JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1997, p. 2706–2708 Vol. 35, No. 10
0095-1137/97/$04.00+0

Proficiency Testing of Clinical Microbiology Laboratories Using Modified Decontamination Procedures for Detection of Nontuberculous Mycobacteria in Sputum Samples from Cystic Fibrosis Patients

SUSAN WHITTIER,[1,2]* KENNETH OLIVIER,[3] PETER GILLIGAN,[4,5] MICHAEL KNOWLES,[3] PHYLLIS DELLA-LATTA,[1,2] AND THE NONTUBERCULOUS MYCOBACTERIA IN CYSTIC FIBROSIS STUDY GROUP†

Clinical Microbiology Service, Columbia-Presbyterian Medical Center,[1] and Department of Pathology, College of Physicians & Surgeons,[2] New York, New York, and Departments of Medicine,[3] Microbiology-Immunology,[4] and Pathology,[5] University of North Carolina School of Medicine, Chapel Hill, North Carolina

Received 12 November 1996/Returned for modification 10 February 1997/Accepted 26 July 1997

An improved decontamination method has been demonstrated to reduce overgrowth of mycobacterial media by *Pseudomonas aeruginosa* and allow the successful recovery of nontuberculous mycobacteria (NTM) from cystic fibrosis (CF) patients. Twenty microbiology laboratories participating in a multicenter investigation designed to determine the significance of NTM in CF patients were required to demonstrate proficiency in the incorporation of this improved method; this was accomplished by successful decontamination and culture workup of a panel of simulated sputum samples seeded with *P. aeruginosa* and various NTM. All laboratories successfully recovered NTM from samples with acid-fast bacillus (AFB) smear scores of 3+/4+ (i.e., 2 to 18 or >18 organisms/field). Low-inoculum samples (1+/2+ AFB smears [2 to 18 organisms in 100 or 10 fields]) were problematic in that processed specimens were often smear and/or culture negative.

The microbiological flora of cystic fibrosis (CF) patients has been studied extensively (2, 3, 10). In recent years, the role of nontuberculous mycobacteria (NTM) in this patient population has been examined. Once considered to be quite rare, NTM are being recovered with increasing frequency from respiratory specimens from adolescent and adult CF patients (5, 8).

Unfortunately, many strains of *Pseudomonas aeruginosa*, an organism isolated from respiratory secretions of approximately 80% of CF patients, can hinder or even prevent the recovery of mycobacteria. These strains are able to survive routine sputum decontamination with NALC-NaOH (*N*-acetyl-L-cysteine–2% sodium hydroxide); therefore, improved decontamination techniques are necessary when processing respiratory specimens from CF patients colonized with *P. aeruginosa.*

The Centers for Disease Control lab manual for the isolation and identification of mycobacteria recommends that 5% oxalic acid be utilized for decontaminating patient specimens which contain *P. aeruginosa* (4). A recent report demonstrated this treatment alone was not sufficient; however, NALC-NaOH decontamination followed by treatment with oxalic acid dramatically reduced overgrowth with *P. aeruginosa* (11). It was recommended that this method of decontamination be utilized for all CF respiratory specimens being processed for mycobacterial cultures.

A multicenter investigation was initiated to examine the impact of NTM on the natural course of CF lung disease in the United States. Participating mycobacteriology laboratories were required to institute the NALC-NaOH–oxalic acid decontamination protocol for the processing of all CF respiratory specimens. In order to validate this new procedure, laboratories participated in a proficiency survey conducted by one of the coinvestigators. The results of that survey and discussion of this improved decontamination method are presented here.

A panel of five simulated sputum samples, prepared according to the New York State of Department of Health protocol for preparation of proficiency test specimens, was sent to each laboratory to assess the proficiency of isolation of NTM by the NALC-NaOH–oxalic acid decontamination procedure. Briefly, a 1% suspension of mucin (Sigma Chemical Co., St. Louis, Mo.) was prepared in 0.0005 M phosphate buffer (pH 6.0), the suspension was autoclaved for 30 min, and the pH of the suspension was adjusted to 7.0 with an aqueous solution of sodium phosphate. A nonmucoid strain of *P. aeruginosa*, isolated from a CF patient, was used to seed all samples at a concentration of 10^3 organisms/ml. Three of five samples were also seeded with one NTM, either *Mycobacterium chelonae*, *Mycobacterium avium* complex (MAC), or *Mycobacterium fortuitum*. A suspension (McFarland standard of 2) was prepared for each mycobacterium and then diluted 1:5 and 1:20 in order to achieve final acid-fast bacillus (AFB) smear results approximately equivalent to scores of 1+ to 2+ (1+/2+) and 3+ to 4+ (3+/4+), respectively, with scores assigned as follows: 1+, 2 to 18 organisms/100 fields; 2+, 2 to 18 organisms/10 fields; 3+, 2 to 18 organisms/field; 4+, >18 organisms/field (450× magnification). The panels, each with a final sample volume of 5 to 10 ml, were sent by overnight express. If participating laboratories were not able to process the specimens upon receipt, they were to be refrigerated for not more than 24 h before processing. Proficiency samples were incorporated into the routine work flow of the laboratory and decontaminated by the NALC-NaOH–oxalic acid method described previously (11).

A total of 20 microbiology laboratories received the first panel of five simulated sputum samples. An additional panel

* Corresponding author. Mailing address: Columbia-Presbyterian Medical Center, 622 W. 168th St., BHS-3-326, New York, NY 10032. Phone: (212) 305-6237. Fax: (212) 305-8971.

† Study group members are listed in the appendix.

TABLE 1. Results of phase 1 proficiency panel[a]

Sample no.	Expected results		No. of sites with correct results (%)	
	AFB smear	AFB culture	AFB smear	AFB culture
1	3+/4+	MAC	20 (100)	19[b] (95)
2	Negative	Negative	18[c] (90)	19[c] (95)
3	1+/2+	*M. chelonae*	10 (50)	11 (55)
4	Negative	Negative	20 (100)	20 (100)
5	3+/4+	*M. fortuitum*	18 (90)	20 (100)

[a] A total of 20 microbiology laboratories or sites.
[b] Misreported due to clerical error; correctly identified as MAC.
[c] Cross-contamination from adjacent specimen.

was prepared and processed in the mycobacteriology laboratory of Columbia-Presbyterian Medical Center to ensure specimen integrity; this panel was held at room temperature for 16 to 20 h to simulate shipping conditions.

The overall results of laboratory performance are summarized in Table 1. Samples 1 and 5 were seeded with sufficient mycobacteria to yield final AFB smear results of 3+/4+. Sample 3 was seeded to yield a final AFB smear result of 1+/2+. Samples 2 and 4 were not seeded (no mycobacteria).

It was unknown whether the low positivity for sample 3 was due to the initial low inoculum of the sample or whether *M. chelonae* was more susceptible to the decontamination process. Therefore, a second panel of samples was devised to examine the question regarding low-inoculum seeding. Eight laboratories received panels of three samples: one negative and two positive for *M. chelonae* (with final AFB smear scores of 1+/2+ and 3+/4+). Data are summarized in Table 2.

An additional panel consisting of similarly prepared MAC samples was processed by one laboratory on three separate occasions (data not shown). The 3+/4+ specimen was AFB smear and culture positive for all three panels. The 1+/2+ specimen was smear and culture positive for two of three panels.

When the effectiveness of enhanced decontamination was analyzed with respect to inhibition of bacterial overgrowth, it was found that only 6% of all samples were contaminated with *P. aeruginosa*. In addition, *P. aeruginosa* did not interfere with the isolation of NTM from any smear-positive specimens.

The issue of apparent increasing rates of NTM isolation in the CF population is confounded by the difficulties in determining the significance of such isolation. Distinguishing colonization of the respiratory tract from actual infection is necessary in order to make sound judgments regarding treatment decisions. This multicenter investigation was initiated to examine more closely the impact of NTM on the natural course of CF lung disease in the United States. In order to accomplish this, improved detection methods are necessary. Combining the NALC-NaOH decontamination method with oxalic acid treatment has been shown to greatly reduce bacterial overgrowth and improve recovery of mycobacteria from all AFB smear-positive specimens (11). Improved detection methods will advance clinical studies aimed at determining the significance of NTM isolation in CF patients.

TABLE 2. Results of phase 2 proficiency panel[a]

Sample no.	Expected results		No. of sites with correct results (%)	
	AFB smear	AFB culture	AFB smear	AFB culture
1	1+/2+	*M. chelonae*	8 (100)	6 (75)
2	Negative	Negative	8 (100)	8 (100)
3	3+/4+	*M. chelonae*	8 (100)	8 (100)

[a] A total of eight microbiology laboratories or sites.

This report describes the validation of laboratory techniques for the isolation of NTM from simulated CF sputum samples. In general, assimilation of the improved method was quite good. Contamination of media with *P. aeruginosa* was low (6%). A similar rate of 4% has been demonstrated during the ongoing clinical investigation, which includes more than 2,000 specimens (8a). Cross-contamination of specimens was shown to occur, as has been demonstrated in routine clinical laboratories (1, 13). This occurrence serves as a reminder that no single culture result can be used independently; multiple specimens are preferable and must be analyzed in conjunction with clinical signs and symptoms. Low-inoculum specimens (AFB smear scores of 1+/2+) were problematic in that they were often smear and/or culture negative. It has been shown that the NALC-NaOH decontamination procedure results in the killing of 28 to 33% of mycobacteria present in a clinical specimen (6). This kill rate is assuredly increased even more by the addition of the oxalic acid treatment. To date, no AFB smear-positive clinical specimens evaluated during the course of this multicenter study have failed to grow NTM; therefore, the organism load is high enough to survive the decontamination procedure. However, the successful recovery of mycobacteria from smear-negative specimens is inherently difficult to evaluate. We can assume that enhanced decontamination methods may prevent the recovery of such low-yield specimens. However, the alternative (less severe decontamination methods which have a high likelihood of bacterial overgrowth) is even less desirable. In addition, the variable acid-fastness of the rapidly growing mycobacteria is problematic when evaluating the efficacy of isolation techniques.

DNA and RNA probe technologies currently being developed promise to enhance the detection of NTM beyond culture. These assays will enable the detection of organisms directly from respiratory specimens, usually in less than 6 h, by a variety of direct amplification techniques. The commercially available direct amplification assays for *M. tuberculosis* complex are extremely sensitive and specific with AFB smear-positive specimens (7, 9, 12). Therefore, they can be of great value when evaluating CF patients in urban areas where tuberculosis is endemic who present with increased respiratory complaints, vague radiologic findings, and smear-positive specimens. The results of these molecular tests, when performed on multiple specimens, can be utilized with increasing confidence when deciding whether isolation is necessary and what treatment regimen to initiate.

Until similar probe techniques are available for the detection of NTM, efforts must be focused on improved isolation strategies. Decontamination with NALC-NaOH–oxalic acid has been shown to be very effective in decreasing bacterial overgrowth and can be readily incorporated into the routine clinical laboratory.

APPENDIX

The NTM in CF Study Group consists of John Besser-Weik, Minnesota State Department of Health, Minneapolis; Patricia Charache, Johns Hopkins Medical Center, Baltimore, Md.; Marie Coyle, Harborview Medical Center, Seattle, Wash.; Mary Jane Ferraro, Massachusetts General Hospital, Boston; Darla Gaskins, Shands Hospital, Gainesville, Fla.; Leonid

Heifits, National Jewish Center, Denver, Colo.; Roy Hopfer, University of North Carolina Hospitals, Chapel Hill; Sue Kehl, Childrens Hospital of Wisconsin, Milwaukee; Peggy Kitterman, Indiana University Medical Center, Indianapolis; Patty Newcomb-Gayman, Associated Regional and University Pathologists, Inc., Salt Lake City, Utah; Ernest Ng, Kaiser Regional Laboratory, Berkeley, Calif.; Carl Pierson, University of Michigan Hospitals, Ann Arbor; Sharon Reed, UCSD Medical Center, San Diego, Calif.; J. William Rourke, Oregon Health Sciences University, Portland; Frances Santorelli, Tulane University Medical Center, New Orleans, La.; Donald Stieritz, Hahnemann University Hospital, Philadelphia, Pa.; Rosemary Verrall, Vanderbilt Medical Center, Nashville, Tenn.; Robert Wadowsky, Childrens Hospital of Pittsburgh, Pittsburgh, Pa.; Tommy Williams, Childrens Memorial Hospital, Chicago, Ill.; and Mary York, UCSF Medical Center, San Francisco, Calif.

This work was supported by a grant from the Cystic Fibrosis Foundation.

REFERENCES

1. **Dunlap, N. E.** 1995. Laboratory contamination of *Mycobacterium tuberculosis* cultures. Am. J. Respir. Crit. Care Med. **152:**1702–1704.
2. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35–51.
3. **Govan, J. R. W., and J. W. Nelson.** 1993. Microbiology of cystic fibrosis lung infections: themes and issues. J. R. Soc. Med. Suppl. **86:**11–18.
4. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology: a guide for the level III laboratory. Centers for Disease Control, U.S. Department of Health and Human Services, Atlanta, Ga.
5. **Kilby, J. M., P. H. Gilligan, J. R. Yankaskas, E. Highsmith, Jr., L. J. Edwards, and M. R. Knowles.** 1992. Nontuberculosis mycobacteria in adult patients with cystic fibrosis. Chest **102:**70–75.
6. **Krasnow, I., and L. G. Wayne.** 1966. Sputum digestion. I. The mortality rate of tubercle bacilli in various digestion systems. Am. J. Clin. Pathol. **45:**352–355.
7. **Moore, D. F., and J. I. Curry.** 1995. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by Amplicor PCR. J. Clin. Microbiol. **33:**2686–2691.
8. **Olivier, K. N., P. H. Gilligan, J. R. Yankaskas, and M. R. Knowles.** 1992. Pulmonary nontuberculous mycobacteria in cystic fibrosis. Pediatr. Pulmonol. **S8:**116–117.

8a. **Olivier, K. N.** Personal communication.

9. **Pfyffer, G. E., P. Kissling, R. Wirth, and R. Weber.** 1994. Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by a target-amplified test system. J. Clin. Microbiol. **32:**918–923.
10. **Rosenfeld, M., and B. Ramsey.** 1992. Evolution of airway microbiology in the infant with cystic fibrosis: role of nonpseudomonal and pseudomonal pathogens. Semin. Respir. Infect. **7:**158–167.
11. **Whittier, S., R. L. Hopfer, M. R. Knowles, and P. H. Gilligan.** 1993. Improved recovery of mycobacteria from respiratory secretions of patients with cystic fibrosis. J. Clin. Microbiol. **31:**861–864.
12. **Whittier, S., E. Waithe, and P. Della-Latta.** 1995. Evaluation of Gen-Probe Amplified MTD and Amplicor (Roche) for the direct detection of *Mycobacterium tuberculosis* from respiratory specimens, abstr. C146, p. 26. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
13. **Wurtz, R., P. Demarais, W. Trainor, J. McAuley, F. Kocka, L. Mosher, and S. Dietrich.** 1996. Specimen contamination in mycobacteriology laboratory detected by pseudo-outbreak of multidrug-resistant tuberculosis: analysis by routine epidemiology and confirmation by molecular technique. J. Clin. Microbiol. **34:**1017–1019.