, incubated, and read for quality and quantity of growth at 24, 48, and 72 h. Five (1.5%) specimens from 4 (1.9%) children and 75 (22.9%) specimens from 16 (14.7%) adults grew *B. cepacia* complex. At 24, 48, and 72 h, BCSA achieved 43, 93, and 100% detection, respectively; OFPBL achieved 26, 84, and 96%, respectively; and PCA achieved 33, 74, and 84% detection, respectively. Quality was assessed as pinpoint or good growth. At 24 h, most cultures growing *B. cepacia* complex had pinpoint colonies. By 48 and 72 h, 48 and 69% of *B. cepacia* complex cultures, respectively, had good growth on BCSA, while on OFPBL 19 and 30%, respectively, had good growth and on PCA 11 and 18%, respectively, had good growth. BCSA was superior to OFPBL and PCA in suppressing organisms other than *B. cepacia* complex; 40 non-*B. cepacia* complex organisms were isolated from BCSA, 263 were isolated from OFPBL, and 116 were isolated from PCA. We conclude that BCSA is superior to OFPBL and PCA in its ability to support the growth of *B. cepacia* complex and to suppress other respiratory organisms.**

Burkholderia cepacia is an important pathogen in pulmonary infections of patients with cystic fibrosis (CF). Because of the resistance of *B. cepacia* to many antimicrobial agents, *B. cepacia* infections are difficult to eradicate once a patient has become infected, and studies have demonstrated that it can spread from patient to patient as well as cause substantial morbidity and mortality (7, 10, 12, 13, 15–17). In the clinical laboratory, *B. cepacia* can be difficult to isolate, as it usually grows more slowly than other organisms frequently found in respiratory secretions from CF patients and, consequently, in culture can be overgrown with bacteria such as mucoid *Pseudomonas aeruginosa*. Once isolated, *B. cepacia* is often difficult to identify, especially when isolated from a patient with long-standing colonization, because the organism can undergo phenotypic changes (1). These auxotrophic changes can cause the strain to no longer react as expected in basic identification tests, resulting in the strain of *B. cepacia* being misidentified as other members of the *Burkholderia* or *Alcaligenes* genus or as *Ralstonia pickettii* (8). Alternatively, these organisms, as well as *Stenotrophomonas maltophilia*, can be misidentified as *B. cepacia* (2, 8, 9).

A taxonomic study on *B. cepacia*-like bacteria and creation of a database comprising all presently known *Burkholderia* species and genomovars, *Ralstonia* species, and *P. aeruginosa*, based on whole-cell protein electrophoresis, have recently been performed (18). This work has indicated that the species previously called *B. cepacia* is actually a complex of closely related organisms that are genetically distinct from each other. The taxonomic studies revealed that whole-cell protein electrophoresis is a very useful method for genomovar differentiation within the *B. cepacia* complex. However, this method is laborious and technically demanding and therefore not available in most laboratories. A variety of DNA-based identification procedures are currently being developed and should allow differentiation of most genomovars within the *B. cepacia* complex in due course (4, 12, 14, 21). As yet, only two of the five members can be phenotypically separated from the group. The complex includes *Burkholderia vietnamiensis* (formerly genomovar V); *Burkholderia multivorans* (formerly genomovar II); and *B. cepacia* genomovars I, III, and IV (6, 18). Accurate discrimination by genomovar typing among members of the *B. cepacia* complex will enable determination of the prevalence of each genomovar in a given epidemiological niche.

In an effort to improve the speed and accuracy of the isolation of the *B. cepacia* complex, we had devised an enriched selective medium. *B. cepacia* selective agar (BCSA) contains 1% lactose and 1% sucrose in an enriched base of casein and yeast extract with 600 U of polymyxin per ml, 10 μg of gentamicin per ml, and 2.5 μg of vancomycin per ml (8). We compared BCSA to oxidation-fermentation polymyxin bacitracin lactose agar (OFPBL; oxidation-fermentation agar supplemented with lactose, 300 U of polymyxin per ml, and 0.2 U of bacitracin per ml) (20), and to *Pseudomonas cepacia* agar (PCA; DeCicco holding medium with 300 U of polymyxin per ml and 100 μg of ticarcillin per ml) (5), in growth and selection of a wide range of bacterial strains from our laboratory collection. In this paper, we describe a study comparing the same

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three media used in three hospitals in the routine setup procedures for respiratory specimens from CF patients. The participating laboratories received guidelines regarding comparison of the selective media but were allowed to incorporate the additional selective agars into their existing CF respiratory protocols as best suited the individual laboratory's procedures. We felt that it was important to incorporate laboratory-to-laboratory and technologist-to-technologist variations into the study, as long as the main criterion was met; that the three selective media within each specimen were treated equally.

(The material reported in this paper was presented at the 12th Annual North American CF Conference, October 1998 [7a].)

MATERIALS AND METHODS

The laboratories participating in the study were at St. Paul's Hospital (SPH), Vancouver, British Columbia, Canada; Children's Hospital and Regional Medical Center (CHRMC), Seattle, Wash.; and The Children's Hospital (TCH), Denver, Colo. Each hospital contributed the selective medium that they were using and was supplied with BCSA made by the University of British Columbia (UBC) laboratory. BCSA was made as preivously described (8), with the following clarification: the phenol red and crystal violet were prepared as 0.8 and 0.02% aqueous solutions, respectively, and 10 ml of each was added per liter. SPH required OFPBL, which was made and supplied by UBC. PCA was purchased from PML Microbiologicals (Tualatin, Oreg.) for SPH and CHRMC. CHRMC purchased prepared OFPBL plates from BBL, Cockeysville, Md. TCH purchased PCA and OFPBL from Remel LP (Lenexa, Kans.). The plates were included as part of the routine and selective media used by the individual laboratories for the culture of CF respiratory pathogens. At SPH, following the routine plates for sputum culture (blood, MacConkey, colistin-nalidixic acid, and inhibition mold agars) the laboratory's usual *B. cepacia* isolation plate (PCA) was inoculated first, followed by the other two plates. TCH inoculated the plates in random order; their routine media included blood, MacConkey, mannitol salt, and *Haemophilus influenzae* isolation agars. CHRMC performed quantitative sputum analyses, and plates were inoculated in random order; their other media included MacConkey, Mycosel, DNase, mannitol salt, streptococcal selective, and *H. influenzae* selective agars. Quantitation of pathogens in sputum was performed by a modification of the technique of Wong et al. (3). Sputa were solubilized by mixing 0.5 g of sputum with 0.5 ml of Sputolysin (Calbiochem, La Jolla, Calif.) and vortexing thoroughly. Dilutions (10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}) were made in phosphate-buffered saline (pH 7.0) with 0.1% gelatin added. One-hundred-microliter aliquots of each of the first three dilutions were plated on media selective for *B. cepacia* complex. Throat swabs were processed by placing them in 0.99 ml of phosphate-buffered saline with gelatin and vortexing thoroughly. Processing of all throat swabs and any sputum samples of less than 0.1 g was only qualitative but was performed on selective media. The number of CFU per gram of sputum was quantified by colony counts on each plate and calculated by the formula (CFU $\times$ 2)/dilution. SPH and CHRMC incubated selective plates between 35 and 37°C (ambient air), and TCH incubated *Burkholderia* selective media at 30°C; observations were made after 1, 2, and 3 days of incubation.

Growth on each of the selective media was graded for quantity and quality of growth. Quantity of growth at SPH and TCH was determined as follows: scant, less than 10 colonies in the main inoculum area; 1+, more than 10 colonies in the main inoculum area; 2+, growth into the second quadrant; 3+, growth into the third or fourth quadrant. The quantitative counts supplied by CHRMC were converted in the following manner: 1 to 100 CFU/ml = scant; 100 to $<10^4$ CFU/ml = 1+; 10^4 to 10^6 CFU/ml = 2+; $>10^6$ CFU/ml = 3+. Quality of growth was determined as either pinpoint or good growth at each time point. At SPH and TCH, the technologist assigned to the CF bench for that day read plates. The technologists reading the cultures were familiar with their own selective medium but had not used the other two media before, and so they graded quality and quantity of growth in as unbiased a manner as possible. One technologist at CHRMC performed all quantitative counts and qualitative observations.

SPH sent most plates with any growth to UBC for further examination and identification. Initially, CHRMC sent pure cultures of all organisms isolated to UBC, but due to the large number of yeast and fungi isolated, the laboratory switched to sending only gram-negative organisms for confirmation of identification. TCH sent only gram-negative organisms for confirmation to UBC. Organisms were identified with the API 20 NE or API 20E (Biomerieux Vitek, Inc., Hazelwood, Mo.) and oxidative-fermentative sugars as previously described (8, 19). Organisms identified as *Burkholderia* species or unusual alkaline nonfermenting gram-negative bacilli were subjected to whole-cell protein electrophoresis as described by Vandamme et al. (18) for confirmation of identification and genomovar determination. At least one *Burkholderia* species per patient was tested by random amplified polymorphic DNA (RAPD) PCR (13) and compared to other isolates previously analyzed for that patient when available, as well as to other isolates from that center. RAPD fingerprinting was performed in order to determine whether the organism was the same clonal strain as isolated previously from that patient and to ascertain the number of distinct strains present in each center.

TABLE 1. Specimens of respiratory secretions submitted for analysis

Category	No.	No growth[a] [no. (%)]	Growth[b] [no. (%)]	
			Not *B. cepacia*	*B. cepacia* complex
Pediatric throat	165	139 (84.2)	26 (15.8)	0
Pediatric sputum	163	77 (47.2)	81 (49.7)	5 (3.1)
Adult sputum	328	144 (43.9)	109 (33.2)	75 (22.9)
Total specimens	656	360 (54.9)	216 (32.9)	81 (12.3)

[a] No growth of organisms on any selective medium.
[b] Growth of organisms on any selective medium.

RESULTS

A total of 656 specimens were cultured; 328 cultures were from 109 patients attending the adult center (SPH) and 328 cultures were from 209 patients attending the two pediatric centers (Table 1). All specimens from adults were sputum cultures. From the pediatric clinics, the specimens were split between throat swabs and sputa (three bronchial alveolar lavage specimens were included with the sputum results). Cultures were examined for growth, and specimens were categorized as to whether there was growth on any of the selective agars, growth of organisms other than *B. cepacia* complex on any of the selective agars, or growth of *B. cepacia* complex. One hundred thirty-seven of 209 (65.6%) pediatric patients had no growth of any organisms on any agar selective for *B. cepacia*: only five (3.1%) sputum cultures from four (1.9%) children grew *B. cepacia* complex. *B. cepacia* complex was not isolated from any throat specimens. Of the 109 adults in the study, 61 (60.0%) had no growth of any organisms on the selective media and 16 (14.7%) had growth of *B. cepacia* complex from 75 (22.9%) sputum cultures.

Table 2 lists the organisms isolated on the *B. cepacia* selective media. The table is divided into three sections; organisms confirmed as *B. cepacia* complex, organisms that were not *B. cepacia* complex but were difficult to separate biochemically from *B. cepacia* complex, and all other organisms.

Sixteen adult patients were infected with *B. cepacia* complex. Isolates from 14 adult patients were identified as *B. cepacia* genomovar III and were subtyped by RAPD PCR to groups 1, 2, 4, 6, and 16. One adult was transiently colonized with an organism that was most likely *B. cepacia* genomovar I. Two adults were infected with *B. multivorans* (genomovar II, different RAPD types), and one became cocolonized with *B. cepacia* RAPD group 6 (genomovar III). This patient was cocolonized with the two organisms for several months, after which only the *B. cepacia* organism was isolated. Fifteen specimens from one adult were received over an 18-week period; *B. cepacia* (RAPD group 02, genomovar III) failed to grow on two OFPBL and six PCA plates after 3 days of incubation. This organism was scored as good growth on all BCSA plates and as pinpoint growth on the remaining OFPBL and PCA plates.

Samples from only five pediatric patients yielded *B. cepacia* complex. Three children had isolates identified as *B. multivorans* with unique RAPD patterns. Of the two other children, one had *B. vietnamiensis* isolated from one culture and an unidentified nonfermenting gram-negative bacillus (UNFB) from another sputum 3 months later; the other child had a UNFB, most likely a *Burkholderia* species, but identification could not be confirmed. Sputa from four children at one center grew *Burkholderia gladioli*; each had a unique RAPD pattern.

TABLE 2. Recovery of organisms from BCSA, OFPBL, and PCA selective media from 296 respiratory specimens from patients with CF

Organism	No. of isolates recovered on:		
	BCSA	OFPBL	PCA
B. cepacia complex (total)	81	78	68
Genomovar I	1	0	1
Genomovar III	73	71	63
B. multivorans (genomovar II)	6	6	3
B. vietnamiensis (genomovar V)	1	1	1
Organisms similar to *B. cepacia* complex (total)	9	13	6
B. gladioli	1	5	1
Probable *Burkholderia* sp.	2	2	1
Unidentified, not *Burkholderia* sp.	1	1	1
R. pickettii	1	1	1
Ralstonia species	3	3	2
Alcaligenes species	1	1	0
Other non-*B. cepacia* complex (total)	31	250	110
Achromobacter xylosoxidans	2	3	2
Alcaligenes denitrificans	1	1	0
P. aeruginosa	3	22	26
S. maltophilia	0	7	10
Flavobacterium indologenes	5	3	2
Other nonfermenters[a]	0	2	4
Coliforms[b]	0	5	5
Staphylococcus sp.	0	23	8
Other gram-positive[c]	0	4	0
Yeast	3	138	18
Fungus	17	42	35

[a] Other nonfermenters: *Pseudomonas putida*, one on OFPBL and two on PCA; *Pseudomonas fluorescens*, one on PCA; *Sphingomonas paucimobilis*, two on OFPBL and one on PCA.

[b] Coliforms: *Proteus* species, three on OFPBL; *Serratia marcescens*, one on OFPBL; *Serratia liquefaciens*, one on PCA; *Klebsiella pneumoniae*, three on PCA; *Serratia* species, one on OFPBL and one on PCA.

[c] Other gram-positive: diphtheroids and *Streptococcus* species.

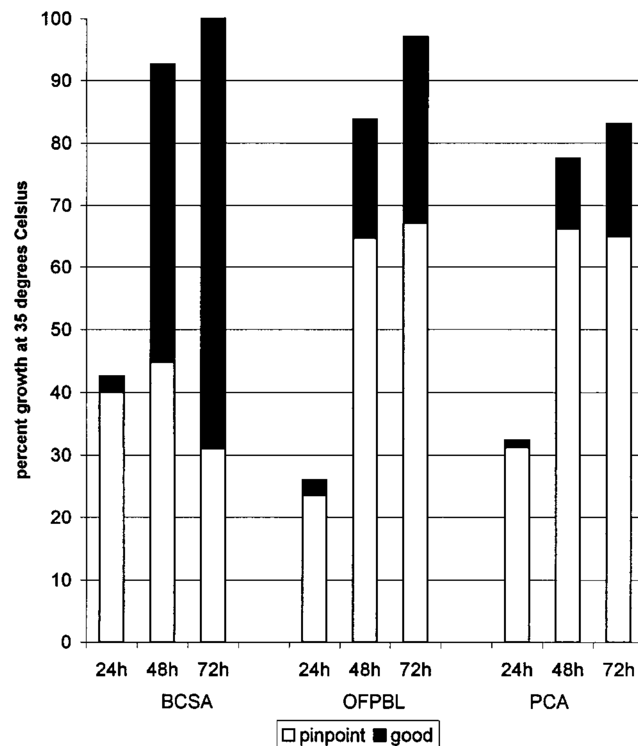

FIG. 1. Quality of growth for 81 cultures with *B. cepacia* complex, expressed as pinpoint or good growth over 3 days of incubation at 35°C. Not all selective plates were scored for quality of growth on each of the three days of incubation, and so the percentages reflect data only for the 67 to 81 plates for each of the three media scored each day.

Quantitative analyses for *B. gladioli* ranged from 6.0×10^2 to 4.1×10^5 CFU/ml. The children's specimens also produced most of the *Ralstonia*-like organisms and the organisms that were difficult to identify.

Most organisms other than *B. cepacia* complex that grew on the three selective media were graded as scant or 1+ (grew in the first quadrant of the main inoculum) and consisted of fungi, yeast, and gram-positive organisms. SPH identified the yeast and fungi to species level and identified most as *Aspergillus fumigatus* and *Candida albicans*. One adult had seven cultures that grew a mixture of *Wangiella dermatitidis*, *C. albicans*, *Scedosporium apiospermum*, *A. fumigatus*, *Acremonium* species, and *Staphylococcus aureus* on the selective plates. The cultures from this patient accounted for two of the fungi (*S. apiospermum*) isolated on BCSA, six yeasts and five fungi on OFPBL, and five fungi on PCA. OFPBL suppressed yeast and fungi poorly. OFPBL and PCA performed similarly in their inability to suppress growth of some gram-negative bacilli, notably *P. aeruginosa* and *S. maltophilia*. The gram-negative organisms and the *Staphylococcus* species frequently grew in clumps of mucus. Ten cultures positive for *B. cepacia* complex also grew other organisms such as *P. aeruginosa*, *Serratia marcescens*, *Staphylococcus* species, yeast, and fungi on OFPBL and PCA; a fungus grew on one BCSA plate. The presence of these contaminating organisms did not appear to hinder the detection of *B. cepacia* complex.

CHRMC used a quantitative sputum culture method on all CF sputa. Specimens from two patients yielded low counts of 20 to 700 CFU (per ml) of a UNFB resembling *Burkholderia* species and *B. multivorans* on BCSA and OFPBL and no growth on PCA. One of these patients also had 2.0×10^3 CFU (per ml) each of *C. albicans* on OFPBL and *Staphylococcus* sp. on PCA. A third patient had two cultures 10 weeks apart, growing *Ralstonia* species (between 1.6×10^4 and 1.0×10^6 CFU/ml). Two other patients had three specimens growing *B. multivorans* on all three plates (between 8.0×10^3 and 2.1×10^8 CFU/ml).

Analysis of quantity of growth of *B. cepacia* genomovar III for the adults (most of whom had been colonized for a number of years) was usually 2+ to 3+. BCSA demonstrated heavier growth more quickly than OFPBL or PCA. By 72 h, 66.7% of BCSA plates demonstrated 3+ growth compared to 61.7 and 48.1% for OFPBL and PCA, respectively.

Quality of growth of *B. cepacia* complex (pinpoint or good growth) is displayed in Fig. 1. *B. cepacia* complex was visible on 42.5% of BCSA plates within the first 24 h. The detection rate improved to 92.6 and 100% after 2 and 3 days of incubation, respectively. *B. cepacia* complex initially appeared as pinpoint colonies, but the quality of growth improved to good growth for 69.0% of the positive cultures on BCSA by the third day. OFPBL and PCA had fewer plates demonstrating any growth after the first day's incubation (26.0 and 32.6%, respectively). This rate improved with additional incubation, but by the third day, 67.1% of OFPBL and 64.9% of PCA plates still demonstrated only pinpoint colonies. Total growth at 72 h for the 81 cultures with *B. cepacia* complex was as follows: BCSA, 100%; OFPBL, 96.2%; and PCA, 83.9%.

DISCUSSION

Three selective agars were compared in clinical laboratory settings for their ability to isolate *B. cepacia* complex from respiratory secretions of patients with CF. *B. cepacia* complex grew more quickly and to a larger colony size on BCSA than on OFPBL or PCA. Quantitative analysis indicated that as few as 20 CFU of *B. multivorans* per ml could be detected on BCSA and OFPBL, demonstrating excellent sensitivity of the media. Several strains of *B. cepacia* genomovar III and *B. multivorans* did not grow, or grew poorly, on PCA. Similarly, one strain each of *B. cepacia* genomovars III and I did not grow on OFPBL. If either OFPBL or PCA had been used as the sole selective medium, potentially up to four patients with *B. cepacia* complex could have missed being detected. BCSA was able to support the growth of different clones within genomovars of the *B. cepacia* complex, since all *B. multivorans* isolates had DNA fingerprints that were different from each other, and the *B. cepacia* genomovar III isolates from 14 adult patients had five distinct RAPD patterns. Barth and Pitt (1) described auxotrophy in isolates of *B. cepacia* from CF patients; it may be that some strains from patients who have been colonized for a long time can no longer grow on minimal media. Carbon assimilation studies performed in an effort to identify to species level within the *B. cepacia* complex have demonstrated auxotrophy (8a). BCSA is more enriched than OFPBL or PCA, as it contains yeast extract, not found in OFPBL or PCA, and five times the pancreatic digest of casein that is found in OFPBL (PCA does not contain casein). Yeast extract and casein provide a rich variety of ingredients to overcome the nutritional deficiencies that may prevent some strains of *B. cepacia* from growth on other selective media.

As described previously (2, 8), we had observed isolates of *S. maltophilia* that had been sent to us, identified as *B. cepacia*; in these clinical trials, no isolates of *S. maltophilia* grew on BCSA. As well, BCSA inhibited significantly more non-*B. cepacia* complex organisms than did OFPBL and PCA. Several organisms that we were unable to identify were isolated but were not *B. cepacia* complex, as determined by whole-cell protein electrophoresis. These were more commonly found in children, or in adults who had not had *B. cepacia* complex before. The appearance of these organisms seemed to be transient, as they were not present in subsequent cultures. It is important that new isolates of suspected *B. cepacia* complex be sent to a reference laboratory experienced in the phenotypic and genotypic identification of this group.

The in-hospital laboratory study demonstrated that, for the culture of CF respiratory specimens, BCSA was superior to OFPBL and PCA for rapidity and quality of recovery of *B. cepacia* complex and was more inhibitory toward organisms other than *B. cepacia* complex. These features would make BCSA an important addition to CF sputum culture protocols.

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