APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1994, p. 1021–1024
0099-2240/94/$04.00+0

Vol. 60, No. 3

Evaluation of Procedures for Isolation of Nontuberculous Mycobacteria from Soil and Water

T. KAMALA, C. N. PARAMASIVAN, DANIEL HERBERT. P. VENKATESAN AND R. PRABHAKAR*

Tuberculosis Research Centre, Indian Council of Medical Research, Madras, India

Received 30 July 1993/Accepted 29 December 1993

Six methods of decontamination each for the isolation of mycobacteria from soil and water were compared. On the basis of the results obtained. three of the six methods for soil and two of the six methods for water were further evaluated. For both soil and water samples, the method using 3% sodium lauryl sulfate in combination with 1% NaOH yielded more positives than the other methods.

Mycobacteria are characterized by their slow rate of growth and have longer generation times than many other bacteria (11). Thus, their isolation from environmental samples such as soil and water is more difficult because such samples are also rich in other microbial flora. Many methods have been reported by different workers for the isolation of mycobacteria from environmental samples such as soil and water (13). A nonstringent method used for isolation could result in overgrowth of contaminants, while too stringent a method could result in loss of mycobacteria. Hence, a suitable method would be one which results in a higher number of positive samples along with a higher number of strains from each sample compared with other methods However, there are not many reports which have compared and evaluated the different methods available (10).

In this study, some of the available methods for processing and decontamination of soil and water samples for the isolation of mycobactetia have been compared and evaluated on the basis of the net number of positive samples and the number of strains obtained by each method.

Inoculation of processed samples. For all of the methods. 10 μl of the processed soil or water sample was inoculated into each of four slopes of Lowenstein-Jensen (LJ) medium and four slopes of Falkinham's selective medium (FSM) (5). Two slopes of each medium were incubated at 30°C; the other two were incubated at 37°C. The slopes were read weekly for 8 weeks. Acid fastness of all positive cultures was confirmed by Ziehl-Neelsen staining. When mixed growth of acid-fast bacilli (AFB) as discrete colonies occurred, individual colonies were picked and subcultured on LJ medium to obtain pure cultures. Where confluent mixed growth was obtained. a portion of the growth was streaked onto a 7H11 agar plate to obtain pure cultures. Cultures were considered positive with contamination where the growth consisted of both AFB and non-AFB as determined by Ziehl-Neelsen and Gram staining. Such cultures were decontaminated with 1% cetrimide (7) in an effort to retrieve them as pure cultures.

Basis of comparison. The net number of positive samples and the number of strains isolated by each method from the total number of samples tested formed the basis for comparison. taking into account those cultures which were initially obtained as positive with contamination but which were retrieved as positive following treatment with 1% cetrimide (7). The number of strains isolated by each method was calculated on the basis of preliminary identification tests, which included smear by Ziehl-Neelsen staining, growth rate, colony morphology, and pigmentation. Kappa statistics, McNamer's test, and the χ^2 test were applied. wherever appropriate, for analysis.

Soil samples. Fifteen soil samples were collected for each of two sets of experiments from different sites within the Tuberculosis Research Centre campus, Madras. From each site, soil scraped up to a depth of 3 cm with a sterile trowel was collected into four sterile McCartney bottles (1). Soil samples each weighing 5 g, were suspended in duplicate in 20 ml of sterile 0.1% nutrient broth (Hi Media, Bombay, India), shaken manually for 60 s, and centrifuged at 1,000 rpm at room temperature for 10 min (Beckman model J-6B centrifuge). Portions of the supernatant were then processed by random allocation by Falkinham's method (1% NaOH, 2% NaOH, and 4% NaOH) (1), Engbaek's method (3% sodium lauryl sulfate [SLS], 1% NaOH), (2) and Gangadharam's method (1% cetrimide) (7). Two modifications of Falkinham's method (1) which employed 2% NaOH and 4% NaOH, respectively, were also tried. In addition, 4 g of soil, in duplicate, was taken and treated with 4% NaOH by the method of Reznikov and Leggo (4% NaOH) (12). The highest net number of positive samples and the highest number of strains were obtained with Engbaek's method on LJ medium at 30°C (12/15; 21 strains) (Table 1). This method also gave good results on LJ medium at 37°C (11/15; 18 strains). followed by the method of Reznikov and Leggo, on LJ medium at 30°C (11/15; 13 strains). As the methods of Engbaek and Reznikov and Leggo, both on LJ medium at 30°C gave good results for the net number of positive samples and the number of strains obtained, further evaluation of these two methods was conducted in a second set of experiments Though Faikinham's methods using 1% NaOH and 4% NaOH gave the same net number of positive samples on FSM at 37°C (10/15), the method using 4% NaOH was selected for further evaluation because it yielded more strains and fewer contaminations. even though the difference was not statistically significant ($P > 0.2$).

In the second set of experiments, the best results were obtained with Engbaek's method (15/15; 20 strains) as cornpared with Reznikov and Leggo's method (8/15: 8 strains) (Table 1), the difference being statistically significant (McNamer's $\chi^2 = 5.14$, $P = 0.03$). However, the comparison of Engbaek's method with Falkinham's method on FSM at 37°C showed that the difference between these two methods was not statistically significant (McNamer's $\chi^2 = 3.2$, $P = 0.07$).

Water samples. Thirteen samples of water were collected for the first set of experiments and 15 samples were collected for

* Corresponding author. Mailing address: Tuberculosis Research Centre, Indian Council of Medical Research, Chetput, Madras 600 031, India Phone: (91) 44 8265425. Fax: (91) 44 8262137.

TABLE 1. Culture results of 15 soil samples processed by different methods in experiments 1 and 2[a]

Method	Expt no..	Medium and temp. (°C)	No. of samples positive		No. of strains isolated
			Before cetrimide treatment	After cetrimide treatment	
Engbaek's method (3% SLS, 1% NaOH)	1	LJ 30	6	12	21
	2	LJ 30	15	15	20
	1	LJ 37	2	11	18
	1	FSM 30	4	4	4
	1	FSM 37	6	6	6
Reznikov and Leggo's method (4% NaOH)	1	LJ 30	8	11	13
	2	LJ 30	5	8	8
	1	LJ 37	7	10	9
	1	FSM 30	3	3	3
	1	FSM 37	3	4	4
Falkinham's method (4% NaOH)	1	LJ 30	1	1	1
	1	LJ 37	2	2	2
	1	FSM 30	4	7	7
	1	FSM 37	5	10	12
	2	FSM 37	4	10	12
Falkinham's method (1% NaOH)	1	LJ 30	0	0	0
	1	LJ 37	0	2	3
	1	FSM 30	3	4	5
	1	FSM 37	6	10	10
Falkinham's method (2% NaOH)	1	LJ 30	0	1	2
	1	LJ 37	0	1	1
	1	FSM 30	5	7	7
	1	FSM 37	5	9	9
Gangadharam's method (1% cetrimide)	1	LJ 30	3	6	7
	1	LJ 37	6	8	9
	1	FSM 30	0	0	0
	1	FSM 37	0	0	0

[a] 15 samples in each experiment.

TABLE 2. Culture results of water samples processed by different methods in experiments 1 and 2[a]

Method	Expt no.	Medium and temp. (°C)	No. of samples positive		No. of strain isolated
			Before cetrimide treatment	After cetrimide treatment	
Engel's method (3% SLS, 1% NaOH)		LJ 30	5	6	11
		LJ 37	7	7	14
	2	LJ 37	10	11	21
	1	FSM 30	0	0	0
	1	FSM 37	0	0	0
Falkinham's method (8% NaOH)	1	LJ30	5	6	7
	1	LJ 37	5	6	10
	2	LJ 37	10	11	13
	1	FSM 30	0	0	0
	1	FSM 37	0	0	0
Goslee and Wolinsky's method (4% H_2SO_4)	1	LJ 30	5	6	7
	1	LJ 37	6	6	9
	1	FSM 30	0	1	1
	1	FSM 37	0	1	1
Goslee and Wolinsky's method (4% NaOH)	1	LJ 30	3	4	4
	1	LJ 37	3	3	4
	1	FSM 30	0	1	1
	1	FSM 37	0	0	0
Goslee and Wolinsky's method (NaOH-NaOCl)		LJ 30	3	3	4
		LJ 37	1	2	3
		FSM 30	0	0	0
		FSM 37	0	0	0
Falkinham's method (4% NaOH)	1	LJ 30	2	2	2
		LJ 37	1	2	2
	1	FSM 30	0	2	2
	1	FSM 37	0	0	0

[a] 13 samples in experiment 1 and 15 samples in experiment 2.

the second set from taps, wells, and water coolers at different sites within the city of Madras. From each site, water was collected into sterile 20-oz (1 oz = 28.350 g) screw-capped glass bottle protected from light. All samples were processed within 3 h of the time of collection (4). Two aliquots of each sample were treated by Falkinham's method (4) using 3% NaOH and 8% NaOH, respectively. in place of the 2% NaOH originally used. In addition, two 100-ml aliquots of the sample were filtered with a 0.22-µm-pore-size membrane filter (Millipore Corp., Bedford, Mass.). The filters with the deposit were transferred to two sterile McCartney bottles containing 5 ml of sterile distilled water and 2 sterile class beads 5 mm in diameter and shaken on a mechanical shaker for 1 h. One of the suspensions was divided into three equal parts and randomly allocated for treatment by Goslee and Wolinsky's methods (6) employing NaOH-NaOCl, 4% NaOH, and 4% H_2SO_4 respectively. The second suspension was treated by Engel's method (3) using 3% SLS and 1% NaOH. The highest net number of positive samples and the highest number of strains were obtained by Engel's method on LJ medium at 37°C (7/13; 14 strains) (Table 2). Though Engel's method on LJ medium at 30°C and Falkinham's (8% NaOH) and Goslee and Wolinsky's (4% H_2SO_4) methods on LJ medium at 30°C and at 37°C yielded the same number of net positive samples (6/13), the number of strains was highest for Engel's method (11 strains), followed by Falkinham's method (10 strains) and Goslee and Wolinsky's method (9 strains). The Kappa test gave an almost perfect agreement between Engel's method and Goslee and Wolinsky's method (K = 0.84). However. on the basis of overall performance, Engel's method on LJ medium at 37°C and Falkinham's method (8% NaOH) on LJ medium at 37°C were selected for further evaluation in a second set of experiments.

In the second set of experiments, Engel's method and Falkinham's method gave similar results for the net number of positive samples (11/15). The Kappa test also gave a good agreement between the two methods ($\kappa = 0.67$). However, Engel's method proved to be better as it yielded more strains (21 strains) as compared with Falkinham's method (13 strains).

Mycobacterial isolates obtained from soil and water by the different methods. All of the strains obtained from soil and water in the second set of experiments were further identified to the species level with the help of numerical taxonomic methods (15), including the 19 tests described earlier (9). The results are presented in Table 3. Strains belonging to eight different species were obtained from soil. Engbaek's method yielded strains of seven species and the highest number of strains belonged to *M. fortuitum.* Reznikov and Leggo's method and Falkinham's method yielded strains belonging to five and few species, respectively, and did not yield any strains belonging to *M. chelonae* subsp. *chelonae, M. aurum,* or *M. thermoresistibile.* Strains isolated from water belonged to seven different species. and the highest number of isolates belonged to the MAIS *(M. avium-intracellulare-scrofulaceum)* complex. Engel's method yielded strains belonging to all of the seven species. while only three. namely, *M. diernhoferi, M. vaccae,* and the MAIS complex, were among the isolates obtained by Falkinham's method.

Of the methods for processing soil samples compared in the

TABLE 3. Species-level identification of the isolates obtained from soil and water In the second set of experiments

Species	No. of strains				
	Soil			Water	
	Engbaek's method	Falkinham's method	Reznikov and Leggo's method	Engel's method	Falkinham's method
MAIS complex	1	1	1	9	10
M. aurum	1	0	0	0	0
M. chelonae subsp. *chelonae*	2	0	0	0	0
M. diernhoferi	1	5	2	1	1
M. fortuitum	12	3	1	3	0
M. gadium	2	1	1	1	0
M. gastri	0	0	1	0	0
M. smegmatis	0	0	0	1	0
M. terrae	0	0	0	2	0
M. thermoresistibile	1	0	0	0	0
M. vaccae	0	0	0	4	1
Others	0	2	2	0	1
Total	20	12	8	21	13

present study. Engbaek's method (2) yieided the best results. namely, the net number of positive samples, the number of species and strains obtained. and the contamination rate. Falkinham's method (1) and Reznikov and Leggo's method (12) were originally developed by these workers for the isolation of mycobacteria from soil. particularly those belonging to the MAIS complex, In the present study, Reznikov and Leggo's method, with inoculation on LJ medium and incubation at 30°C, yielded negative results in 11 samples which were positive for mycobacteria by some of the other methods. It also yielded a consistently lower number of strains as compared with some of the other methods. Falkinham's method (1) gave very high rates of contamination. even with higher concentrations of NaOH when used on LJ medium. Though the contamination rate was lower when FSM (5) was used, the net yield was low. Gangadharam's method (7) originally used for sputum samples. performed poorly when used with FSM. On LJ medium, the number of strains obtained by this method was much lower than with the other methods.

For water sampler though Falkinham's method (4) and Engel's method (3) were equally good according to the number of positive samples. the latter method proved to be better according to the number of species and strains obtained and the contamination rate. Though the number of strains of mycobacteria obtained by the methods of Goslee and Wolinsky (6) was lower than that obtained by some of the other methods. the 4% H_2SO_4 method gave good results for the number of samples positive for mycobacteria

The results of this study indicate that 3% SLS used in combination with 1% NaOH is suitable as an effective decontaminating agent for the isolation of mycobacteria from soil and water in this region. Since the combination requires the use of only a lower concentration of NaOH, the bactericidal effect of NaOH on mycobacteria is probably reduced. Reports by Sula (14) and Langerova and Taquet (8) confirm this finding. This could be one of the reasons why more strains and more species of mycobacteria were obtained when NaOH in combination with SLS was used as in Engbaek's method for soil and in Engel's method for water than when NaOH alone was used as in the other methods. SLS, being a detergent. may also allow for easy homogenization of samples, thereby permitting effective decontamination even with lower concentrations of NaOH.

The present study also showed that treatment with 1% cetrimide is an effective procedure for retrieving pure cultures of mycobacteria from cultures initially positive with contamination.

The Tuberculosis Research Centre is one of the permanent Institutes of the Indian Council of Medical Research (ICMR), and T. Kamala is an ICMR junior research fellow.

REFERENCES

1. **Brooks, R. W, R. C. Parker, H. Gruft, and J. O. Falkinham III.** 1984. Epidemiology of infection by non-mycobacteria V. Numbers in eastern United States soils and correlation with soil characteristics. Am. Rev. Respir. Dis. **130:** 630-633.
2. **Engbaek, H. C. B. Vergmann and B. Weis Bentzon. 1967. The** sodium lauryl sulphate method in culturing sputum for mycobacteria. Scand. J. Respir. Dis. **48:** 268-284.
3. **Engel, H. W. B., L C. Bernald, and A. H. Havelaar.** 1980. The occurrence of *Mycobacterium kansasii* in tapwater. Tubercle **61:** 21-26.
4. **Falkinham, J. O., III, B. C. Parker. and H. Graft.** 1980. Epidemiology of infection by non-tuberculous mycobacteria. I. Geographic distribution in the eastern United States. Am Rev. Respir. Dis. **121:** 931-939.
5. **George, K. L., and J. O. Falkinham III.** 1986. Selective medium for the isolation and enumeration of *Mycobacterium avium-intracellulare* and *M. scrofulaceum.* Can. J. Microbiol. **32:** 10-14.
6. **Goslee. S, and E. Wolinsky.** 1976. Water as a source of potentially pathogenic mycobacteria. Am Rev. Respir. Dis. **113:** 287-292.
7. **Joseph, S., N. G. K. Nair, and P. R J. Gangadharam.** 1969. **A** sputum swab culture method for tubercle bacilli using cetrimide compared with two other swab culture methods and the concentration culture method. Tubercle **50:** 299-303.
8. **Langerova, M, and A. Taquet** 1968. Comparaison de differentes methodes d'homogeneisation et de purification des produits pathologiques en fonction de milieux de culture solides ou liquides. Bull. W.H.O. **39:** 663-680.
9. **Paramasivan, C. N., D. Govindan, R. Prabhakar, P. R Somasundaram, S. Subbammal, and S. P. Tripathy.** 1985. Species level identification of nontuberculous mycobacteria from South Indian BCG trial area during 1981. Tubercle **66:** 9-15.
10. **Portaels, F., A De Muynck, and M. P. Sylla.** 1988. Selective isolation of mycobacteria from soil: a statistical analysis approach. J. Gen. Microbiol. **134:** 849-855.
11. Ratledge, C. 1983. Nutrition, growth and metabolism, p. 186-271. In C. Ratledge and J. Stanford (ed.). The biology of the mycobac-

teria. vol. 1. Academic Press. London.
12. Reznikov, M., and J. H. Leggo. 1971. Examination of soil in the Brisbane area for organisms of the *Mycobacterium avium-intracellulare-scrofulaceum* complex. Pathology **6:** 269-273.
13. Songer, J. G. 1981. Methods for selective isolation of mycobacteria from the environment. Can. J. Microbiol. **27:** 1-7.
14. Sula, S. 1968. Comparative trials with different decontaminating agents for growing *Mycobaterium tuberculosis* from sputum specimens. Bull. W.H.O. **39:** 647-655.
15. Tsukamura, M. 1984. Identification of mycobacteria. Mycobacterioses Research Laboratory of the National Chubu Hospital, Aichi, Japan.