Improved Recovery of Mycobacteria from Respiratory Secretions of Patients with Cystic Fibrosis

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Pulmonary colonization and infection of patients with cystic fibrosis by *Mycobacterium* spp. has recently been recognized as a potentially important clinical problem. However, frequent contamination of mycobacterial cultures by pseudomonads has hampered efforts to define the extent of this problem. This study was done to evaluate current techniques and to establish a more efficient method of recovering mycobacteria from respiratory secretions of patients with cystic fibrosis. Decontamination of respiratory specimens ($n = 121$) with 0.25% *N*-acetyl-L-cysteine and 1% sodium hydroxide (NALC-NaOH) was associated with a high rate of pseudomonas overgrowth for both Lowenstein-Jensen slants (74%) and BacTec vials supplemented with PANTA (polymyxin B [50 U/ml], amphotericin B [5 µg/ml], nalidixic acid [20 µg/ml], trimethoprim [5 µg/ml], azlocillin [10 µg/ml]) (36%). This overgrowth limited recovery of mycobacteria to only 64% (9 of 14) of specimens positive by smear for acid-fast bacilli (AFB). Decontamination of specimens ($n = 441$) with NALC-NaOH, followed by 5% oxalic acid treatment, resulted in contamination of only 5% of Lowenstein-Jensen slants and 3% of BacTec vials. AFB were recovered from all 90 AFB smear-positive specimens following the use of this decontamination technique. We recommend that respiratory secretions be decontaminated with NALC-NaOH and oxalic acid to decrease the incidence of *Pseudomonas aeruginosa* overgrowth.

Mycobacteria are being recovered with what appears to be increasing frequency from the respiratory tracts of patient with cystic fibrosis (CF). Six previous studies have been done to assess the recovery rate of mycobacteria from CF patients. Wood et al. (16) isolated *Mycobacterium tuberculosis* from 2 of over 700 CF patients; *M. fortuitum* was recovered from three additional patients. Boxerbaum (1) recovered *M. chelonae* from 6 and *M. fortuitum* from 2 of 430 patients. Hoiby (7) reported that 2 of 70 CF patients had a mycobacterium other than *M. tuberculosis* recovered over a 1-year period. Hjelte et al. (6) recovered mycobacteria from 6 of 54 CF patients. *M. tuberculosis* was recovered from one patient, the *M. avium* complex was recovered from three, and two patients had unidentified isolates of mycobacteria. Smith et al. (14), in cultures from 223 patients, found seven that were positive for mycobacteria, including three with *M. tuberculosis*, one each with *M. chelonae* and *M. fortuitum*, and two with unidentified mycobacteria. Most recently, we reported the recovery of mycobacteria, primarily the *M. avium* complex, from 20% of 87 adult CF patients at the University of North Carolina Hospitals (9).

Other organisms which colonize or infect the respiratory tracts of CF patients greatly complicate the recovery of mycobacteria. *Pseudomonas aeruginosa* is a particular problem, because it is present in the respiratory tracts of approximately 80% of CF patients (4) and selected isolates can survive the most commonly used sputum decontamination method (0.25% *N*-acetyl-L-cysteine [NALC]-1% NaOH) (9, 14). When *P. aeruginosa* survives NALC-NaOH decontamination, it can result in overgrowth and liquefac-

tion of Lowenstein-Jensen (LJ) slants (9, 14) and false positive-readings in BacTec vials. We (9) and Smith et al. (14) have both reported that bacterial overgrowth by *P. aeruginosa* prevented isolation of mycobacteria from specimens positive for acid-fast bacilli (AFB) by smear. In our study, we found that 67 of 297 cultures (22.6%) were overgrown by bacteria, primarily *P. aeruginosa*. Therefore, it is important to optimize decontamination conditions to recover mycobacteria from this patient population.

This study was initiated to evaluate current techniques used to decontaminate respiratory secretions obtained from CF patients. In phase one, a retrospective analysis, we determined the contamination rate of CF specimens treated with NALC-NaOH. We compared those findings with results from a prospective study (phase two) using NALC-NaOH followed by 5% oxalic acid for decontamination. In addition, we investigated whether supplementation of BacTec vials with additional antimicrobial agents would decrease contamination rates. Recovery of mycobacteria from smear-positive and smear-negative patients was determined following the use of each technique.

MATERIALS AND METHODS

Patient population. Specimens were obtained from approximately 100 patients seen in the CF clinic, as well as from CF inpatients, at the University of North Carolina Hospitals between September 1990 and February 1992. Respiratory specimens, including sputa, tracheal aspirates, and specimens obtained by bronchoscopy, were sent to the clinical laboratory for AFB smear and culture. A total of 562 specimens were studied: 121 specimens in phase one of the study and 441 specimens in phase two. All specimens were processed within 24 h of receipt.

Decontamination techniques. (i) NALC-NaOH. Respiratory

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specimens were combined with an equal volume of 0.5% NALC-2% NaOH in a 50-ml screw-top conical tube and vortexed for at least 20 s. After 15 min of room temperature incubation, the specimen volume was brought to 50 ml with sterile distilled water and the resulting mixture was centrifuged for 5 min at $4,500 \times g$. The supernatant was poured off, leaving approximately 1 ml of the concentrated specimen for inoculation of media and smear preparation.

(ii) **NALC-NaOH-oxalic acid.** Respiratory specimens were decontaminated as described above. An equal volume of 5% oxalic acid was added to the concentrated sediment. This mixture was vortexed and allowed to incubate at room temperature for 30 min, with vortexing every 10 min. The specimen volume was brought to 50 ml with sterile distilled water, and the mixture was centrifuged for 5 min at $4,500 \times g$. The supernatant was decanted, leaving approximately 1 ml of the specimen. With pH paper, the specimen was neutralized by addition of 4% NaOH to a pH of approximately 7 prior to inoculation of media (12).

Culture techniques. Each specimen was inoculated onto two LJ slants (BBL, Cockeysville, Md.) and into one BacTec 7H12B vial (Becton Dickinson Diagnostic Instrument Systems, Cockeysville, Md.). Slides were also prepared from concentrated sediment and stained by the Truant fluorochrome technique (15). BacTec vials were supplemented with PANTA (polymyxin B [50 U/ml], amphotericin B [5 µg/ml], nalidixic acid [20 µg/ml], trimethoprim [5 µg/ml], azlocillin [10 µg/ml]) in accordance with manufacturer recommendations. One portion of this study compared two different antibiotic supplementations of the BacTec vials. The first consisted of regular supplementation of the BacTec vials with PANTA, while in the second method the BacTec vials were supplemented with both PANTA and 50 µg of piperacillin per ml immediately prior to specimen inoculation. LJ slants were examined once a week for 8 weeks. BacTec vials were monitored twice weekly for the first 2 weeks and once a week for weeks 3 and 4.

Seeding experiments. Sputum specimens from 30 CF patients known to be negative for AFB were used for seeding experiments. A cell suspension of *M. avium* was adjusted to a no. 1 McFarland standard, and 0.1 ml of this suspension was added for every 1.0-ml specimen volume. This was done to ensure that each specimen received a standardized number of organisms which was sufficient to allow consistent recovery. Each specimen was divided into three equal aliquots and decontaminated by one of the following methods previously described: NALC-NaOH, oxalic acid, or NALC-NaOH followed by oxalic acid.

Organism identification. Identification of rapidly growing acid-fast organisms was performed by standard techniques as outlined in reference 12. *M. avium* complex isolates were identified by the Accuprobe technique (Gen Probe, San Diego, Calif.) (5).

Identification and susceptibility testing of bacterial contaminants. *Pseudomonas* species were identified by standard methods (3). Susceptibilities were determined by the Bauer-Kirby method in accordance with National Committee for Clinical Laboratory Standards disk diffusion standards for aerobic bacteria (11).

RESULTS

In our laboratory, the standard NALC-NaOH method for decontaminating respiratory secretions cultured for mycobacteria is used. In the initial phase of this study, 121 respiratory specimens from CF patients were decontami-

TABLE 1. Recovery of AFB from 121 CF respiratory specimens decontaminated by NALC-NaOH (phase one results)

Culture result	No. of isolates that were:			
	AFB smear positive		AFB smear negative	
	LJ	BacTec	LJ	BacTec
<i>M. avium</i> complex	3	4	1	3
<i>M. chelonae</i>	4	5	0	1
Bacterial overgrowth	7 ^a	5 ^b	82 ^c	38 ^d
Negative	0	0	24	65

^a All 7 of these isolates were *P. aeruginosa*.

^b All 5 of these isolates were *P. aeruginosa*.

^c All 82 of these isolates were *P. aeruginosa*.

^d Thirty-four of these isolates were *P. aeruginosa*, and four were *P. cepacia*.

nated by this method (Table 1). Of these, 89 (74%) LJ slants and 43 (36%) BacTec vials were contaminated. The bacterial contaminants recovered included 85 isolates of *P. aeruginosa* and 4 of *P. cepacia*. We were able to isolate mycobacteria from only 9 of 14 AFB smear-positive specimens. Cultures from the other five specimens were overgrown with *P. aeruginosa*. Mycobacteria were isolated from 4 of 107 AFB smear-negative specimens. The demonstration of such high rates of contamination in these respiratory specimens led to the examination of alternative decontamination methods.

Studies were designed to examine the recovery of mycobacteria from 30 seeded respiratory specimens obtained from CF patients following the use of each of three decontamination methods: treatment with NALC-NaOH alone, oxalic acid alone, or NALC-NaOH followed by oxalic acid. Oxalic acid treatment, alone and in combination with NALC-NaOH, was used in these pilot studies because of its reported ability to eliminate *P. aeruginosa* as a contaminant from sputum specimens (12). Treatment with NALC-NaOH alone resulted in the highest contamination rate, with 70% (21 of 30) of LJ slants and 47% (14 of 30) of BacTec vials growing *P. aeruginosa*. Oxalic acid treatment alone yielded 53% (16 of 30) contamination of LJ slants and 50% (15 of 30) contamination of BacTec vials. Treatment of specimens with NALC-NaOH followed by oxalic acid resulted in 13% (4 of 30) and 10% (3 of 30) contamination of LJ slants and BacTec vials, respectively.

On the basis of these results, a total of 441 respiratory specimens were treated with NALC-NaOH followed by oxalic acid (Table 2). Of these, 20 (5%) of the LJ slants and 13 (3%) of the BacTec vials were contaminated with

TABLE 2. Recovery of AFB from 441 CF respiratory specimens decontaminated by NALC-NaOH-oxalic acid (phase two results)

Culture result	No. of isolates that were:			
	AFB smear positive		AFB smear negative	
	LJ	BacTec	LJ	BacTec
<i>M. avium</i> complex	34	34	11	15
<i>M. chelonae</i>	56	56	12	13
Bacterial overgrowth	0	0	20 ^a	13 ^b
Negative	0	0	308	310

^a All 20 of these isolates were *P. aeruginosa*.

^b All 13 of these isolates were *P. aeruginosa*.

P. aeruginosa. No other contaminants were isolated. Mycobacteria were recovered from all 90 AFB smear-positive specimens, as well as from 28 smear-negative specimens.

Although NALC-NaOH-oxalic acid treatment had a low contamination rate in BacTec vials (3%), we determined whether a change in antimicrobial supplementation of the vials might reduce contamination further. A total of 54 isolates of *P. aeruginosa* were recovered from BacTec vials despite the presence of the standard antibiotic supplementation (PANTA). Forty-two of the 54 isolates were available for susceptibility testing by disk diffusion. Thirty-seven of these isolates were recovered post NALC-NaOH decontamination, and five were recovered post NALC-NaOH-oxalic acid treatment. A total of 35 (83%) were susceptible to piperacillin. Other susceptibilities were ceftazidime (66%), colistin (69%), imipenem (69%), ticarcillin-clavulanic acid (52%), tobramycin (36%), gentamicin (36%), amikacin (33%), ciprofloxacin (36%), chloramphenicol (10%), and trimethoprim-sulfamethoxazole (5%).

On the basis of these antibiograms, we supplemented the PANTA-containing BacTec vials with piperacillin (50 µg/ml). These bottles were processed in parallel with bottles containing PANTA alone. A total of 238 CF respiratory specimens were studied, and all were initially decontaminated with NALC-NaOH-oxalic acid. Of these, 38 were positive for mycobacteria. The BacTec vials supplemented with PANTA detected all 38 positive specimens and had a contamination rate of 3.3% (8 of 238). The vials containing piperacillin detected only 22 of the 38 positives and had a contamination rate of 1.7% (4 of 283). Of the 22 samples that were positive in both vials, 9 became positive on the same day. However, the remaining 13 were detected in the PANTA-supplemented vials an average of 7 days prior to those in vials supplemented with PANTA and piperacillin (range, 3 to 29 days).

DISCUSSION

Several surveys have shown that mycobacteria are present in the lungs of CF patients (1, 6, 7, 9, 14, 16). The pathogenic role of mycobacteria other than *M. tuberculosis* in the lung disease of these patients is not understood, although (9, 10, 14) pathogenic, nontuberculous mycobacterial pulmonary infection is clearly present in some patients. To understand the role of these organisms in CF lung disease more fully, accurate methods for detecting them are needed. Further, the importance of identifying patients infected or colonized with these organisms has increased, with lung transplantation becoming a therapeutic option in this patient population. Posttransplant immunosuppressive therapy increases the risk for opportunistic infections, including mycobacterial infections (13). Colonized CF patients may be at even greater risk for developing posttransplant mycobacterial infection. Accurate identification of CF lung transplant candidates who are infected or colonized with mycobacteria is required for better definition and management of this clinical problem.

The rate of recovery of mycobacteria from CF respiratory secretions is greatly complicated by the presence of *P. aeruginosa* in the respiratory tracts of approximately 80% of CF patients. We and others (9, 14) have had difficulty isolating mycobacteria from smear-positive specimens because of bacterial overgrowth, usually due to the mucoid phenotype of *P. aeruginosa*. Because of this problem of bacterial overgrowth, nonculture methods, such as staining or molecular techniques, seem to be ideal. However, acid-

fast staining techniques are not useful in determining the species of mycobacterium. They also are not as sensitive as culture (12). Gene probes have been shown to identify isolates of both *M. tuberculosis* and the *M. avium* complex accurately (5, 8). However, this technique requires initial isolation of the mycobacteria before identification and has not proven useful in detecting these organisms directly in clinical specimens (5). The polymerase chain reaction may prove to be an ideal method for detecting mycobacteria directly from patients' specimens, but technical difficulties in specimen processing make this technique unreliable (2). Therefore, detection and identification of mycobacteria in respiratory secretions still require isolation of the organisms.

We investigated decontamination and culture approaches which could improve the recovery of mycobacteria from respiratory specimens of CF patients. The standard NALC-NaOH method of decontamination resulted in bacterial overgrowth in 74% of LJ slants and 36% of antibiotic-supplemented BacTec vials. Modification of this technique to include 30 min of incubation with 5% oxalic acid following NALC-NaOH digestion resulted in contamination rates of only 3% with BacTec vials and 5% with LJ slants. We do not attribute these lower contamination rates to the presence of fewer specimens containing *P. aeruginosa* in the NALC-NaOH-oxalic acid-treated group than in the NALC-NaOH-treated group. All specimens were obtained from the same, primarily adult, CF population, of whom approximately 70% are chronically colonized with *P. aeruginosa* (data not shown). Further, seeding studies yielded similar results. Our attempts to reduce bacterial overgrowth further, by addition of piperacillin to the PANTA-supplemented BacTec vials, revealed that contamination was slightly decreased (3.3 to 1.7%). However, recovery rates of mycobacteria were reduced by addition of piperacillin, and time to detection was delayed for many isolates. Therefore, addition of piperacillin to PANTA-supplemented BacTec vials is not warranted.

Bacterial decontamination of respiratory specimens from CF patients with NALC-NaOH-oxalic acid yielded an impressive reduction in bacterial overgrowth and improved recovery of mycobacteria from all acid-fast stain-positive specimens, as well as a number of smear-negative specimens. We recommend this decontamination technique for isolation of acid-fast organisms from this microbiologically complex patient population.

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