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Value of the Chlorhexidine Decontamination Method for Recovery of Nontuberculous Mycobacteria from Sputum Samples of Patients with Cystic Fibrosis

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The chlorhexidine method was compared to the *N*-acetyl-L-cysteine-NaOH-oxalic acid decontamination method currently recommended for the recovery of nontuberculous mycobacteria (NTM) from patients with cystic fibrosis. Sputum samples ($n = 827$) treated with chlorhexidine yielded twice as many NTM-positive cultures as those treated by the reference method (54 [6.50%] versus 27 [3.25%]; $P < 0.0001$) despite a higher contamination rate (20% versus 14.2%; $P = 0.0017$).

Nontuberculous mycobacteria (NTM) are a growing problem for patients with cystic fibrosis (CF) (8, 9). A recent North American multicenter study showed a prevalence (at least one sputum sample positive for NTM) of between 7 to 24% in CF patients, depending on the center (10). Members of the *Mycobacterium avium-intracellulare* complex (MAC) are the most frequently isolated NTM in CF adults (9), whereas *Mycobacterium abscessus*, a rapidly growing mycobacterium that is often multidrug resistant, is the most common in children (6, 11, 16).

The recovery of NTM from CF sputum samples is hampered by the presence of *Pseudomonas aeruginosa* in the respiratory tracts of 80% of CF patients. This bacterium rapidly overgrows mycobacterial cultures, so specific decontamination methods are required. The most widely used decontamination method for NTM isolation from CF patients is the two-step *N*-acetyl-L-cysteine-NaOH-oxalic acid (NALC-NaOH-OxA) method. This method substantially decreases the contamination rate of CF sputum cultures and allows better recovery of NTM than is possible by decontamination with NALC-NaOH alone (1, 17, 18). However, some reports suggest that the NALC-NaOH-OxA method may affect the viability of mycobacteria (2) and consequently lead to false-negative results for samples with low mycobacterial loads (1, 18). Moreover, this double decontamination method has not been extensively validated for *M. abscessus*, a mycobacterium prevalent in pediatric CF patients. Indeed, different mycobacterial species resist various decontamination agents to different extents (12).

A decontamination method based upon the use of chlorhexidine (CHX) has been proposed for the recovery of mycobacteria. This method is inexpensive, rapid, and easy to perform. Chlorhexidine has a broad spectrum of activity against common contaminating bacteria and seems to have minimal effects on the viability of mycobacteria (3, 4, 13). A study using clinical sputum samples (containing *M. tuberculosis*) and sputum samples artificially seeded with various mycobacteria reported that the CHX method performed better than other decontamination methods (10). However, clinical samples from CF patients with NTM organisms and, in particular, *M. abscessus* were not included in this study, and such samples are often heavily contaminated. We therefore compared the CHX method to the NALC-NaOH-OxA reference method to test sputum samples from CF patients.

The chlorhexidine method was performed as follows. Samples were incubated with an equal volume of 0.1% dithiothreitol (Sigma, France) and vortexed for 15 min at room temperature. Three volumes of 1% chlorhexidine digluconate (Sigma, France) were then added, and the mixture was vortexed for 15 min at room temperature. The samples were washed in phosphate-buffered saline (PBS) and centrifuged at 3,000 × *g* for 20 min at room temperature. The pellets were resuspended in 1 ml of PBS and cultured. The NALC-NaOH-OxA method was performed as described by Whittier et al. (17). Briefly, specimens were combined with an equal volume of 0.5% NALC-2% NaOH and vortexed for at least 15 min at room temperature. The specimens were centrifuged at 3,000 × *g* for 20 min at room temperature and washed with PBS and then mixed with an equal volume of 5% oxalic acid. This mixture was vortexed and allowed to incubate at room temperature for 30 min, with vortexing every 10 min. It was made up to 50 ml with PBS and centrifuged at 3,000 × *g* for 20 min. The pellet was resuspended in 1 ml of PBS, and aliquots were stained and cultured. Samples obtained by either method were cultured by inoculating four Löwenstein-Jensen (LJ) slants (Bio-Rad, Marnes la

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TABLE 1. Treatment of sputum samples artificially seeded with *M. abscessus* or *M. avium* by the NALC-NaOH-OxA or chlorhexidine method

Species and dilution	Result for smear	No. of CFU of *M. abscessus* or *M. avium*/ml detected by indicated decontamination method[a]	
		NALC-NaOH-OxA	Chlorhexidine
M. abscessus			
1/10	Positive	1.5×10^2	8×10^4
1/1000	Negative	10	10^3
1/10000	Negative	0	80
M. avium			
1/10	Positive	6×10^4	8×10^5
1/1000	Negative	5×10^2	4×10^4
1/10000	Negative	2.2×10^2	5×10^3

TABLE 2. Detection of mycobacteria by the CHX and NALC-NaOH-OxA methods based on the positivity of AFB smears

NTM (no. of samples)	No. of culture-positive samples detected by the indicated method					
	CHX only		NALC-NaOH-OxA only		Both CHX and NALC-NaOH-OxA	
	Smear positive	Smear negative	Smear positive	Smear negative	Smear positive	Smear negative
M. abscessus (43)	4	18	0	1	15	5
M. avium (3)	0	3	0	0	0	0
Other NTM (14)	0	8	0	5	0	1
All organisms	4	29	0	6	15	6

Coquette, France). Two LJ slants were incubated at 30°C and two at 37°C. The slants were examined twice weekly for 2 weeks and then weekly for a further 10 weeks. Acid-fast bacilli (AFB) smears were stained by the auramine-rhodamine method (Merck).

Preliminary experiments were performed with CF sputum samples negative for NTM and artificially seeded with *M. avium* Nck2133911 or *M. abscessus* Nck2136817 (Necker-Enfants Malades Microbiology Laboratory collection). Mycobacteria were collected from LJ slants and resuspended in PBS (MacFarland 1). The mycobacterial suspensions were serially diluted, and dilutions of 1/10, 1/1,000, and 1/10,000 (volume = 0.05 ml) were used to inoculate 1-ml aliquots of mycobacteria-free CF sputum samples. Each seeded sample was divided into two equal aliquots, one decontaminated by the CHX method and the other by the NALC-NaOH-OxA method. Decontaminated aliquots were cultured on LJ slants, and CFU were counted. The CHX method was clearly more sensitive for both organisms tested (Table 1). Samples seeded with the three dilutions of *M. avium* were all culture positive with both decontamination methods, but samples treated with the CHX method yielded between 10 and 100 times more CFU. The superiority of the CHX method was even more apparent with *M. abscessus*: samples inoculated with the 1/10,000 dilution were found to be culture positive by this method and culture negative by the NALC-NaOH-OxA method.

These preliminary results prompted us to compare the CHX and reference methods under conditions of clinical use. The study was performed between January 2004 and January 2005 at the microbiology laboratories of three French CF centers (center I, Necker-Enfants Malades hospital, Paris; center II, Trousseau hospital, Paris; center III, Bretonneau hospital, Tours). The three participating laboratories are experienced in culturing NTM for cases of CF and have collaborated in this field for several years. At each hospital, samples were sent to the microbiology laboratory and stored at −20°C. Within 2 weeks of collection, the samples were rapidly thawed, vortexed, and divided equally into two aliquots (1 to 2 ml in volume). The aliquots were then processed for AFB smears and NTM cultures as described above. NTM were subjected to species identification using the Accuprobe technique (Gen Probe) and *hsp65* sequencing (14). Fisher's exact test was used for statistical analysis.

A total of 827 sputum samples consecutively collected from 289 CF patients were studied. The patients were 149 males and 140 females, aged from 5 months to 42 years (mean age, 12.4 years). Samples were scored as contaminated if there was bacterial overgrowth of the four LJ slants: 166 (20%) specimens were contaminated after being tested by the CHX method, whereas 118 (14.2%) were contaminated after treatment with NALC-NaOH-OxA ($P = 0.0017$). A total of 55 samples were not interpretable with either method due to massively contaminated cultures. A total of 60 samples (from 32 patients) were found to be NTM positive by one or both decontamination methods. The recovered NTM were 43 (72%) *M. abscessus*, 7 (11%) *M. gordonae*, 3 (5%) *M. avium*, 3 (5%) *M. chelonae*, 1 *M. peregrinum*, 1 *M. immunogenum*, and 2 undefined rapidly growing mycobacteria. Table 2 shows the results obtained with the CHX and NALC-NaOH-OxA methods. Of the 60 positive samples, 33 were positive by the CHX method alone, 6 by the NALC-NaOH-OxA method, and 21 by both methods. Overall, samples treated with the CHX method yielded significantly more positive NTM cultures than those treated with the NALC-NaOH-OxA method (54/827 [6.50%] versus 27/827 [3.25%]; $P < 0.05$). The rate of false-negative results observed with the NALC-NaOH-OxA method was 55% overall (33/60 versus 6/60 [10%] with the CHX method; $P < 0.0001$) and exceeded 70% for smear-negative samples (29/41 versus only 6/41 [14.6%] for the CHX method; $P < 0.0001$). Twenty-two of the 43 samples (39.5%) positive for *M. abscessus* (from 17 patients) were positive by the CHX method alone (Table 2), and 18 (81.8%) of these were smear negative. All of the 19 AFB smear-positive samples evaluated during this study grew *M. abscessus* and all were found to be culture positive by the CHX method (versus 15/19 [78.9%] with the NALC-NaOH-OxA method). Finally, the three samples (from 3 patients) found to be positive for *M. avium* during this study, all smear-negative, were detected by the CHX method and scored negative by the NALC-NaOH-OxA method.

This study clearly shows that the CHX decontamination method is better than the NALC-NaOH-OxA method for the recovery of NTM in CF samples. This is probably because CHX better preserves the viability of mycobacteria, as has been suggested by several authors (3, 4, 10, 13). However, the rate of contamination is slightly higher with the CHX method

than with the reference method. Nevertheless, the advantages of the CHX method seem to outweigh the disadvantages, because more than half of the culture-positive samples appeared falsely negative by the NALC-NaOH-OxA method (versus only 10% with the CHX method). The superiority of the CHX method was also evident for the smear-negative samples, with the rate of false-negative samples exceeding 70% with the NALC-NaOH-OxA method (versus only 15% with the CHX method). Despite several attempts using various approaches (centrifugation-washing, neutralization with lecithin), we were not able to make the CHX method compatible with the Mgit system (Becton Dickinson, Le pont de Claix, France), which could be particularly valuable for the isolation of MAC. Indeed, chlorhexidine needs to be neutralized by an agent like lecithin (Sigma, St. Quentin Fallavier, France) to stop its action before Mgit flasks are inoculated (this step is unnecessary with Löwensten-Jensen medium, which contains high amounts of lecithin). Unfortunately, the addition of lecithin to Mgit medium generates nonspecific fluorescence, making the Mgit system useless. We are thus currently testing other neutralizing agents that would be compatible with the Mgit system.

Consistent with previous studies in pediatric CF centers (11, 16), most NTM-positive samples evaluated during our study were positive for *M. abscessus*. The number of MAC-positive samples was too small for meaningful statistical analysis of the benefits of the CHX decontamination method with MAC infections. It would be interesting to continue this evaluation with adult CF patients.

In conclusion, the CHX decontamination method is faster and easier to perform than the reference NALC-NaOH-OxA method, has a similar cost, and is much more sensitive for the recovery of *M. abscessus* from CF patients. The potential severity of *M. abscessus* infections in patients with CF, particularly in cases of lung transplantation (7, 9, 15), and the therapeutic difficulties caused by the frequent multiresistance of this species (5) have been described recently. Therefore, we recommend this method of decontamination when testing for NTM in children and adolescents with CF and in patients of any age with suspected *M. abscessus* infections.

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