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Novel Method for Processing Respiratory Specimens for Detection of Mycobacteria by Using C_{18}-Carboxypropylbetaine: Blinded Study

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A novel method for processing respiratory specimens to improve culture and acid-fast staining of mycobacteria is introduced. This new method utilized *N,N*-dimethyl-*N*-(*n*-octadecyl)-*N*-(3-carboxypropyl)ammonium inner salt (Chemical Abstract Service no. 78195-27-4), also known as C_{18}-carboxypropylbetaine (CB-18). In a blinded, five-center study, CB-18-based processing was compared to the standard method combining NALC and NaOH (NALC/NaOH). A total of 573 respiratory specimens were tested. Individual specimens were split approximately equally; the host institutions processed half of each specimen by the NALC/NaOH method, while the other half was processed with CB-18 at Quest Diagnostics—Baltimore. A total of 106 specimens were culture positive for acid-fast bacilli (AFB). Replacement of the primary decontamination agent with CB-18 caused changes in all diagnostic parameters. Aggregate culture sensitivity improved by approximately 43% ($P < 0.01$), and smear sensitivity improved by approximately 58% ($P < 0.01$). The sensitivity of smear relative to that of *M. tuberculosis* isolates exceeded 93% ($P < 0.01$) when specimens were processed with CB-18. The average times to a positive result were reduced by 7.3 days in liquid culture ($P < 0.01$) and 5.3 days on solid media ($P < 0.05$); however, the CB-18 method had a 20.8% contamination rate in liquid culture versus a rate of approximately 7.5% with NALC/NaOH processing. There were also unusual reductions in liquid culture sensitivity and smear specificity among CB-18-processed specimens. The characteristics of the latter parameters suggested that refinement of the CB-18 processing method should allow further improvements in culture sensitivity. This study showed that the CB-18 method has the potential to improve both smear and culture detection for these important human pathogens.

The World Health Organization estimates that approximately one-third of the world's population is infected with tuberculosis and that in this decade alone, almost 90 million people will contract tuberculosis (8). During the same time period, approximately 3 million people will die annually from this disease, resulting in more deaths than are caused by any other single pathogen (2). Early diagnosis of tuberculosis remains one of the primary hurdles in curtailing the spread of this disease.

There are myriad problems associated with the laboratory detection of mycobacterial infections. These problems can be grouped into three general categories. First, the nature of both the specimen and the processing method compromises detection. For example, the specimens and solutions used to process specimens inhibit nucleic acid amplification. Most specimens contain large numbers of saprophytic and/or infectious microorganisms that interfere with culture methods; hence, a decontamination step is essential. Decontamination compromises the viability of the mycobacteria (6, 9, 10, 23, 25, 26), and thus processing impacts the sensitivity of detection by culture as well.

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Second, the innate nature of the disease produces low copy number and intermittent shedding of the organisms. Recent submissions to the Food and Drug Administration for amplification-based tuberculosis detection kits illustrate a significant number of specimens presenting with low copy number (1). Amplification sensitivities on smear-positive specimens exceeded 95%, but sensitivities of approximately 50% on smear-negative specimens were reported (i.e., high false-negative rate). The studies of Clarridge et al. (3) and Wobeser et al. (24) demonstrate typical results from studies with significant *n* values, and the panels described by Noordhoek et al. (11, 12) show the diverse results between academic studies evaluating amplification tests.

The third problem relates to the inherent physiological nature of the mycobacteria. Attributes of mycobacteria include (i) aggregation, cording, and clumping; (ii) surface tension caused by the waxy cell wall; (iii) buoyancy; (iv) slow growth; and (v) a thick cell wall. All methods approved by the Centers for Disease Control and Prevention (CDC) for preparing clinical specimens for detection involve a centrifugation step (6). Surface tension and buoyancy combine to significantly compromise the efficacy of concentrating specimens for analysis (i.e., enrichment of mycobacteria in the pellet), independent of detection method (5–7, 13–18, 21). The consequence of inefficiently concentrating these organisms is intensified by both cording and the innate low-copy-number phenomena, and this

effect is further complicated by viability and/or slow growth (e.g., culture methods) or the difficulty associated with lysing these organisms (e.g., amplification methods). The net effect is that mycobacteria are so scarce in processed sediments that Poisson distribution dominates sampling (e.g., some sediment aliquots have no target molecules or bacilli), and the few organisms that are collected must be efficiently lysed or must be viable to compete with contaminating bacteria. In addition, the low copy number requires large specimen volumes, which in turn demands a concentration-decontamination step.

Therefore, because the specimens, the solutions used to process specimens, and the processing itself, as well as the inherent nature of the disease and the innate physiological nature of these organisms, complicate detection, a new method for specimen processing is needed. The method must negate surface tension and buoyancy to facilitate more efficient collection of the bacilli. The method should maintain the viability of the mycobacteria and alleviate cording to enhance distribution in processed sediments. Finally, the method should not interfere with the diagnostic technique itself.

We introduce a new processing method based on the use of a zwitterionic detergent that simultaneously solves many of these problems. This detergent, *N,N*-dimethyl-*N*-(*n*-octadecyl)-*N*-(3-carboxypropyl)ammonium inner salt (Chemical Abstract Service no. 78195-27-4), also known as C_{18}-carboxypropylbetaine (CB-18), was initially presented in 1996 (20) and was completely described in U.S. patent 5,658,749 (21). The purpose of this laboratory-based study was to compare the recommended processing method combining NALC and NaOH (NALC/NaOH) (6) with a CB-18-based method with respect to processing respiratory specimens for detection of mycobacteria. The results of a blinded, five-center study are discussed wherein these two processing methods were compared by smear and culture on split specimens.

MATERIALS AND METHODS

Specimen collection. Five-hundred seventy-three respiratory specimens were tested. Specimens were collected from five sites: the District of Columbia Department of Human Services Bureau of Laboratories, Washington, D.C.; Johns Hopkins Medical Institutes, Baltimore, Md.; Quest Diagnostics—Baltimore (Quest), Baltimore, Md.; Quest Diagnostics—Teterboro, Teterboro, N.J.; and the University of Maryland, Baltimore, Baltimore, Md.. All specimens entered into the study were originally submitted to the host institutions for routine analysis for the presence of acid-fast bacilli (AFB). Only respiratory specimens with greater than 5 ml were entered into the study. Daily specimens were split approximately equally each day. Each site used a modified version of the NALC/NaOH procedure recommended by the CDC (6) to process half of each specimen, while the other half of each specimen was sent to Quest, where it was processed by the CB-18 procedure described below.

Specimens to be processed by the CB-18 procedure were placed in sterile 50-ml conical tubes for transport. Specimens from sites 2, 3, and 5 were transported by courier to Quest daily. Specimens from sites 2 and 3 were stored at 4°C for 6 h and 30 h, respectively, and specimens from sites 1 and 5 were stored at room temperature for 3 h and 36 h, respectively, prior to being processed with CB-18. Specimens from site 4 were shipped overnight at room temperature and were delayed for approximately 48 h before being processed. The results of processing by NALC/NaOH were blinded until the end of the study, whereas the CB-18 processing results were reported to each site in real time.

Smear analysis. All processed specimens were subjected to acid-fast staining by the recommended auramine-rhodamine method (6). Smear values were recorded according to guidelines recommended by the CDC (6).

An abnormally large number of specimens presented with smear-positive and ± smear values when processed by the CB-18 method. Many of these results could not be confirmed by culture. In order to discuss the relevance of these findings, the following decision was made regarding smear results: if a ± smear value was recorded for a given specimen by a particular processing method and if an AFB culture-positive result was obtained with the same specimen by any method (e.g., NALC/NaOH or CB-18, on liquid or solid media), then the ± smear result was deemed smear positive by that processing method for that specimen. Furthermore, if a smear value of 1+ or higher was recorded for a given specimen by a particular processing method, then that specimen was reported as smear positive by that processing method, independent of the culture results.

Culture analysis. All processed specimens were subjected to analysis by culture on both liquid and solid media according to recommended procedures (6). The amount of NALC/NaOH-processed sediment was approximately equal to the amount of CB-18-processed sediment analyzed for each specimen. All sites except site 4 used the BACTEC 12B/460TB culture system (Becton Dickinson, Cockeysville, Md.) as the primary culture method. Site 4 used the MGIT culture system (Becton Dickinson) as the primary method. All liquid cultures were supplemented with PANTA (Becton Dickinson). CB-18 liquid cultures were further modified as described below. Liquid cultures were checked for growth every other day for 2 weeks and then weekly thereafter before being discarded. NALC/NaOH cultures were monitored for 8 weeks. In an effort to evaluate the effects of this new processing procedure on culture, liquid cultures of specimens processed with CB-18 were monitored for 16 weeks. All sites used Lowenstein-Jensen (L-J) slants as the secondary culture method, with the exception of site 2, which used SeptiCheck as the backup culture method. Site 5 used 7H11 slants in addition to L-J slants. Sediments from CB-18-processed specimens were planted on 7H11-selective slants as the secondary culture method. All solid cultures were checked weekly for 8 weeks. All positive cultures were subjected to acid-fast staining, according to recommended procedures (6), to evaluate the presence of AFB.

Mycobacterial identification. Mycobacterial isolates were identified by AccuProbe (Gen-Probe, San Diego, Calif.) or standard biochemical analyses (6). In some instances, isolates were identified to the species level by lipid profiles. Lipid profiles were determined by gas-liquid chromatography according to procedures recommended by the manufacturer (MIDI, Newark, Del.).

NALC/NaOH processing. The NALC/NaOH protocol (6) varied slightly from site to site. Sites 2 through 5 used 4% NaOH (i.e., 2% final concentration), while site 1 used 3% NaOH (i.e., 1.5% final concentration). In general, specimens were typically treated with an equal amount of a solution containing the appropriate amount of NaOH supplemented with 0.5% NALC and 1.45% citrate. Specimens were incubated for 20 min at room temperature, diluted with water (site 1) or buffer (sites 2 to 5), and then subjected to centrifugation at 3,800 × *g* for 20 min at 4°C. Following centrifugation, specimens were decanted, and the sediment was then resuspended in 1 ml of sterile, filtered water. All specimens were then subjected to acid-fast staining and cultured as described above. Sites that performed redigestion of contaminated liquid cultures used the same NALC/NaOH procedure described above, or other recommended procedures (6), and the sediments were analyzed as described above.

Preparation of CB-18 reagents. CB-18 was added to all specimens as a 10-fold concentrate. The final concentration of added components was 50 mM Tris-HCl (pH 8.0), 0.1 mM NaCl, 1.0 mM CB-18, and 5 mM NALC. Tris buffer was manufactured as a 20-fold stock by using 1 M Tris-HCl (pH 8.0) at 25°C and then adding NaCl to 2 mM. CB-18 (Ecochem Research, Inc., Chaska, Minn.) was made as a 100-fold concentrate (100 mM) in 1:1 isopropanol-water. The 10-fold CB-18 concentrate was made immediately prior to use by diluting the 20-fold buffer in half and then mixing it with NALC to 50 mM. The 100-fold CB-18 concentrate was diluted 1:10 into this solution, and the complete mixture was added to the specimen. Unused portions were discarded. Tissue culture-grade water (Life Technologies, Inc., Rockville, Md.) was used in all CB-18 reagents and for all aspects of specimen processing.

CB-18 processing. Specimens were first treated with an equal volume of 0.5% NALC–1.45% citrate for 10 min at room temperature, and then they were brought to a final volume of 40 ml with 4 ml of a 10-fold concentrate of CB-18 and sterile, filtered water. The specimen–CB-18 mixtures were shaken at 140 rpm for 90 min at 37°C, and then they were subjected to centrifugation at 3,800 × *g* for 20 min at 30°C. Specimens were decanted, and sediments were resuspended in 0.5 ml of sterile, filtered water. In order to analyze the same amount of sediment as the corresponding NALC/NaOH-processed sediments, a smaller volume of resuspension fluid was added to the CB-18-processed sediments, because these sediments were typically less compact and more care was required during decanting. As such, larger volumes of supernatant remained in these tubes following decanting.

All sediments were then subjected to acid-fast staining and were cultured on both liquid (BACTEC 12B) and solid (7H11-selective) media (Becton Dickinson). All positive smears were checked by Quest's AFB laboratory personnel to ensure reporting accuracy. CB-18 liquid cultures used the BACTEC 12B/460TB system according to the manufacturer's instructions, except for the addition of ceftazidime (Sigma, St. Louis, Mo.), which was included in an attempt to combat contamination (22). BACTEC 12B bottles were supplemented with PANTA, which had been fortified with ceftazidime such that the final concentration of ceftazidime was 8 μg/ml in the 12B bottle (CB-18/12B/PANTA/*caz*).

Ceftazidime was formulated as a 36-mg/ml stock in 10% sodium bicarbonate (wt/wt with respect to ceftazidime) and stored at −20°C in 50-μl aliquots. Immediately prior to use, a 50-μl aliquot was thawed and mixed with 1 ml of PANTA reconstitution fluid (Becton Dickinson). One milliliter of the diluted cephalosporin was then added to lyophilized PANTA with an additional 4 ml of reconstitution fluid. The PANTA/*caz* mix was then added to the 12B culture bottles according to the instructions of the manufacturer.

CB-18/Acid processing. Contaminated cultures were redigested with a modified CB-18 protocol involving acid digestion (CB-18/Acid). The culture fluid (≈4.5 ml) was mixed with sterile water to a final volume of 9 ml. One milliliter of the 10-fold CB-18 concentrate was added, and the culture–CB-18 mixture was

TABLE 1. Summary of AFB isolation by site

Site	No. of specimens	Total no. of culture-positive AFB specimens (% incidence)	No. of specimens with culture results by decontamination method			P
			NALC/NaOH only	CB-18 only	Both methods	
1	171	24 (14.0)	5	7	12	>0.05
2	44	14 (31.8)	0	3	11	>0.05
3	90	16 (17.8)	2	4	10	>0.05
4	162	36 (22.2)	3	19	14	<0.01
5	106	16 (15.1)	3	8	5	>0.05
Total	573	106 (18.5)	13	41	52	<0.01

shaken at 140 rpm for 90 min at 37°C. Following CB-18 treatment, 500 μl of 10 M sulfuric acid was added, and this mixture was then incubated at room temperature for 15 min. The specimen was then neutralized with 500 μl of 10 M NaOH and immediately brought to a final volume of 40 ml with 50 mM Tris-HCl (pH 8.0). Specimens were then subjected to centrifugation and processed as described above and cultured on 12B/PANTA/*caz* medium only.

RESULTS

Culture recovery. The study samples consisted of 573 respiratory specimens: 416 sputa (72.6%) and 157 bronchial washes (27.4%). A total of 106 specimens (18.5%) were positive for mycobacteria. Of the positive specimens, 82.1% were derived from sputa, while the remaining 17.9% were derived from bronchial washes. Comparison by site showed that improvements in culture positivity were achieved at all sites, with site 4 showing the greatest improvement (Table 1).

Of the 106 AFB isolates, 29 (27.4%) were *M. tuberculosis* complex (MTBC), and 77 (72.6%) were mycobacteria other than tuberculosis (MOTT) (Table 2). There were five "other" MOTT isolates: two were derived from NALC/NaOH-processed specimens (one each of *M. gastri* and *M. terrae*), and three were derived from CB-18-processed specimens (two *M. neoaurum* [identified by the CDC] and one *M. szulgai*). Three isolates were classified as *M. avium-M. intracellulare-M. scrofulaceum*. All three of these isolates were negative when examined with the MAC AccuProbe test, but generated biochemical results commonly shared by MAC and *M. scrofulaceum* species. Three specimens were unidentifiable (one was sent to the CDC and reported as an "unidentified pattern"). In the MOTT category, four isolates were lost prior to purification and/or identification. Two of these isolates would not grow on solid media, stopped growing in liquid culture, and eventually could not be recovered. The other two were lost because of complications related to contamination (one of these was from a patient positive for *M. chelonae*). Only two positive cultures from CB-18-processed specimens were identified past the 8-week incubation (one MAC and one unknown [both identified at 10 weeks]).

Processing of specimens with CB-18 increased aggregate culture sensitivity by approximately 43%, and increased recovery in all species, with the exception of MTBC (Table 2). Among MTBC-positive specimens, culture sensitivity was reduced by 12.1% when processed by the CB-18 method, but this was not statistically significant. Among MOTT-positive specimens, culture sensitivity was increased by approximately 83.6%. The most significant difference in AFB isolation was among rapid growers (i.e., *M. chelonae*, *M. fortuitum*, and *M. neoaurum*). Of the 19 NALC/NaOH-processed specimens missed by site 4, 10 were rapid growers.

Three of the 13 culture-positive specimens missed when processed by the CB-18 method presented with contamination in the 12B bottle and were redigested with the CB-18/Acid procedure. Five of these 13 specimens presented with contamination on the 7H11-selective slants and were lost. All three specimens requiring CB-18/Acid treatment presented with contamination on the slant. Overall, contamination was involved in 5 (38.5%) of these 13 discrepant specimens.

Of the 41 culture-positive specimens that were missed when processed by the NALC/NaOH method (Table 2), 16 (39.0%) could be associated with previous infection, other positive specimens from the same patient, or other risk factors, such as AIDS. Only 6 (14.6%) of the liquid cultures from these 41 specimens were contaminated (none of these specimens presented with contamination on solid media.) Of these 41 discrepant specimens, there were only five instances wherein two or more isolates of the same species were processed with CB-18 on the same day (1 pair of MAC, 1 pair of *M. fortuitum*, and 2 pairs and 1 triplet of *M. chelonae*). All 11 of these isolates were from site 4. Each of the *M. chelonae* clusters was processed on different days, and each cluster was consistent with a given group originating from the same patient. The MAC pair were from two different patients, but both patients had a history of MAC infection. Only the *M. fortuitum* pair could not be excluded as resulting from laboratory contamination. The fact that these pairings were all derived from site 4 skewed the apparent discrepant isolation rate from this site.

Time to positive. When specimens were processed with CB-18, the average times to a positive result were reduced by 7.3 days in liquid culture and 5.3 days on solid media (Table 3), and the times to a positive MTBC result were reduced by 12.4 and 8.1 days in liquid culture and on solid media, respectively. All of these differences were statistically significant. Among MOTT isolates, the average times to a result were reduced by 5.0 and 2.8 days on liquid and solid culture, respectively; however, these reductions were not statistically significant. Among the different MOTT species, only the reduction among rapid growers on solid media was statistically significant.

Detection by smear. There were 39 positive AFB smear results when processed with NALC/NaOH and 97 positive

TABLE 2. Summary of culture-positive isolates classified according to species and processing method

Species	Total no. of culture-positive AFB specimens	No. of specimens with culture result by decontamination method[a]			P
		NALC/NaOH only	CB-18 only	Both methods	
MTBC	29	4	1	24	>0.05
MAC	29	3	8	18	>0.05
M. kansasii	5	0	0	5	
M. chelonae	12	0	8	4	<0.01
M. fortuitum	8	0	7	1	<0.01
M. gordonae	8	4	4	0	
M. avium-M. intracellulare-M. scrofulaceum	3	0	3	0	>0.05
Other	5	2	3	0	>0.05
Unknown	3	0	3	0	>0.05
Lost	4	0	4	0	<0.05
Total	106	13	41	52	<0.01

[a] The overall culture sensitivities were 61.3% for NALC/NaOH and 87.7% for CB-18 ($P < 0.01$). The MTBC culture sensitivities were 96.6% for NALC/NaOH and 86.2% for CB-18 ($P > 0.05$). The MOTT culture sensitivities were 48.1% for NALC/NaOH and 88.3% for CB-18 ($P < 0.01$).

TABLE 3. Summary of time-to-positive data for different mycobacterial groups analyzed by culture method and processing method

Species	Total no. of culture-positive specimens	Time to detection by decontamination and culture method[a]							
		NALC/NaOH				CB-18			
		Solid media		Liquid media		Solid media		Liquid media	
		n	Avg ttp (days)	*n*	Avg ttp (days)	*n*	Avg ttp (days)	*n*	Avg ttp (days)
MTBC	29	22	27.5 ± 11.9[b]	28	19.6 ± 13.4	24	19.4 ± 11.1	19	7.2 ± 6.8
MAC	29	13	27.7 ± 17.1	21	17.3 ± 17.8	21	19.5 ± 18.6	20	8.9 ± 7.4
M. kansasii	5	5	16.2 ± 9.7	5	13.8 ± 10.9	5	16.0 ± 5.1	4	29.2 ± 22.9
Rapid growers[c]	22	4	7.0 ± 0.0	5	31.8 ± 24.6	20	14.4 ± 13.2	7	3.4 ± 4.2
All other AFB	21	4	24.8 ± 4.8	5	21.2 ± 13.7	7	32.7 ± 13.0	8	30.5 ± 23.8
Total	106	48	24.4 ± 13.7	64	19.4 ± 15.9	77	19.1 ± 14.5	58	12.1 ± 14.9

[a] ttp, time to positive. Statistical analyses were performed by using a two-tailed, heteroscediastic Student's *t* test. Calculated *t* test values for all isolates, MTBC isolates, and all MOTT isolates were <0.01, <0.01, and >0.05, respectively, in liquid culture and 0.041, 0.022, and >0.05, respectively, on solid media. All *t* test values for the various MOTT groups were >0.05 in liquid culture. For the various MOTT groups on solid media, only the reduction in the time to a positive result for rapid growers was statistically significant ($P = 0.023$).

[b] Mean ± standard deviation.

[c] Rapid growers include all *M. chelonae*, *M. fortuitum*, and *M. neoaurum* isolates.

AFB smear results when processed with CB-18 (see Materials and Methods for smear reporting criteria). Only 1 of the 39 AFB smear results generated by the NALC/NaOH processing method could not be confirmed by a culture result. In contrast, of the 97 smear results generated by the CB-18 processing method, only 60 could be confirmed by culture: 56 of these specimens were culture positive when processed with CB-18, while the other 4 culture results were confirmed among NALC/NaOH-processed specimens. Therefore, there were 37 specimens that generated a smear result when processed with CB-18, but that had no confirming culture result by any processing method. Of these 37, 15 had smear values of 1+ or greater and were classified as smear positive (regardless of culture results), while 22 had ± smear values and were considered smear negative.

Of the 106 AFB culture-positive specimens, 61 (57.6%) were smear positive when processed by either NALC/NaOH, CB-18, or both methods. CB-18 processing increased aggregate smear sensitivity by 58.1%, with a 3.1% reduction in specificity (Table 4). Improvements in smear positivity were observed for all mycobacterial species, including MTBC, CB-18 processing increased smear sensitivity among MOTT isolates by 83.3% and among MTBC isolates by 34.9%. Analysis of the isolates that were smear positive only when processed with CB-18 revealed that these discrepancies were not limited to any one group or species. Consistent with this was the observation that when the smear values by the different processing methods were compared (Table 5), increases in smear values were not unique to either the MTBC or MOTT groups, but spread uniformly between both groups. This uniform distribution of increased smear values among CB-18-processed specimens was also true even within the MOTT group (Table 5).

Smear specificity. Forty-one specimens reported a smear result (i.e., ±, 1+ and 2+) when processed with CB-18, but were culture negative by the same method. This discrepancy in smear results prompted an examination of each processing method, independent of one another (Table 6). According to this analysis, the "relative" increase in smear sensitivity provided by CB-18 processing was only 5.8%, whereas the reduction in smear specificity was 8.8%. In this group of 41 specimens, 4 were culture positive when processed by NALC/NaOH, and an additional 9 could be associated with disease, the presence of mycobacteria, or other risk factors (e.g., AIDS). At least 13 (31.7%) of these 41 smear results appeared legitimate.

Similarly, the validity of the significant number of smear ± results ($\Sigma = 30$) was examined. Of these 30 specimens, 2 were culture positive when processed by both the CB-18 and NALC/NaOH methods, 3 were culture positive only when processed by the CB-18 procedure, and 3 were culture positive only when processed by the NALC/NaOH method. Of the remaining 22 smear ± specimens, 6 could be associated with previous disease, the presence of acid-fast bacteria, or other risk factors.

TABLE 4. Summary of data for smear-positive isolates classified according to species and processing method

Species	Total no. of culture-positive AFB specimens	No. of specimens with smear result by decontamination method[a]		
		NALC/NaOH only	CB-18 only	Both methods
MTBC	29	0	7	20
MAC	29	1	7	8
M. kansasii	5	0	0	5
M. chelonae	12	0	2	4
M. fortuitum	8	0	2	0
M. gordonae	8	0	0	0
M. avium-M. intracellulare-M. scrofulaceum	3	0	0	0
Other	5	0	2	0
Unknown	3	0	2	0
Lost	4	0	1	0
Total (no. smear positive and culture negative)	106	1 (0)	23 (14)	37 (1)

[a] A specimen was considered smear positive if the smear value was 1+ or greater, independent of the culture results, or if a smear result was reported as ± by that method and the specimen could be confirmed by culture (solid or liquid media) by any processing method (either NALC/NaOH, CB-18, or both). See Materials and Methods for smear reporting criteria. The overall smear sensitivities were 35.8% for NALC/NaOH and 56.6% for CB-18 ($P < 0.01$). The overall smear specificities were 99.8% for NALC/NaOH and 96.8% for CB-18. The MTBC smear sensitivities were 69.0% for NALC/NaOH and 93.1% for CB-18 ($P < 0.01$). The MOTT smear sensitivities were 23.4% for NALC/NaOH and 42.9% for CB-18 ($P < 0.01$).

TABLE 5. Evaluation of smear values and smear sensitivities by method and mycobacterial group

Result	No. of specimens with result in group		
	All	MOTT	MTBC
Identical smear values	18[a]	8	10
CB-18 smear value > NaOH smear value	19	9[b]	10
NaOH smear value > CB-18 smear value	0	0	0
CB-18 smear positive and NaOH smear negative	23	16	7
NaOH smear positive and CB-18 smear negative	1	1	0
Total no. smear positive	61	34	27

[a] The maximum smear value (4+) was reported by both methods in 14 instances.
[b] These nine specimens were comprised of four MAC, three *M. kansasii*, and two *M. chelonae* isolates.

Therefore, of all of these smear ± results, 14 (46.7%) appeared legitimate.

Smear and culture. CB-18 processing did not significantly increase culture sensitivity among smear-positive specimens (7.8%), but it did significantly increase culture sensitivity among smear-negative specimens (184%) (Table 7). CB-18 processing increased culture sensitivity by 28.0% among specimens classified as MOTT smear positive and by 200% among specimens classified as MOTT smear negative. Among specimens classed as MTBC smear positive, culture sensitivity was reduced by 12.5% ($P > 0.05$) when processed with CB-18. Culture sensitivity among MTBC smear-negative specimens was equivalent (50%), but statistically insignificant.

TABLE 6. Independent evaluation of smear versus culture for each processing method[a]

Smear value	No. of specimens with smear value			
	NALC/NaOH		CB-18	
	AFB positive	AFB negative	AFB positive	AFB negative
−	28	506	37	439
±	3	0	5	25[d]
1+	7	1[b]	8	14[e]
2+	8	1[c]	6	2[f]
3+	5	0	7	0
4+	14	0	30	0
Total	65	508	93	480

[a] In this analysis, the smear and culture data from NALC/NaOH-processed specimens were considered independent of the results generated from CB-18 processing (e.g., all smear and culture results generated from processing with CB-18 were ignored), and the results were first segregated as either NALC/NaOH culture positive or NALC/NaOH culture negative. The smear results after NALC/NaOH processing were then overlaid on these culture results to categorize the specimen data. The analogous breakdown was then performed with results from processing with CB-18. The smear sensitivities (AFB positive) were 56.9% for NALC/NaOH and 60.2% for CB-18. The smear specificities (AFB negative) were 99.6% for NALC/NaOH and 91.5% for CB-18.
[b] This specimen was from a patient previously culture positive for MAC and *Nocardia* sp. This specimen was also culture positive for MAC when processed by the CB-18 method.
[c] This specimen was from a patient previously positive for MTBC. In addition, this specimen was also 2+ smear positive when processed by the CB-18 method.
[d] One of these specimens was culture positive for *M. gastri*, and two of these specimens were culture positive for MTBC when processed with NALC/NaOH.
[e] One of these specimens was culture positive for MTBC when processed with NALC/NaOH.
[f] One of these specimens was from a patient previously positive for MTBC. The same specimen was also 2+ smear positive and culture negative when processed with NALC/NaOH.

TABLE 7. Comparison of culture results according to smear result and mycobacterial group

AFB type	Smear category[a]	Total no. of culture-positive AFB specimens	No. of specimens with culture result by decontamination method[b]		
			NALC/NaOH only	CB-18 only	Both methods
MTBC	Positive	27	3	0	24
	Negative	2	1	1	0
Total		29	4	1	24
MOTT	Positive	34	2	9	23
	Negative	43	7	31	5
Total		77	9	40	28

[a] A specimen was considered smear positive if it was reported as positive when processed by either the NALC/NaOH method, the CB-18 method, or both processing methods.
[b] The culture sensitivities among all smear-positive specimens were 85.2% for NALC/NaOH and 91.8% for CB-18 ($P > 0.05$). The culture sensitivities among all smear-negative specimens were 28.9% for NALC/NaOH and 82.2% for CB-18 ($P < 0.01$). The culture sensitivities among smear-positive MTBC specimens were 100% for NALC/NaOH and 88.9% for CB-18 ($P > 0.05$). The culture sensitivities among smear-negative MTBC specimens were 50.0% for NALC/NaOH and 50.0% for CB-18 ($P > 0.05$). The culture sensitivities among smear-positive MOTT specimens were 73.5% for NALC/NaOH and 94.1% for CB-18 ($P < 0.05$). The culture sensitivities among smear-negative MOTT specimens were 27.9% for NALC/NaOH and 83.7% for CB-18 ($P < 0.01$).

Among the 106 culture-positive specimens, the CB-18 processing method identified 56 as both smear and culture positive, 37 as culture positive but smear negative, 4 as smear positive but culture negative (3 MTBC and 1 *M. gastri*), and 9 as both smear and culture negative. The NALC/NaOH processing method identified 37 specimens as both smear and culture positive, 28 specimens as culture positive but smear negative, 1 specimen as smear positive but culture negative (MAC), and 40 specimens as both smear and culture negative.

Sensitivity of liquid versus solid media. The sensitivity of the primary culture system was compared with the sensitivity of the secondary culture system within each processing method, independent of the results of the other method (Table 8). Based on this analysis, there was a significant reduction in liquid culture sensitivity when specimens were processed with CB-18, most notably among smear-positive specimens. In contrast, the relative sensitivities of solid media among smear-positive and smear-negative specimens for the two processing methods were almost identical. For example, the sensitivity of solid media among smear-positive specimens was 38.1% higher than that among smear-negative specimens when processed with NALC/NaOH and 37.4% higher when processed with CB-18. Scrutiny of the group of 35 solid-medium-only isolates among CB-18-processed specimens revealed that of the 21 smear-negative specimens, 12 (57.1%) were rapid growers, whereas only 2 (21.4%) of the 14 smear-positive specimens were rapid growers (Table 8). Examination of the corresponding CB-18/12B/PANTA/*caz* bottles of these 35 solid-medium-only isolates revealed that 11 (31.4%) were contaminated and subjected to CB-18/Acid treatment.

Discrepant MTBC isolates. The four MTBC-positive specimens missed by culture when processed with CB-18, as well as four other specimens from these same patients, were analyzed

TABLE 8. Independent comparison of culture sensitivities for each processing method

Method	Smear result	No. of specimens with culture result on media			Total no. of culture-positive AFB specimens	% Sensitivity on media	
		Liquid and solid	Liquid only	Solid only		Liquid	Solid
NaOH	+	31	6[a]	0	37	100	83.8
	−	16	11[b]	1[d]	28	96.4	60.7
Total		47	17[c]	1[e]	65	98.5	73.8
CB-18	+	38	4[f]	14[i]	56	75.0	92.9
	−	4	12[g]	21[j]	37	43.2	67.6
Total		42	16[h]	35[k]	93	62.4	82.8

[a] These six included four MTBC and two MAC isolates.
[b] These 11 included 2 MTBC, 6 MAC, 1 *M. fortuitum*, and 2 *M. gordonae* isolates.
[c] None of the corresponding solid-medium cultures were lost to contamination.
[d] This specimen was the *M. gastri* isolate.
[e] The corresponding liquid culture was not contaminated.
[f] These four included one MTBC and two unknown isolates and one of the lost isolates.
[g] These 12 included 5 MAC, 2 *M. avium-M. intracellulare-M. scrofulaceum*, and 2 lost isolates and 1 *M. gordonae*, 1 *M. fortuitum*, and 1 of the *M. chelonae* isolates.
[h] Only two (12.5%) of the corresponding solid-medium cultures were lost to contamination.
[i] These 14 included 5 MTBC, 4 MAC, and 2 *M. fortuitum* isolates and 1 *M. chelonae*, 1 *M. szulgai*, and 1 of the *M. kansasii* isolates.
[j] These 21 included 1 MTBC, 2 MAC, 5 *M. fortuitum*, 5 *M. chelonae*, 3 *M. gordonae*, and 2 *M. neoaurum* isolates and 1 *M. avium-M. intracellulare-M. scrofulaceum*, 1 unknown, and 1 of the lost isolates.
[k] A total of 11 (31.4%) of the corresponding liquid cultures were contaminated and subjected to redigestion by the CB-18/Acid procedure.

(Table 9). Of the eight MTBC isolates analyzed, seven were smear positive when processed with CB-18, but only three were reported as smear positive when processed with NALC/NaOH. In contrast, only the initial specimen from patient 2 (512-S3) grew in the 12B/PANTA/*caz* system when processed with CB-18, whereas all eight of the isolates grew in 12B/PANTA when processed with NALC/NaOH. The two specimens from patient 1 were both submitted on the same day, and both processing methods reported a smear-positive result, but only the NALC/NaOH-processed specimens grew in liquid media. The first specimen from patient 2 (512-S3) was reported as smear positive within the mandatory 24-h reporting period by the site. The second specimen from this patient (527-S3) was submitted 10 days after the initial specimen and 5 days after the patient was started on drug therapy. The three specimens submitted after initiation of drug therapy were all negative in 12B/PANTA/*caz* when processed with CB-18, whereas two were positive on the 7H11 slant. While contamination played a role in the discrepancies between both patient 1 and patient 3, contamination was not involved in the discrepancies between patient 2 or patient 4.

Contamination. The contamination rate for CB-18-processed specimens was almost 2.8-fold higher in liquid culture, and it was almost 4.8-fold higher on 7H11-selective medium (Table 10). Of the 43 specimens that presented with liquid culture contamination when processed with NALC/NaOH and the 119 specimens that presented with liquid culture contamination when processed with CB-18, only 19 specimens were concordant.

The acid redigestion procedure (CB-18/Acid) was performed with the liquid media of all CB-18-processed specimens that presented with contamination. Among these 119 specimens, 18 (15.1%) were eventually seen to harbor mycobacteria. Four culture-positive results originated from NALC/NaOH-processed specimens, 11 culture-positive results were derived from the corresponding 7H11 slants (CB-18/7H11) of these contaminated liquid cultures, and only 1 specimen was culture positive by both CB-18/7H11 and culture following NALC/NaOH processing. The liquid media that had been subjected to the CB-18/Acid redigestion procedure were culture positive in only 2 of these 16 instances, but 2 additional culture-positive specimens were identified following CB-18/Acid redigestion that were not positive by any other method. Therefore, the CB-18/Acid redigestion procedure identified 4 (22.2%) of these 18 AFB-positive specimens. Of the 119 redigested cultures, only 8 (6.7%) presented with subsequent contamination.

TABLE 9. Analysis of the four *M. tuberculosis* specimens missed by culture when processed with CB-18, as well as four additional specimens from two of these patients

Patient	Specimen identification	Result by decontamination method[a]						Mo/day/yr of collection
		NALC/NaOH			CB-18			
		Smear	Culture		Smear	Culture		
			Liquid	Solid		12B	7H11	
1	571-S1	1+	+/28	+/42	1+	−	−/Cont	2/7/96 (initial specimen)
	573-S1	1+	+/28	−	3+	−	+/20	2/7/96 (initial specimen)
2	512-S3	2+	+/4	−	4+	+/3	+/12	2/8/96 (first specimen)
	527-S3	−	+/24	−	±	−	−	2/17/96 (on therapy)
	538-S3	−	+/31	+/33	2+	−	+/49	2/27/96 (on therapy)
	541-S3	−	+/31	+/33	2+	−	+/20	2/27/96 (on therapy)
3	016-S4	−	+/56	+/42	−	−/R	−/Cont	
4	089-S5	−	+/13	−	±	−	−	

[a] Smear values were reported as per CDC guidelines (6). Positive MTBC cultures are indicated by + followed by a number (e.g., +/4). This number represents the total time to a culture-positive result by that method (i.e., liquid or solid media) in days. Negative cultures and smears are highlighted with −. Slants lost to contamination are marked with Cont, and liquid cultures subjected to redigestion are marked with R.

TABLE 10. Summary of site-specific contamination results

Contamination site (n)	No. of contaminated specimens by processing and culture method (rate [%])			
	NALC/NaOH		CB-18	
	Liquid culture	Solid media	BACTEC 12B	7H11-selective medium
1 (171)	11 (6.4)	10 (5.8)	31 (18.1)	17 (9.9)
2 (44)	0	3 (6.8)	4 (9.1)	3 (6.8)
3 (90)	5 (5.6)	2 (2.2)	8 (8.9)	1 (1.1)
4 (162)	19 (11.7)	0	56 (34.6)	41 (25.3)
5 (106)	8 (7.6)	0	20 (18.9)	9 (8.5)
Total (573)	43 (7.5)	15 (2.6)	119 (20.8)	71 (12.4)

DISCUSSION

Increases in both smear and culture sensitivity were achieved when specimens were processed by the CB-18 method. These increases are believed to result from a reduced impact on viability, altering the buoyant density of the mycobacteria to enhance collection efficiency during centrifugation, and dispersion of those mycobacteria that cord (20, 21, 22). While numerous performance variables were affected by switching from NaOH-based to CB-18-based processing, the results of this study indicate that the improvements in sensitivity accrued to specimens processed with CB-18 were the result of multivariable factors.

The overall increase in smear sensitivity was possibly the most significant finding in this study. Improvements in smear sensitivity with the CB-18 processing method resulted from increases in the identification of both MOTT- and MTBC-positive specimens. Because tuberculosis control programs in developing countries depend almost exclusively on diagnosis by smear, the CB-18 method could potentially identify more infectious patients earlier, thus reducing transmission of tuberculosis.

It could be argued that subjecting the mycobacteria to CB-18 processing affected the cell wall in such a way as to improve the technique of staining (i.e., causing the bacteria to adhere to the slide more efficiently), or that in the context of processing with CB-18, the cell wall might not be degraded, as may be the case with NaOH-based processing. However, the relative sensitivities of smear to culture for the two different processing methods were similar, indicating that the smear was unaffected by CB-18 processing at a technical level.

A statistically significant increase in culture sensitivity was achieved when specimens were processed by the CB-18 method. The increased frequency in the isolation of MOTT from smear-negative specimens was the single most significant contributing factor to this increase, and this in turn was primarily a consequence of an increased frequency in the recovery of rapid growers. Most remarkable was the fact that the rapid growers comprised 41.5% of the culture-positive specimens missed when processed with NALC/NaOH, but only 17.4% of specimens reported as smear positive when processed with CB-18 and smear negative when processed with NALC/NaOH. Similarly, only 22.2% of specimens that reported higher smear values when processed with CB-18 were from specimens positive for rapid growers. In contrast to the culture results, the increased smear sensitivity accrued with CB-18 processing appeared to be independent of group or species.

We do not know the clinical significance of the increase in MOTT isolation. The issue of the validity of these discrepant specimens can be approached from several perspectives. First, of the specimens missed by culture when processed with NALC/NaOH, almost 40% could be associated with previous infection, other positive specimens from the same patient, or other risk factors. The second perspective highlights the fact that the CB-18 method is simply a processing procedure and is not a diagnostic technique per se. Regardless, there was no indication of laboratory contamination (i.e., crossover contamination).

When specimens were processed with CB-18, reductions in the time to detection averaged approximately 1 week in liquid culture. Reductions in the time to a positive result were even greater among MTBC-positive specimens. Faster reporting times represent a significant advance, because decisions regarding therapy and isolation could be made more expeditiously.

Improvements in culture sensitivity with CB-18 processing might be due to either increased recovery, enhanced viability, dispersion, or a combination of any of these (21). Similarly, reductions in the time to detection might be due to either increased recovery, enhanced viability, or both. Alternatively, increased smear sensitivity could only result from increased recovery. While it would be nearly impossible to determine the various components, the increased sensitivities and reductions in time to positivity support the hypothesis that the CB-18 method provided multivariable improvements in specimen processing.

The processing and culture system (i.e., CB-18/12B/PANTA/*caz*) had a profound impact on the results of this study. This was exemplified by the increased frequency in the isolation of MOTT from smear-negative specimens (200%), compared to the liquid culture sensitivity of smear-positive specimens when processed with CB-18 (75%). These results are in contrast to those from the study by Stone et al. (19), wherein the BACTEC 12B sensitivity among NALC/NaOH-processed smear-positive specimens was 99.3% (n = 439). While contamination may partially explain the loss in liquid culture sensitivity, it does not explain the significant number of CB-18-processed specimens that were deemed smear positive, but that failed to grow in liquid culture. The fact that missed isolates from these smear-positive specimens were both MOTT and MTBC suggested that there was a wide variation in susceptibility among mycobacteria to this processing and culture system.

Analysis of the toxicity of CB-18 has suggested an induced susceptibility to the PANTA/*caz* formulation caused by the presence of CB-18 in the liquid culture (22). While processing with CB-18 appears to have a tuberculocidal component, culture sensitivity was more dependent on the CB-18 concentration present in liquid culture and on the isolate than on the actual processing step itself. The impact of CB-18 on viability in culture can be overcome with lecithin (22).

If CB-18 caused an induced susceptibility, this could explain the loss in smear specificity. For example, if CB-18 processing provided enhanced viability, recovery, and distribution of mycobacteria in the resulting sediments, but the combination of CB-18 with PANTA/*caz* in liquid culture had a synergistically deleterious effect on the viability of only a few isolates, then increased numbers of acid-fast bacteria would have been observed by microscopy, but a confirming culture result would not have been forthcoming (i.e., an increase in culture sensitivity with an apparent loss in smear specificity.) Alternatively, these results may indicate a subpopulation of fastidious mycobacteria that are difficult to grow in culture (e.g., *M. genavense*). Nevertheless, almost half of the ± smear results observed with CB-18-processed specimens appeared legitimate, indicating that many of these specimens may have been

false negative by culture. Had further refinement of the interface between the CB-18 processing method and culture allowed capture of this class of specimens by culture, the results of this study would have been significantly different.

The CB-18 processing method missed a total of four MTBC isolates, three of which were smear positive. Contamination potentially played a role in only two of these discrepancies. Discrepancies in general appeared to be related to contamination and/or the combination of CB-18 and PANTA/*caz* in the culture system. Additionally, there does appear to be a difference in behavior between naïve specimens and specimens derived from patients on drug therapy in the context of this new processing and culture system. As such, the utility of the CB-18 processing method for monitoring drug therapy by culture or directly performing susceptibility testing in the presence of CB-18 needs to be evaluated further or subsequently refined.

Ceftazidime was originally included in the 12B/PANTA culture system to control gram-negative contamination (22). Given the high contamination rate in liquid culture, it was difficult to discern whether ceftazidime had any impact on contamination. Analysis indicated that the specimens presenting with contamination were different for the two processing methods. Because exchanging NaOH for CB-18 alters the dynamics of processing, these differences were anticipated. Other critical factors related to contamination appeared to be the length of time to specimen processing and the conditions under which the specimens were stored. Those specimens that were held at room temperature had the highest rates of contamination, whereas specimens held at 4°C had the lowest contamination rates. The nature of the contaminants plaguing CB-18 culture and approaches to resolving this problem are discussed in detail in a separate publication (22).

The CB-18 processing method is a novel approach to improving the diagnostic sensitivity of any detection platform through enhancements at the level of processing respiratory specimens. These improvements are the result of enhancements in recovery, viability, and dispersion (21). The unusual loss in smear specificity and liquid culture sensitivity among smear-positive specimens suggests that further improvements in culture sensitivity are possible. The results of smear analysis are as promising as the sodium hypochlorite (NaOCl) results of Gebre et al. (4); however, contrary to NaOCl-based methods, CB-18 processing also permits sediments to be subjected to culture for eventual susceptibility testing. Of the 1.7 billion people presumed to be infected with tuberculosis (8), the majority are in countries where smear is the primary diagnostic tool. The method described herein is simple, inexpensive, and easy to perform, requiring only a bench top centrifuge with the appropriate tubes. The method should be evaluated in an area in which tuberculosis is endemic, where it could potentially enhance smear sensitivity. Incorporation of CB-18 in the clinical laboratory should provide improved diagnostic capabilities and decisions concerning treatment and isolation.

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