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Comparison of the Sodium Hydroxide Specimen Processing Method with the C_{18}-Carboxypropylbetaine Specimen Processing Method Using Independent Specimens with Auramine Smear, the MB/BacT Liquid Culture System, and the COBAS AMPLICOR MTB Test

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A study was performed to diagnose tuberculosis by smear, culture, and nucleic acid amplification. The study was comprised of two independent arms. Each arm used a different specimen processing method; in one arm, all specimens were processed with *N*-acetyl-L-cysteine–sodium hydroxide, and in the other arm, all specimens were processed with C_{18}-carboxypropylbetaine and lytic enzymes. In each arm, all processed sediments were split for analysis by auramine smear, by culture using the MB/BacT liquid culture system and solid media, and by nucleic acid amplification using the COBAS AMPLICOR MTB test. In the *N*-acetyl-L-cysteine–sodium hydroxide arm, 1,468 specimens were analyzed: 65 were smear positive; 88 and 42 were culture positive for *Mycobacterium tuberculosis* and nontuberculous mycobacteria, respectively; and 103 were PCR positive. Relative to cultures positive for *M. tuberculosis*, the sensitivity and specificity of the smear were 68.2% and 99.6%, respectively, and those of PCR were 75.0% and 97.3%, respectively. In the C_{18}-carboxypropylbetaine study arm, 1,423 specimens were analyzed: 44 were smear positive; 82 and 31 were culture positive for *M. tuberculosis* and nontuberculous mycobacteria, respectively; and 91 were PCR positive. The sensitivity and specificity of the smear were 48.8% and 99.7%, respectively, and those of PCR were 78.0% and 98.0%, respectively. When the two arms were compared, C_{18}-carboxypropylbetaine specimen processing significantly increased the number of smear-negative and culture-positive specimens and significantly increased the PCR sensitivity among this same group of specimens while at the same time significantly reducing the inhibition rate.

There are myriad problems associated with identifying the approximately 30 million individuals worldwide with active tuberculosis. In 2001, it was estimated that only one-third of the 3.6 million smear-positive cases of tuberculosis were reported (30). While smear microscopy is the most widely used screening tool, it is estimated that microscopy can miss two-thirds of culture-positive cases (12, 17). The Centers for Disease Control and Prevention recommend that three separate morning sputum specimens be taken on three separate days because some specimens contain few, if any, bacilli (9). Because these samples might contain so few bacilli, large specimen volumes are needed. The large volumes and mucoid character of sputum necessitate that specimens be processed and concentrated prior to analysis. The currently recommended specimen processing methods (9) are harsh and can kill up to 90% of the bacilli present in the specimen (5, 31). The buoyant density of *Mycobacterium tuberculosis* (which ranges from 0.79 to 1.07, with the average below 1.0) (23) complicates collection by centrifugation due to inefficient sedimentation of bacilli: the net result is that the integrity of processed specimens is compromised following centrifugation because bacilli are poured off with the supernatant fraction. When bacilli clump or cord, large numbers of bacilli can partition as a single bacillus. In those specimens in which few bacilli are present, these factors can leave the sediment virtually devoid of bacilli (10). Therefore, specimen processing has a significant impact on diagnostic sensitivity.

The specimen processing method based on the use of the zwitterionic detergent C_{18}-carboxypropylbetaine (CB-18) has been used in a variety of settings (11, 14, 24, 26). This method is unique in that it compromises buoyancy, disperses cords to some degree, and is less harsh than other processing methods. The present study was a follow-up to a previous study that used split specimens and that had shown increased culture sensitivity following CB-18 specimen processing but no significant difference in smear sensitivity (14, 15). In the study described here, the CB-18 specimen processing method was compared with a method that combines *N*-acetyl-L-cysteine (NALC) with sodium hydroxide (NaOH) (NALC-NaOH); each method was performed on an independent group of specimens to minimize the effects of splitting. In each arm of the study, processed sediments were split for analysis by acid-fast staining, culture, and PCR using the COBAS AMPLICOR Mycobacterium tuberculosis (MTB) test. This is the first comprehensive study to evaluate the effects of CB-18 specimen processing on the sensitivity of PCR for the detection of tuberculosis. Statistical

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comparisons of the arms were done, and the implications of the results are discussed.

MATERIALS AND METHODS

Clinical specimens. This study was comprised of two separate and independent arms that coincided in space and time. All specimens were collected between February 2001 and December 2002 from the clinical microbiology laboratory of the Hospital Universitari Germans Trias i Pujol, Barcelona, Spain. In one arm of the study, specimens were processed solely by the NALC-NaOH method (see below), and in the other arm, specimens were processed solely by the CB-18/lytic-enzyme method (see below). The different processing methods were alternated on a daily basis. Specimens were selected randomly. The only criteria for entry into the study were sufficient specimen volume or mass. Only those specimens with a >4-ml volume or 2 g of mass were selected for processing. A total of 1,468 specimens were processed in the NALC-NaOH arm of the study (1,282 sputa, 168 bronchial washes, and 18 nonrespiratory specimens), and a total of 1,423 specimens were processed in the CB-18 arm of the study (1,268 sputa, 144 bronchial washes, and 11 nonrespiratory specimens).

NALC-NaOH decontamination procedure. The NALC-NaOH specimen processing method was a modified version of the recommended procedure (9). Briefly, specimens were decontaminated with an equal volume of 2% NaOH with 0.5% NALC, incubated at room temperature for 20 min, neutralized with 67 mM phosphate buffer (pH 6.8), and then subjected to centrifugation at 3,300 × *g* for 20 min at 4°C. The specimens were decanted following centrifugation, and the sediments were resuspended in the remaining backwash. From the resuspended sediment, a portion was taken for acid-fast staining as described below, and approximately 100 μl was removed for analysis by the AMPLICOR test and prepared for analysis as described below. To the remaining sediment was added 1 ml of the same phosphate buffer. Portions of the diluted sediments were also used to inoculate two tubes of Löwenstein-Jensen (L-J) medium (approximately 200 μl each), and approximately 500 μl was used to inoculate one MB/BacT culture bottle (bioMérieux, Marcy l'Etoile, France) supplemented with Mycobacteria Antibiotic Supplement (MAS) (bioMérieux). Statistical comparisons were performed using standard χ^2 analysis.

CB-18 decontamination procedure. The CB-18 processing procedure was a modified version of that originally used in our laboratory (14). In this modified procedure (Integrated Research Technology, LLC, product literature), an equal volume of buffered CB-18 solution (2 mM CB-18, 100 mM Tris-HCl, 25 mM citrate, pH 6.0, 3 mM NaCl, and 0.5% NALC) was added directly to each specimen in a 50-ml conical centrifuge tube. The specimens were vortexed and then incubated at 37°C for 90 min. After incubation, sterile water was added to a final volume of 40 ml and mixed, and then the specimen was subjected to centrifugation at 3,300 × *g* for 20 min at 25°C (centrifugation at 4°C is not required with the CB-18 specimen processing method). The sediments were resuspended in the remaining backwash, and a portion was subjected to acid-fast staining as described below. A 100-μl portion was also prepared for analysis by the AMPLICOR test as described below. To the remaining sediment was added 1 ml of a modified version of the lytic-enzyme resuspension previously described (25). The LYTIC DECON II formulation (Integrated Research Technology, LLC) contained lysozyme, *Lysobacter* extract, and *Trichoderma* extract in 50 mM Tris-HCl, 12.5 mM citrate, pH 6.0, 3 mM NaCl, and 0.5% NALC. The sediments were incubated for 30 min at 37°C. A 200-μl portion was used to inoculate a single 7H11 selective plate, and a 500-μl portion was used to inoculate one MB/BacT bottle supplemented with MAS. This lytic enzyme formulation cannot be used in conjunction with inspissated media, such as L-J medium, as the proteases present liquefy solidified media.

Microscopy, culture, and mycobacterial identification. Acid-fast staining using auramine-rhodamine was performed according to recommended procedures (9). Specimens positive by fluorochrome staining were confirmed using the Ziehl-Neelsen technique (9). MB/BacT culture vials were incubated at 35 to 37°C for 8 weeks or until they were flagged as positive by the BacT/Alert instrument (bioMérieux). After 2 months, all MB/BacT vials considered negative were subcultured onto L-J medium as previously described (14). All L-J slants were incubated at 35 to 37°C in a humidified atmosphere for 2 months and inspected twice per week. The 7H11 plates were incubated in a 5% CO_2 atmosphere at 35 to 37°C for 2 months and read twice per week under an optical microscope at ×100 magnification. Identification of mycobacterial isolates used standard procedures (9, 13, 16) and/or the Inno-LiPA Mycobacteria Test (Innogenetics, Ghent, Belgium) (18).

Those specimens processed by the CB-18 method that were negative for the presence of acid-fast bacteria or that appeared to be contaminated were further analyzed. A portion of these cultures was subcultured onto blood agar plates. Contaminating microorganisms were subjected to Gram staining and identified to the species level by conventional methods (16).

Nucleic acid amplification and sequencing of discordant PCRs. All nucleic acid amplification analyses used the COBAS AMPLICOR MTB Test (Roche Molecular Systems, Inc.). The procedure used to prepare the 100-μl portion of each sediment strictly adhered to the method given by the manufacturer. Only those specimens that were validated by the positive and negative controls were included in comparative analyses. Those specimens that were deemed inhibited by the internal positive control were not purified but simply reamplified. A significant number of specimens in both processing methods were negative by culture but positive following analysis by PCR (37 following NALC-NaOH processing and 27 following CB-18 processing). Amplification products from 14 of these specimens were chosen at random. These samples were purified and sequenced by Lark Technologies (Houston, Texas).

RESULTS

Results from the NALC-NaOH study arm. The NALC-NaOH study arm processed a total of 1,468 specimens. This group produced 88 specimens positive for *M. tuberculosis* complex (MTBC) by culture (6.0%) and 42 specimens positive for nontuberculous mycobacteria (NTM) (2.9%) (Table 1). The NTM group of isolates included the following: 17 *Mycobacterium xenopi* isolates, 12 *Mycobacterium gordonae* isolates, four *Mycobacterium avium-M. intracellulare-M. scrofulaceum* complex isolates, three *Mycobacterium chelonae* isolates, three *Mycobacterium kansasii* isolates, one *Mycobacterium fortuitum* isolate, one *Mycobacterium mucogenicum* isolate, and one rapid grower of unknown species. The NTM were treated as a single group in all analyses. There were 39 (2.7%) specimens contaminated in MB/BacT liquid culture vials, and 67 (4.6%) L-J slants were contaminated. The average time to positive (ttp) in the MB/BacT system was over 1 week faster than on solid media (Table 2). The liquid culture system was also approximately 15% more sensitive than solid media (Table 3). This was due exclusively to isolation of mycobacteria from smear-negative specimens.

A total of 65 specimens were positive by auramine staining in the NALC-NaOH study arm (Table 1). The overall sensitivity and specificity of the smear relative to culture were 46.9% and 99.7%, respectively. Among specimens culture positive for *M. tuberculosis*, the sensitivity and specificity of the smear were 68.2% and 99.6%, respectively. Of the four specimens that were smear positive and culture negative, two were from patients who were on antituberculosis therapy at the time the specimens were taken (both were PCR positive), and the other two were from the same patient and were determined to be false positive.

Analysis of NALC-NaOH-processed specimens by PCR identified 103 (7.0%) positive specimens and 123 (8.4%) inhibited specimens. After repeat testing, the number of inhibited specimens decreased to 57 (4%). Of the 103 PCR-positive specimens, 66 were MTBC culture-positive specimens (75.0% sensitivity), and 37 specimens were MTBC culture negative and were initially considered false positive (Table 1). A detailed analysis of these patients showed that 7 of these 37 culture-negative and AMPLICOR-positive samples were from patients on antituberculosis therapy (all smear negative). Three of these same 37 specimens (again, all smear negative) came from patients who had another specimen(s) culture positive for *M. tuberculosis*. These 10 samples were classified as true positive. Seven of these 37 culture-negative and AMPLI-

TABLE 1. Summary of smear, culture, and amplification results in the NALC-NaOH study arm

Specimen	Culture results (no.)			Diagnostic value (%)			
	MTBC positive	NTM positive	Negative	Sensitivity	Specificity	PPV[a]	NPV[b]
All							
AMPLICOR positive	66	0	37				
AMPLICOR negative	14	40	1,188	75.0	97.3	64.1	98.4
AMPLICOR inhibited	8	2	113				
Total	88	42	1,338				
Smear positive							
AMPLICOR positive	57	0	2				
AMPLICOR negative	0	1	2	95.0	60.0	96.6	50.0
AMPLICOR inhibited	3	0	0				
Total	60	1	4				
Smear negative							
AMPLICOR positive	9	0	35				
AMPLICOR negative	14	39	1,186	32.1	97.5	20.5	98.6
AMPLICOR inhibited	5	2	113				
Total	28	41	1,334				

[a] PPV, positive predictive value.
[b] NPV, negative predictive value.

COR-positive samples were also randomly selected for sequencing of the amplicons (data not shown). All seven revealed sequences that were consistent with the 16S rRNA gene of *M. tuberculosis*. Therefore, the resolved specificity was calculated to be 98.0%.

Among smear-positive specimens, the AMPLICOR test had excellent positive predictive value (96.6%), but among smear-negative specimens, the positive predictive value dropped markedly (20.5%). When the eight MTBC culture-positive specimens that were inhibited were retested, two of the three smear-positive specimens retested as positive and one remained inhibited. Of the five smear-negative specimens, only one retested as positive; one was negative, while the other three remained inhibited. Therefore, the resolved sensitivity overall became 78.4%, whereas the resolved sensitivity among smear-positive and smear-negative specimens became 98.3% and 35.7%, respectively.

Results from the CB-18 study arm. A total of 1,423 specimens were processed in the CB-18 study arm. This group produced 82 MTBC culture-positive specimens (5.8%) and 31 specimens positive for NTM (2.2%) (Table 4). The NTM isolates included 14 *Mycobacterium gordonae* isolates, six *Mycobacterium avium-M. intracellulare-M. scrofulaceum* complex isolates, five *M. fortuitum* isolates, four *M. xenopi* isolates, and two *M. kansasii* isolates. In MB/BacT liquid culture vials, 23 specimens were reported as contaminated (1.6%), and 33 specimens were reported as contaminated on 7H11 selective plates (2.3%). The ttp in MB/BacT culture vials was also 1 week shorter than on solid media (Table 5). The most unusual result in the CB-18 study arm was the difference between the liquid- and solid-medium sensitivities: the MB/BacT system was almost three times more sensitive than the 7H11 selective plates (Table 6). There was no temporal relationship between processing dates and low sensitivity (i.e., the results were not due to a bad lot of 7H11 selective plates).

A total of 44 specimens were positive by staining in the CB-18 study arm (Table 4). Relative to culture, the overall sensitivity and specificity of the smear were 37.2% and 99.8%, respectively. Relative to those specimens culture positive for *M. tuberculosis*, the sensitivity and specificity of the smear were 48.8% and 99.7%, respectively. The two smear-positive specimens that were culture negative were from patients on antituberculosis therapy (both were also PCR positive).

Analysis of CB-18-processed specimens by AMPLICOR identified 91 specimens as positive (6.4%) and another 91 specimens as inhibited (6.4%). After repeat testing, the number of inhibited samples decreased to 68 (4.8%). Of the 91 AMPLICOR-positive specimens, 64 (sensitivity = 78.0%) were culture positive for *M. tuberculosis* (Table 4), and 27 were culture negative. None of the specimens that were culture positive for NTM were PCR positive. An examination of pa-

TABLE 2. Summary of time-to-positive results in the NALC-NaOH study arm

Group	Total no. culture positive	LJ		MB/BacT	
		n	Avg ttp (days)	*n*	Avg ttp (days)
All AFB	130	101	25.3 ± 12.8	116	17.5 ± 10.7
MTBC	88	75	21.5 ± 7.0	85	13.6 ± 6.3
Smear positive	60	60	20.4 ± 6.1	60	10.7 ± 3.6
Smear negative	28	15	25.8 ± 8.5	25	20.5 ± 6.1
NTM	42	26	36.1 ± 18.7	31	28.4 ± 12.6

TABLE 3. Comparison of liquid- and solid-medium culture sensitivities in the NALC-NaOH study arm

Group	Culture results (no.)			Sum of AFB culture positive	Sensitivity (%)		
	Liquid and solid	Liquid only	Solid only		Liquid	Solid	*P*
Smear positive	61	0	0	61	100	100	
Smear negative	26	29	14	69	79.7	58.0	<0.05
Total	87	29	14	130	89.2	77.7	<0.05

TABLE 4. Summary of smear, culture and amplification results in the CB-18 study arm

Specimens	Culture results (no.)			Diagnostic value (%)			
	MTBC positive	NTM positive	Negative	Sensitivity	Specificity	PPV[a]	NPV[b]
All							
AMPLICOR positive	64	0	27				
AMPLICOR negative	15	30	1,196	78.0	98.0	70.3	98.6
AMPLICOR inhibited	3	1	87				
Total	82	31	1,310				
Smear positive							
AMPLICOR positive	38	0	2				
AMPLICOR negative	0	2	0	95.0	50.0	95.0	50.0
AMPLICOR inhibited	2	0	0				
Total	40	2	2				
Smear negative							
AMPLICOR positive	26	0	25				
AMPLICOR negative	15	28	1,196	61.9	98.1	51.0	98.8
AMPLICOR inhibited	1	1	87				
Total	42	29	1,308				

[a] PPV, positive predictive value.
[b] NPV, negative predictive value.

tient histories showed that three of the 27 culture-negative and AMPLICOR-positive samples were from patients on antituberculosis therapy (all smear negative), and four specimens (again, all smear negative) came from two patients with another specimen(s) culture positive for *M. tuberculosis*. These seven culture-negative and AMPLICOR-positive samples were classified as true positive. Seven of these same 27 were also randomly selected for sequencing, and again, all 7 revealed sequences consistent with the *M. tuberculosis* 16S rRNA gene (data not shown). Therefore, the resolved specificity of the AMPLICOR test was calculated to be 98.5%. Three specimens that were culture positive for *M. tuberculosis* but PCR inhibited were retested: two smear-positive specimens remained inhibited, and the smear-negative specimen was reported as PCR negative.

A previous report described the spectrum of contaminants in MB/BacT culture vials following CB-18 processing (15). Analyses of the contaminants in the CB-18 arm of this study were also characterized. The spectrum of contaminants in the present study was markedly different from that in the previous study. Over half of the contaminants observed in MB/BacT vials were mycological in origin, and well over three-quarters of the contaminants identified on 7H11 selective plates were mycological (Table 7).

Comparison of the NALC-NaOH and CB-18 study arms. While the possibility of crossover contamination could not be eliminated as a contributing factor to the results reported here, all possible care was taken by laboratory personnel. In addition, no trend was observed to suggest that crossover contamination played a role in the results of this study. Regardless, the results of the two study arms were compared using chi-square analysis (Table 8). The incidences of *M. tuberculosis* and NTM were not significantly different, but the contamination rates in both MB/BacT and solid media were significantly different. The sensitivity of the smear following NALC-NaOH processing was significantly greater, but the difference in specificity was not statistically significant. The isolation of *M. tuberculosis* from smear-negative samples was significantly greater following CB-18 processing, but the isolation of NTM from smear-negative samples was not significantly different. The ttps were slightly shorter following CB-18 processing for *M. tuberculosis*-positive specimens and slightly longer with NTM-positive specimens (a result generally consistent with previous findings) (14); however, due to the design of this study (i.e., these were not split specimens), direct comparisons of ttps were not possible. Specimen processing did not significantly affect the overall sensitivity and specificity of the AMPLICOR test relative to culture, but the sensitivity of the AMPLICOR test among smear-negative and culture-positive specimens was significantly greater following CB-18 processing. Finally, the inhibi-

TABLE 5. Summary of time-to-positive results in the CB-18 study arm

Group	Total culture positive	7H11 selective		MB/BacT	
		n	Avg ttp (days)	*n*	Avg ttp (days)
All AFB	113	33	26.7 ± 10.9	108	19.1 ± 8.4
MTBC	82	18	27.8 ± 11.9	79	18.6 ± 8.1
Smear positive	40	13	26.6 ± 11.5	39	14.9 ± 7.4
Smear negative	42	5	30.8 ± 13.8	40	22.2 ± 7.1
NTM	31	15	25.3 ± 9.6	29	20.5 ± 9.1

TABLE 6. Comparison of liquid- and solid-medium culture sensitivities in the CB-18 study arm

Group	Culture results (no.)			Sum of AFB culture positive	Sensitivity (%)		
	Liquid and solid	Liquid only	Solid only		Liquid	Solid	*P*
Smear positive	13	28	1	42	97.6	33.3	<0.05
Smear negative	15	52	4	71	94.4	26.8	<0.05
Total	28	80	5	113	95.6	29.2	<0.05

TABLE 7. Spectrum of contaminants isolated from specimens cultured in the MB/BacT system with MAS or on 7H11 selective plates following CB-18 processing and lytic-enzyme decontamination

Contaminant	No. (%) of contaminants identified	
	MB/BacT with MAS	7H11 selective
Gram negative		
Pseudomonas aeruginosa	1	2
Other gram-negative rods	2	2
Total	3 (12.0)	4 (12.1)
Gram positive		
Streptococcus spp.	4	1
Staphylococcus spp.	1	
Other gram-positive cocci	2	
Other gram-positive rods	1	
Total	8 (32.0)	1 (3.0)
Yeast (*Candida*)	9 (36.0)	12 (36.4)
Mold (*Aspergillus*)	4 (16.0)	10 (30.3)
Other fungi		5 (15.2)
Unknown	1	1
Total no. of contaminants isolated	25	33
Total no. of specimens contaminated	23	33
Total no. of specimens processed	1,423	1,423
Contamination rate (%)	1.6	2.3

tion rate following CB-18 processing was also significantly lower.

DISCUSSION

The results of the present study show that when specimens are processed with CB-18, there is a statistically significant increase in PCR sensitivity among culture-positive and smear-negative specimens. This interpretation, however, is not without complicating factors. First, the sensitivity of the smear following NALC-NaOH processing was significantly greater than smear sensitivity following CB-18 processing. This suggests that either the smear following NALC-NaOH processing was more sensitive or culture sensitivity among smear-negative specimens was enhanced following CB-18 processing. In the previous study in our laboratory, using split specimens, the smear sensitivities following sodium dodecyl sulfate-NaOH and CB-18 processing were virtually identical, and the observed increase in culture sensitivity was due exclusively to isolation of mycobacteria among smear-negative specimens (14). The first major study comparing CB-18 processing with NALC-NaOH processing reported higher smear sensitivity following CB-18 processing, and the reported increase in culture sensitivity following CB-18 processing was primarily among smear-negative specimens (24). Therefore, the latter explanation, that CB-18 processing enhances culture sensitivity among smear-negative specimens, is probably correct.

If CB-18 specimen processing enhanced culture sensitivity among smear-negative specimens, then it is reasonable to expect a significant increase in the total number of specimens positive for AFB (i.e., either *M. tuberculosis* complex, NTM, or both). An increase in the total number of AFB isolated was not observed following CB-18 processing. There are two possible explanations. The first simply points to the disadvantages of study designs such as that used here (i.e., two independent arms) and the complexities and/or uncertainties of interpreting data that arise as a result (i.e., whether specimens were truly entered into the study randomly or whether some type of bias was inadvertently introduced into the study, thereby skewing the results). The second explanation is related to the deficient sensitivity of solid media following CB-18 processing. This is in striking contrast to all previous reports (14, 24, 25, 27). These reports demonstrated that one of the primary challenges following CB-18 processing was optimizing the sensitivity of liquid culture due to the interaction of CB-18 and antibiotics in liquid media. The importance of solid-medium sensitivity can be seen in the NALC-NaOH arm of this study: approximately 11% of all AFB isolated, and 20% of AFB isolated from smear-negative specimens, were positive on solid media only.

There were several differences between the CB-18/lytic-en-

TABLE 8. Statistical comparisons of significant study results by chi-square analysis

Category	Results of each independent arm		*P* value
	NALC-NaOH	CB-18	
Total no. of specimens processed	1,468	1,423	
Incidence of *M. tuberculosis* (total no. of specimens culture positive for MTBC)	88	82	>0.05
Incidence of NTM (total no. of specimens culture positive for NTM)	42	31	>0.05
Liquid medium contamination (total no. of MB/BacT vials lost to contamination)	39	23	<0.05
Solid-medium contamination[a] (total no. of plates/slants lost to contamination)	67	33	<0.05
Smear sensitivity (total no. of smear-positive specimens that were also MTBC culture positive)	60	40	<0.05
Smear specificity (total no. of smear-positive specimens that were culture negative)	4	2	>0.05
Total no. of smear-negative and MTBC-culture positive specimens	28	42	<0.05
Total no. of smear-negative and NTM-culture positive specimens	41	29	>0.05
Total no. of AMPLICOR-positive specimens	103	91	>0.05
AMPLICOR sensitivity (no. of MTBC-culture positive specimens positive by AMPLICOR)	66	64	>0.05
Smear-positive sensitivity (among specimens both MTBC culture positive and smear positive)	57	38	>0.05
Smear-negative sensitivity (among specimens MTBC culture positive but smear negative)	9	26	<0.05
AMPLICOR specificity (total no. of culture-negative specimens positive by AMPLICOR)[b]	37	27	>0.05
Inhibition rate (total no. of specimens where the IPC[c] indicated that inhibitors were present)[b]	123	91	<0.05

[a] Specimens processed by the NALC-NaOH method were plated on L-J, whereas specimens processed by CB-18/lytic enzymes were plated on 7H11 selective plates.
[b] This is the unresolved AMPLICOR specificity.
[c] IPC, internal positive control.

zyme decontamination method used in the previous study in our laboratory (14) and the present study that may be related to the loss of solid-medium sensitivity: (i) the pHs of the CB-18 and lytic-enzyme buffers were set at 7.6 in the initial study, whereas in the present study, they were set at 6.0; (ii) zymolyase was omitted from the decontamination mixture (it was very expensive); (iii) the *Trichoderma harzianum* extract used in the initial study was Novozyme 234 (Novo Nordisk), but in the present study, this was replaced with the Novo Nordisk *T. harzianum* extract known as Glucanex (the Novozyme 234 product was discontinued; the Glucanex product is manufactured by a different process from the same organism and is marketed as a replacement product); (iv) the *Cytophaga* extract (Sigma L-1784) used in the original study was no longer commercially available—additional extract was obtained contractually using *Lysobacter* sp. strain NCIMB 9497 and the method of Andrews and Asenjo (1) (this is the same strain and method used to manufacture the original product)—the *Cytophaga* sp. strain used, NCIMB 9497, was renamed *Lysobacter* sp. strain NCIMB 9497 (personal communication from Judith E. P. Young, National Collections of Industrial and Marine Bacteria Ltd., Scotland).

The contamination rates following CB-18 processing may also be related to the deficient solid-medium sensitivity. In the initial study, the contamination rates in MB/BacT vials and on 7H11 selective slants were 8.7% and 4.3%, respectively (14), whereas in the present study, the contamination rates were 1.6% and 2.3%, respectively. This suggests that one of the aforementioned factors might have been causing more efficient decontamination of specimens. The most striking difference between the spectrum of contaminants observed in this study and that in the previous report (15) was the notable reduction in both gram-positive and gram-negative microorganisms, especially *Streptococcus* species, in liquid culture. CB-18 is in the class of zwitterionic detergents known as the *n*-alkyl betaines. Under acidic conditions, the anion in these detergents is neutralized, and they begin to take on the character of a cationic detergent (e.g., a quaternary ammonium detergent) (28, 29). While this may explain the reduced contamination rate, it does not explain the loss in solid-medium sensitivity. In liquid media, the processed sediment is diluted approximately 20-fold into the MB/BacT vial (the pH or other component[s] would be significantly diluted). On solid media, however, the sediment components remain present during incubation. The impacts of the changes listed above on solid-medium sensitivity are being investigated.

The final factor that complicates any interpretation that concludes that CB-18 processing improved PCR sensitivity among culture-positive and smear-negative specimens is the actual sensitivity values themselves. For example, while PCR sensitivity among culture-positive and smear-negative specimens following CB-18 processing is approximately double the sensitivity following NALC-NaOH processing, the reported sensitivities are only 61.9% and 32.1%, respectively. The latter sensitivity seems especially low, but sensitivities of 40% or below among culture-positive and smear-negative specimens in studies that use the AMPLICOR test on respiratory specimens are not uncommon (2, 4, 21, 32). In addition, amplification sensitivities are known to vary markedly from site to site. In one study that involved six sites, AMPLICOR sensitivities among culture-positive and smear-negative specimens ranged from 57.1% to 87.5%, with the average being 71.7% (3). In any case, the real issue is that a sensitivity of 61.9% is unremarkable: it is far from a sensitivity that would enable PCR to become a diagnostic screening tool.

If CB-18 specimen processing enhances culture sensitivity among smear-negative specimens, then CB-18 processing may exacerbate the phenomenon that is responsible for the defective amplification sensitivities among this group of specimens. There are several explanations for the substandard amplification sensitivity among culture-positive and smear-negative specimens. First, the false-negative results could be due to inhibition; however, the AMPLICOR test uses an internal positive control—inhibition played no role in the results reported here. The most plausible explanation is related to the presence of extremely low numbers of bacilli. For example, in the present study, culture analyzed approximately 10-fold more sediment than PCR. If fewer than 100 bacilli per ml are present in the sediment, the probability of a false negative by nucleic acid amplification increases significantly (8). This phenomenon of "sample bias," "sampling error," or "statistical dropouts" has been discussed at length in the literature (7, 22), and it is aggravated by the tendency of *M. tuberculosis* to cord or clump—clumps of bacilli partition as a single organism (3). If CB-18 processing is more efficient at collecting bacilli by centrifugation and these bacteria are more viable (26), then the population of specimens that would be culture positive but that experience statistical dropouts by amplification may be increased. A complicating factor is the fact that the AMPLICOR test uses the 16S rRNA gene as its target (a single-copy gene)—there is a one-to-one correspondence between bacilli and amplification targets. Amplification tests based on multicopy genes (e.g., insertion sequences, such as IS*6110*) or on gene products (e.g., 16S rRNA) may have a better chance of approaching desirable sensitivities in the face of sampling error if the lysis step precedes removing an aliquot for analysis.

There are several other observations worth noting. First, in this study, we reported a higher rate of PCR inhibition than in other studies (8.4% and 6.4% by NALC-NaOH and CB-18, respectively) (19). This was probably due to the fact that sediments were resuspended in supernatant backwash and an aliquot was taken prior to being diluted (i.e., the sediment, and therefore the inhibitors, was more concentrated). The second observation is related to the higher percentage of false-positive PCR results than in other studies (42% and 33% by NALC-NaOH and CB-18, respectively) (6, 20). Possible explanations include sample bias (see above) and carryover contamination; however, the AMPLICOR test uses uracil-DNA glycosylase to prevent such contamination. Reischl et al. reported that a high number of false-positive results may come from patients who have undergone X-ray therapy or chemotherapy; these treatments partially destroy the lung tissue and the mycobacteria therein (20). In our study, 17% of the patients with false-positive PCR results were diagnosed with lung carcinoma, but none of these patients had evidence of active tuberculosis. Further studies will be needed to assess the relationship between such cancer therapy and false-positive PCR results.

The arguments presented above strongly suggest that the sensitivity of the AMPLICOR test was significantly increased among culture-positive and smear-negative specimens. The

complications outlined were unexpected and blur any unequivocal statements. What is clear is that specimen processing does have a significant impact on diagnostic sensitivity in general. In any case, further examination of specimen processing as a means to enhance the diagnostic sensitivity of nucleic acid amplification is warranted.

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