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Comparison of Four Decontamination Methods for Recovery of *Mycobacterium avium* Complex from Stools

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The presence of *Mycobacterium avium* complex (MAC) in stool specimens may be a predictor of disseminated MAC infection, yet the methods for decontaminating stools have not been evaluated for their usefulness in recovering MAC organisms. In the present study, four decontamination methods commonly used to recover acid-fast bacteria from respiratory specimens were compared for their utility in recovering MAC from stool specimens. Ten strains of MAC were used at a level of 10^4 to 10^6 CFU to seed the stool specimens. Specimens were divided into four portions and were decontaminated by using the following treatments: (i) *N*-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH), (ii) cetylpyridinium chloride-sodium chloride (CPC-NaCl), (iii) oxalic acid, or (iv) benzalkonium chloride-trisodium phosphate (BC-TSP). The specimens were then plated onto a total of five pieces of selective and nonselective egg- and agar-based media. The oxalic acid method yielded the greatest number of MAC CFU from seeded stool samples; this was followed by NALC-NaOH, BC-TSP, and CPC-NaCl. The difference between the oxalic acid method and each of the other methods was statistically significant (analysis of variance at the 95% significance level). Although more MAC CFU was recovered from seeded stool samples by using oxalic acid than NALC-NaOH, no difference in culture positivity rates was observed when the two methods were used to test 368 clinical stool specimens processed with either oxalic acid (164 specimens) or NALC-NaOH (204 specimens) ($P = 0.07$) or 67 specimens processed by both methods ($P = 0.77$). The oxalic acid and NALC-NaOH decontamination methods both appear to be useful for the recovery of MAC organisms from stool specimens.

Disseminated infection caused by the *Mycobacterium avium* complex (MAC) in patients with AIDS is thought to originate in the gastrointestinal or respiratory tract (3, 4, 6, 14). The presence of MAC in stool specimens from patients with AIDS may be a predictor of disseminated MAC infection (3, 5). Thus, a simple and effective method for decontaminating stool specimens and recovering MAC organisms is needed. A number of methods have been developed for the decontamination of nonsterile specimens for recovery of acid-fast bacilli (AFB), but these were designed primarily for use on respiratory specimens and to enhance the recovery of *Mycobacterium tuberculosis*. There is limited information in the literature on the methods used for recovering AFB from stool samples (1, 8, 12), and no studies have been done comparing different decontamination methods for the recovery of MAC.

Decontamination methods take advantage of the resistance of mycobacteria to acids, bases, or other antibacterial agents. Perhaps the most commonly used decontamination procedure for sputum specimens involves the use of sodium hydroxide-sodium citrate along with the mucolytic agent *N*-acetyl-L-cysteine (NALC; the NALC-NaOH method) (10, 11). This method is widely used because it is rapid and relatively effective in reducing the number of contaminants.

The use of cetylpyridinium chloride (CPC)-sodium chloride (the CPC-NaCl method) was proposed for the decontamination of sputum specimens that are in transport to the laboratory for more than 24 h (15). Contamination rates associated with treatment of sputum specimens by the CPC-NaCl method are lower than those associated with the NALC-NaOH method (15). However, decontamination with CPC-NaCl requires a longer treatment period than is needed with NALC-NaOH and residual CPC has been reported to partially inhibit mycobacterial growth on 7H10 agar medium (7, 15).

Mycobacteria are relatively more resistant than non-acid-fast bacterial contaminants to strongly acidic decontamination solutions (7). In 1930, Corper and Uyei (2) described the use of oxalic acid as a decontaminant for the recovery of *M. tuberculosis* from respiratory specimens. Later, oxalic acid treatment was recommended for use in recovering AFB from heavily contaminated specimens such as those consistently overgrown with *Pseudomonas* species (7). The method involves a 30-min incubation of the specimen with 5% oxalic acid, centrifugation, and neutralization of the sediment.

Treatment of respiratory specimens with benzalkonium chloride (BC; Zephiran)-trisodium phosphate (TSP; the BC-TSP method) has also been described (9, 16). BC is a quaternary ammonium compound that acts as a decontaminating agent. Trisodium phosphate is included in the decontamination solution as a mucolytic agent to assist in homogenization of the specimen (16). The BC-TSP method is a somewhat laborious procedure, since it involves a 30-min treatment, centrifugation, resuspension in neutralization buffer, and a second centrifugation step (7).

Although all the treatment procedures described above have been used on sputum specimens, no comparison of

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these methods has been done to assess their efficacies in recovering MAC from stool samples. The study described here was performed to compare the effectiveness of treatment of stool samples by the NALC-NaOH, CPC-NaCl, oxalic acid, or BC-TSP methods in the recovery of MAC organisms from seeded stool samples.

MATERIALS AND METHODS

Bacterial cultures. Ten strains of MAC were used to seed stool preparations. All isolates were from patients at San Francisco General Hospital who had disseminated MAC infection. Nine of the isolates were identified as *M. avium* by DNA probe analysis and one was identified as *Mycobacterium intracellulare*. All MAC isolates were grown in 7HSF broth (17) to a concentration of approximately 3×10^7 CFU/ml. 7HSF broth is composed of Bacto Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) with 1 g of pancreatic digest of casein (BBL Microbiology Systems, Cockeysville, Md.) per liter, 0.5% glycerol, and 100 ml of OADC enrichment (Difco) per liter; this medium is the equivalent of 7H11 agar without malachite green. The cultures were centrifuged, and the organisms were resuspended in sterile water. Dilutions were prepared to obtain solutions containing approximately 10^5, 10^6, and 10^7 CFU/ml for use in the decontamination procedures. The concentration of MAC in these solutions was confirmed by plating dilutions of each solution onto 7H10 agar.

Preparation of seeded stool samples. Stools from five randomly selected patients who submitted specimens to the Clinical Microbiology Laboratory at San Francisco General Hospital were used to prepare seeded stool samples. Each stool sample was used to test two different MAC strains by each of the four decontamination procedures. Approximately 0.2 g of stool was placed in a 15-ml conical centrifuge tube containing 11 ml of sterile water. The mixture was vortexed for 30 s, and large particulate material was allowed to settle. After 15 min, 10 ml of the supernatant was transferred to a 50-ml conical centrifuge tube and 0.1 ml of a MAC suspension containing 10^5, 10^6, or 10^7 CFU/ml was added. For each bacterial concentration tested, four separate seeded stool preparations were made (one for each of the four decontamination methods). Each preparation was then decontaminated by one of the procedures described below. As a control, stool samples from all five patients were also cultured without seeding.

NALC-NaOH method. NALC–2% sodium hydroxide-sodium citrate solution was prepared as described by Kent and Kubica (7). To the 50-ml tube containing 10 ml of seeded stool sample, an equal volume (10 ml) of NALC-NaOH citrate reagent was added and the tube was vortexed briefly. Following 15 min of incubation at room temperature, the volume was brought to 50 ml with 0.067 M phosphate buffer (pH 6.8) (7) and the contents were mixed by inversion. Bacteria were pelleted by centrifugation at $3,000 \times g$ for 15 min. The supernatant was discarded, and the pelleted material was resuspended in 1 ml of 0.2% bovine serum albumin fraction V (7).

CPC-NaCl method. Ten milliliters of a solution containing 1% CPC and 2% NaCl (7) was added to a 50-ml tube containing 10 ml of seeded stool sample. The contents were mixed by vortexing for 15 s and were then incubated for 24 h at room temperature. The volume was brought to 50 ml with sterile distilled water, and then the tube was centrifuged at $3,000 \times g$ for 15 min. The pelleted material was resuspended in 1 ml of bovine serum albumin.

The 5% oxalic acid method. Ten milliliters of 5% oxalic acid (7) was added to a 50-ml tube containing 10 ml of seeded stool sample. The contents were mixed by vortexing for 15 s, and then the tube was incubated at room temperature for 30 min with occasional mixing. The volume was brought to 50 ml with sterile saline, mixed by inversion, and then centrifuged at $3,000 \times g$ for 15 min. The supernatant was discarded, and the remaining material was brought to neutrality by using 4% NaOH (approximately 0.08 ml) and pH paper strips. The use of phenol red as an indicator for the neutralizing step, as described by Kent and Kubica (7) for respiratory specimens, was determined not to be practical because of interference from the color of the fecal material. After neutralization, the solution was thoroughly mixed before plating.

BC-TSP method. Ten milliliters of a solution containing 0.032% BC (Sigma Chemical Co., St. Louis, Mo.) and 25% TSP (7) was added to a 50-ml tube containing 10 ml of seeded stool sample. The contents were mixed by vortexing for 15 s, and then the tube was incubated at room temperature for 60 min. Bacteria were pelleted by centrifugation at $3,000 \times g$ for 15 min, and then 20 ml of neutralizing buffer (0.067 M phosphate buffer [pH 6.6]) (7) was added to the pellet and the contents were mixed by vortexing for 15 s. The tube was again centrifuged at $3,000 \times g$ for 15 min, and the pelleted material was resuspended in 0.5 ml of neutralizing buffer before plating.

Media. Portions (0.1 ml) of the decontaminated specimens were inoculated onto each of five pieces of media: Lowenstein-Jensen medium (Difco), Mycobactosel Lowenstein-Jensen medium (BBL), Middlebrook 7H11 agar (Difco) plates, Mitchison's selective Middlebrook 7H11 agar plates (13), and Middlebrook 7H11 agar plates containing Gruft's antibiotics (13). All agar media were prepared in disposable plastic plates (50 by 9 mm) with snap-tight lids (Falcon no. 1006; Becton Dickinson Labware, Lincoln Park, N.J.). These plates retard evaporation and, therefore, need not be placed in sealed bags. They also permit easy microscopic examination of colonial growth. All media were incubated at 35°C for 2 weeks in a 5% CO_2 atmosphere and then for an additional 6 weeks at 35°C in an ambient atmosphere. The media were examined for MAC colony counts and for contamination after 2, 4, 6, and 8 weeks of incubation.

Effect of pH on recovery of MAC after oxalic acid decontamination. Stool specimens from nine additional patients were each divided into three portions, and then each specimen was seeded with one of nine different MAC strains. The specimens were then decontaminated with oxalic acid as described above. After centrifugation, the three portions of each stool sample were treated as follows: one was plated onto nonselective 7H11 agar without neutralization (pH $<$ 2), one was neutralized with NaOH before plating, and the other was overalkalinized (pH $\geq$ 11) by adding 0.1 ml of 4% NaOH before plating.

Culture of MAC from clinical stool specimens. For culture of MAC from clinical stool specimens, approximately 0.2 g of stool (i.e., a portion about the size of a pea) was emulsified in 11 ml of sterile water. The mixture was vortexed for 30 s, and the particulate material was allowed to settle for 15 min. Ten milliliters of the supernatant was then transferred to a 50-ml conical centrifuge tube and was decontaminated as described above by using either the oxalic acid method or the NALC-NaOH method, or both methods. The media used in the clinical stool studies were the same as those used in the seeded stool study, except that Gruft's selective Middlebrook agar was not used on clinical specimens.

TABLE 1. Recovery of MAC from seeded stool samples by using four decontamination methods, five different media, and 10 MAC strains

Medium	Mean MAC CFU/ml recovered after treatment with:			
	NALC-NaOH	Oxalic acid	CPC-NaCl	BC-TSP
Nonselective 7H11 agar	1,020	3,160	90	300
Mitchison's selective 7H11 agar	800	1,740	7	0.7
Gruft's selective 7H11 agar	400	510	4	3
Lowenstein-Jensen	450	770	81	420
Mycobactosel Lowenstein-Jensen	990	880	71	300
All media (mean)	732	1,412	51	205

Statistical analysis. Statistical analysis of the data for the seeded stool study was performed by analysis of variance for repeated measures (StatView II; Abacus Concepts, Inc., Berkeley, Calif.). Chi-square contingency table analysis (StatView II) was performed on data from clinical stool specimens.

RESULTS

Culture results for the five stool specimens used in the seeding study confirmed that all five stool samples were negative for MAC prior to seeding. Table 1 shows data for the mean number of MAC CFU per milliliter recovered from seeded stool samples on each type of medium after treatment by each of the four decontamination methods. An analysis of variance for repeated measures showed no significant difference among the 10 MAC strains used in the study ($P = 0.38$), but it did show a significant difference among the four methods in the mean number of MAC CFU recovered per milliliter ($P = 0.001$). The oxalic acid method yielded a significantly greater (95% significance level) number of MAC CFU per milliliter than the NALC-NaOH, CPC-NaCl, or BC-TSP methods. The difference in CFU per milliliter between the oxalic acid method and the CPC-NaCl method and the difference between the oxalic acid method and the BC-TSP method were highly significant (99% significance level). The difference between the oxalic acid and NALC-NaOH methods, although statistically significant, represented only a twofold difference in MAC CFU per milliliter counts (1,412 versus 732, respectively).

The four procedures were also evaluated for their abilities to prevent contamination of media by non-acid-fast organisms. A piece of medium was considered to be contaminated if it was nonevaluable because of the presence of contaminants. Nonevaluable was defined as complete overgrowth by bacterial or fungal contaminants before 8 weeks of incubation and no AFB present on the medium. Any piece of medium containing colonies of AFB was considered to be evaluable. By these criteria, the CPC-NaCl and BC-TSP methods had the lowest overall contamination rates (each had 10 contaminated pieces of media of the 150 pieces inoculated [7%]); this was followed by the oxalic acid (11%) and NALC-NaOH (22%) methods. The contamination rates varied among the media included in the study (Table 2). The selective Mycobactosel Lowenstein-Jensen medium had the lowest overall contamination rate (2%); this was followed by Mitchison's selective 7H11 agar (3%), Lowenstein-Jensen medium (15%), Gruft's selective 7H11 agar (19%), and nonselective 7H11 agar (21%). In no case was an entire specimen lost because of contamination on every piece of medium. Although nonselective 7H11 agar had the highest contamination rate, it also permitted recovery of the greatest number of CFU of MAC. The ranking of the media on the basis of the number of MAC CFU recovered was nonselective 7H11 agar > Mitchison's selective 7H11 agar, Mycobactosel Lowenstein-Jensen, and Lowenstein-Jensen medium > 7H11 agar containing Gruft's antibiotics.

It has been reported that specimens treated with CPC should be inoculated only onto egg-based media because of a bacteriostatic effect of CPC for mycobacteria on agar

TABLE 2. Contamination rates of media after decontamination of stool specimens by four different methods

Medium	Type of study	Contamination rate (%) by the following method[a]:			
		NALC-NaOH	Oxalic acid	CPC-NaCl	BC-TSP
Nonselective 7H11 agar	Seeded	41	20	13	10
	Clinical	39	22		
Mitchison's selective 7H11 agar	Seeded	3	3	3	0
	Clinical	4	6		
Gruft's selective 7H11 agar	Seeded	20	23	17	17
	Clinical				
Lowenstein-Jensen	Seeded	43	10	0	7
	Clinical	6	7		
Mycobactosel Lowenstein-Jensen	Seeded	7	0	0	0
	Clinical	0	4		

[a] Contamination was defined as complete overgrowth of the medium before 8 weeks of incubation and the lack of AFB by the time that the medium was discarded. The contamination rate was calculated as (number of plates or tubes contaminated/total number of plates or tubes inoculated) × 100.

media (7). The data obtained in the present study (Table 1) revealed that after decontamination with CPC, there was no observable difference in the number of MAC CFU per milliliter recovered on egg-based (Lowenstein-Jensen) medium versus that recovered on nonselective Middlebrook 7H11 agar (81 versus 90 CFU/ml; paired *t* test [$P = 0.64$]).

Effect of final pH on recovery of MAC after oxalic acid treatment. A time-consuming and laborious step in the oxalic acid decontamination procedure is the neutralization of the specimen with NaOH following decontamination with oxalic acid. During the addition of NaOH, inadvertent overalkalinization occurs easily. To test the effect of the final pH on the recovery of MAC, portions of nine different oxalic acid-decontaminated stool specimens were (i) neutralized before plating, (ii) plated without neutralization, or (iii) overalkalinized with NaOH before plating. The results showed that the number of MAC recovered from samples plated without neutralization (mean, 74 CFU) was greater than the number recovered after neutralization (mean, 58 CFU) or overalkalinization (mean, 50 CFU). An analysis of variance for repeated measures showed that these differences were not significant ($P = 0.06$), although the trend was toward significance in favor of plating without neutralization. These results demonstrate that recovery of MAC is not improved by neutralization of oxalic acid-treated stool specimens prior to plating.

Recovery of MAC from clinical stool specimens by oxalic acid or NALC-NaOH decontamination. Prior to the present study, 204 stool samples were cultured for MAC by the NALC-NaOH method, and MAC was recovered from 39 (19%) of these specimens. After completing the seeded stool study, we began culturing stools using the oxalic acid decontamination method. To date, 164 stool samples have been tested by the oxalic acid method, and MAC has been recovered from 20 (12%) of these. Chi-square contingency table analysis of the data showed no significant difference ($P = 0.07$) between the two methods in the proportion of MAC-positive stools. Recently, 67 stool samples were also processed by both the oxalic acid and NALC-NaOH methods. Six specimens were positive by both methods, and one specimen was positive by the NALC-NaOH method only. The difference in the positivity rate between the two methods was not statistically significant ($P = 0.77$). The one specimen that was positive by the NALC-NaOH method and negative by the oxalic acid method grew only a single colony of MAC. Acid-fast smear results for the 435 clinical specimens included in the three clinical stool sample studies revealed that nine of the specimens were positive for AFB. All nine smear-positive specimens were culture positive for MAC by both of the decontamination methods.

The contamination rates of the various media used in the clinical studies were similar to the contamination rates obtained in the seeded stool study, except that Lowenstein-Jensen medium was contaminated less often by the NALC-NaOH method in the clinical studies than in the seeded stool study (Table 2).

DISCUSSION

In a comparison of four decontamination methods that are commonly used for the recovery of AFB from respiratory specimens, the oxalic acid method yielded the greatest number of CFU of MAC from seeded stool specimens. Treatment of stool specimens by the NALC-NaOH method yielded about half the number of CFU per milliliter as that obtained by treatment of stools by the oxalic acid method. This difference, however, might not be important if stool specimens typically contain relatively large numbers of MAC. An analysis of culture results for 368 clinical specimens processed with either oxalic acid (164 specimens) or NALC-NaOH (204 specimens) showed no statistical difference between these two methods in the positivity rate for MAC. In addition, no difference in the MAC positivity rate was seen among 67 stool specimens processed by both the oxalic acid and NALC-NaOH methods.

Two interrelated factors play important roles in the recovery of MAC from stool specimens. One is the ability of decontaminating agents to destroy or inhibit non-acid-fast contaminants, whose presence could mask and prevent the detection of viable MAC organisms. The other is the direct effect of decontaminating agents on the viability of MAC organisms themselves. Since the decontaminating agents that are widely used today are not completely harmless to AFB, the procedure that is chosen for use on stool specimens should be one that achieves a good balance between effective removal of contaminants and a high rate of survival of AFB. In the present study, the CPC-NaCl and BC-TSP methods had the best (lowest) rates of contamination, but they also yielded the lowest number of CFU of MAC. These results suggest that treatments by the CPC-NaCl and BC-TSP methods significantly affect the viability of MAC organisms in stools as well as suppress the growth of non-acid-fast organisms.

The poor results obtained in the present study by the CPC-NaCl method differ from the results reported by Smithwick et al. (15), who found the CPC-NaCl method to be superior to the NALC-NaOH method for the recovery of *M. tuberculosis* and non-tuberculosis mycobacteria from sputum samples that were transported to the laboratory through the mail. In the study of Smithwick et al. (15), non-tuberculosis mycobacteria (including 25 *M. avium-M. intracellulare* isolates) were recovered from 31 of 1,602 sputum specimens by the CPC-NaCl method, while non-tuberculosis mycobacteria (no data on the species were given) were isolated from 17 of the specimens by the NALC-NaOH procedure. In the previous study (15), NaOH was used at a final concentration of 2%, whereas in our study the final NaOH concentration was 1%. The difference between the results of the two studies may be due to (i) the different NaOH concentrations, (ii) differences in sputum versus stool specimens, or (iii) the use of transported specimens versus seeded specimens.

On the basis of the results of the present study, either the oxalic acid method or the NALC-NaOH method can be recommended for recovery of MAC from stool specimens. We found that the oxalic acid method, although more laborious to use than the NALC-NaOH method, can be made as simple as the NALC-NaOH method by omitting the neutralization step. Regardless of the decontamination method used, the most useful media for recovering MAC from stool specimens were Mitchison's selective 7H11 agar and Mycobactosel Lowenstein-Jensen medium. These media had low contamination rates, and there was relatively good recovery of MAC compared with recovery of MAC from nonselective media.

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