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Improved Technique for Isolation of *Mycobacterium kansasii* from Water

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A new concentration procedure, together with a new isolation medium, resulted in a 10- to 100-fold increase in the possibility of identifying *Mycobacterium kansasii* from water samples in comparison to a previously used procedure. In a survey which included both potable and natural water samples from many sites within the state of Texas, nine isolations of high-catalase strains of *M. kansasii* were obtained from 232 water samples tested. Acid-fast smear results were compared with mycobacterial isolations. An isolate from a river in central Texas is the first high-catalase strain of *M. kansasii* encountered in a natural water supply. Surveys of water samples from two Texas towns indicate that chlorine levels may influence the numbers of recoverable mycobacteria in water supplies.

Mycobacterium kansasii produces pulmonary and extrapulmonary infections in both humans and animals (2). The disease has not been demonstrated to be communicable, but the environment appears to be the source of the infection (18). Many surveys have been conducted to ascertain data on the presence of mycobacteria in the environment (1, 4, 5, 8, 14, 15), but there have been only a few reported isolations of the potentially pathogenic, high-catalase strains of *M. kansasii* from the environment (1, 10, 11, 14). The frequent encounters of the various *Mycobacterium* species in the above studies and yet the limited success in the isolation of *M. kansasii* from environmental samples prompted an attempt to improve techniques that have been employed. Attention was focused on water since it appears to be a common source of mycobacteria (1, 4, 5, 8, 10, 14, 15).

MATERIALS AND METHODS

Experimental phase: media. (i) Middlebrook 7H-10 medium. Middlebrook 7H-10 medium (12) was prepared by adding 20 g of Middlebrook 7H-10 Agar Special (Difco) to 1,000 ml of cold distilled water which contained 0.5% glycerol. The mixture was sterilized at 121°C for 15 min and allowed to cool to 56°C in a water bath, and 100 ml of oleic acid-albumin-dextrose-catalase enrichment (Difco) was added. The ingredients were mixed gently to avoid formation of bubbles, and approximately 30 ml was poured into each sterile petri dish (100 by 15 mm). The final pH of the medium was 6.8 ± 0.1. The medium was stored in the dark at 4°C and was used within 14 days of preparation.

(ii) Decreased enrichment (DE) 7H-10 medium. One-liter amounts of Middlebrook 7H-10 Agar Special were prepared as described above, except only 5 ml of oleic acid-albumin-dextrose-catalase enrichment was added. Petri dishes were poured, stored, and used in the same manner as the Middlebrook 7H-10 medium. The final pH of the medium was 6.8 ± 0.1.

(iii) Lowenstein-Jensen medium. Lowenstein-Jensen medium, prepared as described by Vestal (16), was used for maintenance of stock strains and for study of single-colony picks from agar media.

Experimental phase: procedure. (i) Evaluation of DE 7H-10 medium. Suspensions of a variety of *Mycobacterium* species, including known strains of *M. kansasii*, *M. gordonae*, *M. avium* complex, *M. fortuitum*, and *M. chelonei* (obtained from Centers for Disease Control, Atlanta, Ga.), were prepared in 3-ml volumes of sterile physiological saline to a density that would yield individual colonies on media tested. Petri dishes of DE 7H-10 medium and Middlebrook 7H-10 medium were inoculated with equal volumes of suspensions prepared of each organism listed above and were incubated at 35°C with 5 to 10% carbon dioxide in air. Plates were examined for growth at 7, 10, 14, and 21 days, and descriptions of colonial morphologies were recorded.

(ii) Test procedure. In the filtration-scrub-sedimentation (FSS) test procedure, a 4-liter sample of sterile distilled water was seeded with 1.5×10^5 viable units of *M. kansasii*. The entire sample was passed through a membrane filter (type HA, 0.45-μm pore size; Millipore Corp., Bedford, Mass.). The filter was aseptically removed and placed in a sterile petri dish (60 by 15 mm). Approximately 0.5 ml of sterile distilled water was added to the filter, and the surface was abraded vigorously with a sterile capillary pipette which had been heated in a burner flame and bent to a 90° angle approximately 1 in. (ca. 2.5 cm) from the tip (16). The water with the dislodged material was then removed from the petri dish with another sterile capillary pipette and placed in a sterile screw-capped tube (18 by 125 mm). After allowing the heavier particles to settle for 15 min, part of the supernatant was removed with a sterile capillary pipette and discarded.

A drop of the remaining sediment was removed with a sterile capillary pipette and placed on a microscope slide (3 by 1 in.; ca. 7.5 by 2.5 cm). The same capillary pipette was used to spread the material on the slide, forming a smear which was approximately 1 cm square. The smear was air dried, heat fixed at 65°C for 15 min, and examined by the Truant procedure (16). Approximately 0.5 ml of 4% sodium hydroxide, or an amount equal to the specimen sediment, was added to the sediment in the screw-capped tube (16). The mixture was agitated for 15 to 20 s on a Vortex mixer and then allowed to stand for 10 min to aid in decontamination. The treated specimen was neutralized with 1 N hydrochloric acid containing 0.4% phenol red and back-titrated to a light pink color (slightly alkaline pH) with 0.1 N sodium hydroxide. The treated-neutralized sediment was then inoculated onto DE 7H-10 medium.

(iii) Spread-plate technique. The bent capillary pipette described earlier was used to spread the inoculum evenly on one-half of the surface of the DE 7H-10 medium, and then the spreader was lifted from the surface of the medium and without decontamination was used to spread the other half of the surface of the plate.

(iv) Comparative studies. Four liters of sterile distilled water was seeded with 4.3×10^9 viable units of *M. kansasii* as a control sample. Four other samples were tested in an effort to properly evaluate the procedure and the DE 7H-10 medium (Table 1). Sterile flasks were used to collect two 4-liter samples, A and B, from the taps at the Texas Department of Health, after the taps were first flushed for 15 to 20 s. Samples C and D, 2 liters each, came from previously positive sites in Texas (14).

Each of the five samples described above was divided equally. One portion of the sample was processed by the filtration-agitation-centrifugation method (14), and the other portion of each sample was processed by the FSS method described above. One-half of each treated sediment from each method was inoculated onto DE 7H-10 medium plates, and the spread-plate technique was used to disperse the inoculum. The other half of each treated sediment was serially diluted (10-fold) and inoculated onto Middlebrook 7H- medium plates for total counts. All media were incubated at 35°C with 5 to 10% carbon dioxide in air and were examined for growth with a dissecting microscope at 7, 10, 14, and 21 days. Total counts and descriptions of colony morphologies were recorded (Table 1). Representative colony types were subcultured to L-J medium and were identified by standard procedures (16).

TABLE 1. *Tabulation of comparison of methods*

Sample[a]	Total colony counts		FSS/FAC ratio
	FAC[b] method	FSS method	
Control	2.8×10^4	3.0×10^7	1,071:1
A	2.0×10^3	5.0×10^4	25:1
B	3.0×10^3	3.5×10^4	12:1
C	1.8×10^4	1.0×10^5	5:1
D	4.1×10^3	1.5×10^5	37:1

[a] Control: 4 liters of sterile distilled water seeded with 4.3×10^9 viable *M. kansasii* cells; A and B, 4-liter samples of tap water from the Texas Department of Health; C and D, 2-liter water samples from previously positive sites in Texas.

[b] Filtration-agitation-centrifugation method.

Practical application phase. (i) Water samples tested. The pertinent data on samples are contained in Table 2.

(ii) Procedure. All natural water samples were allowed to stand for 24 h at room temperature in the 1-gallon (ca. 1.9-liter) polyethylene bottles in which each sample was received, to permit the sedimentation of extraneous materials which might be present. The supernatant fluids were decanted into sterile containers without disturbing the settled material and were then processed in the same manner as potable water samples. The sediments were discarded. All samples were processed by the FSS method, and the inocula were dispersed evenly on DE 7H-10 medium plates by using the spread-plate technique. All inoculated plates were incubated and read, and representative colonies were subcultured as described above.

RESULTS

Experimental phase. (i) Evaluation of DE 7H-10 medium. As shown in Fig. 1, *M. kansasii* colonies on Middlebrook 7H-10 medium ranged in size from 1.9 to 2.1 mm at 14 days, but on DE 7H-10 medium the range was from 0.8 to 0.9 mm. It was noted that *M. gordonae* produced two types of colonial morphology, smooth and rough. Since the smooth colonial forms of *M. gordonae* were not confused with *M. kansasii* colonies, no further studies were performed, and attention was focused on the rough forms. On Middlebrook 7H-10 medium the size of the rough colonies of *M. gordonae* was approximately 2.0 mm in diameter, but on DE 7H-10 medium they were approximately 0.6 mm. Colonies of *M. kansasii* had more distinct, dark centers and were more regular in shape than the colonies of *M. gordonae*. The colony size of *M. avium* complex, a frequent isolate from water (5, 8), was restricted on DE 7H-10 medium, but *M. fortuitum* and *M. chelonei* colony size and morphology were not altered.

(ii) Comparative studies. Initial studies using the FSS method developed in this paper showed recovery of 1.4×10^4 cells of the 1.5×10^5 cells seeded in the water sample, a recovery rate of 9.3%. When the two methods were compared for isolating acid-fast bacilli from tap water, the FSS procedure was more efficient (Table 1). The seeded water sample gave a 1,000-fold difference in isolation rates (3.0×10^7 versus 2.8×10^4), but the average ratio of recovery by FSS versus the filtration-agitation-centrifugation method was 20:1. It was noted that even though there was a wide range in the ratios, the FSS

TABLE 2. *Water sample information*

Type of water sample	Source	Type of water	Collection technique	Volume	Chlorine level	Storage temp (°C)	Time from collection until sample processed (days)
For chemical analysis	Lakes, streams, bayous, and city water supplies	Natural and potable	Not aseptic	2 to 3 liters	Not done	22–27	<30
For fecal coliforms	Lakes and streams	Natural	Aseptic	100 ml	Not done	<10	<14
For coliforms	Private water systems	Potable	Aseptic	100 ml	Not done	<10	<14
From central Texas town survey	Public water system	Potable	Aseptic	3 to 4 liters	DPD[a]	22–27	<1
From east Texas town survey	Public water system	Potable	Aseptic	3 to 4 liters	DPD[a]	22–27	<1
From central Texas lake survey	Surface lake water	Natural	Not aseptic	3 to 4 liters	Not done	22–27	<1

[a] Calculated by DPD colorimetric method (13).

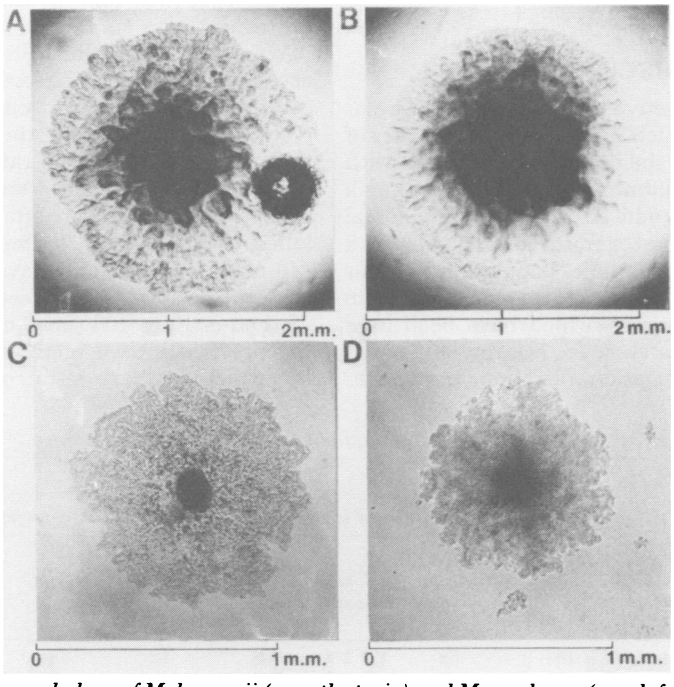

FIG. 1. *Colonial morphology of M. kansasii (smooth strain) and M. gordonae (rough form). (A) M. kansasii on Middlebrook 7H-10 medium at 14 days (×32); (B) M. gordonae on Middlebrook 7H-10 medium at 14 days (×32); (C) M. kansasii on DE 7H-10 medium at 14 days (×50); (D) M. gordonae on DE 7H-10 medium at 14 days (×50).*

method consistently gave higher yields of mycobacteria. In addition, high-catalase strains of *M. kansasii* were isolated from the two samples which had been previously positive (14).

Practical application phase. (i) Water samples tested. As can be seen in Table 3, of 83 total samples with positive smears, *Mycobacterium* spp. were isolated from 70 samples, or 84%. In contrast, of 123 samples tested which had negative smears, only 25, or 20%, had posi-

tive cultures. A similar correlation can be seen between positive smears and the isolations of potentially pathogenic, high-catalase strains of *M. kansasii*. Eight of nine isolations of *M. kansasii* were made from samples with positive smears. Note that the *M. kansasii* isolation data are included in the *Mycobacterium* sp. column of Table 3. Only 26 of the water samples tested were unsatisfactory due to overgrowth with non-acid-fast bacteria or fungi.

Detailed data on the chlorine levels, smears, and culture results of the samples from the central Texas and east Texas towns are presented, respectively, in Tables 4 and 5. The chlorine levels of the water samples from the central Texas town ranged from 0.0 to 2.6 mg/l. Acid-fast bacilli were observed in the smears of four samples, and high-catalase strains of *M. kansasii* were isolated from three samples. The five water samples from the east Texas town, shown in Table 5, had chlorine levels which ranged from 0.0 to 2.5 mg/l. Although three positive smears were found, only one isolation of *M. kansasii* was made.

DISCUSSION

The initial objective was to develop an isolation procedure which would (i) allow recovery of a high number of the organism sought, (ii) with an acceptable contamination rate (17), (iii) on a medium which produced a distinctive colonial morphology of the organism sought even in a large and diverse microbial population. Various methods for the isolation of mycobacteria from environmental sources, which have been used with some success (1, 8, 10, 14), rely on combinations of centrifugation, filtration, and chemical treatment. We found membrane filtration to be more efficient than centrifugation for the removal of mycobacteria from water samples. Once the organisms were trapped on the membrane filter, studies revealed that physically rubbing the surface of the filter removed more of the organisms than simply agitating the filter in saline. Even potable water samples contain numerous microorganisms, so a method of decontamination was required which allowed rapid killing of non-acid-fast bacteria and fungi with minimal effects on mycobacteria. The desire to use a transparent agar-based isolation medium, which enhanced the possibility of recognizing various colony types, eliminated the use of quaternary ammonium compounds as a decontamination agent. Mycobacteria are more resistant to extreme pH changes than most microorganisms (7), so the 4% sodium hydroxide method was employed. Additionally, the use of the time-limited sodium hydroxide procedure, as opposed to a dilution method, maintained the low volume of the specimen and eliminated the need for centrifugation.

We realize that the FSS method does not accomplish the goal of recovery of a high percentage of the organism sought (only 1 to 10% recovery rate). Some of the possible explanations for the low recovery rate include: (i) many of the trapped organisms were not removed from the filter, (ii) a high percentage of the cells were killed by the sodium hydroxide treatment, or (iii) numerous cells may have been discarded in the supernatant after sedimentation. This method is 10 to 100 times more sensitive than the previously used method (14), and both of these methods have resulted in the isolation of

TABLE 3. *Correlation of smear-culture results*

Type of water sample	No. of samples tested	Isolation results						
		Positive smears			Negative smears			
		Mycobacterium spp.[a]	*M. kansasii*[b]	Negative	*Mycobacterium* spp[a]	*M. kansasii*[b]	Negative	Contaminated
For chemical analysis	163	58	2	5	12	0	66	22
For fecal coliforms	29	4	1	7	4	0	14	0
For coliforms	11	2	2[c]	0	4	0	2	3
From central Texas town survey	14	3	2	0	4	1	6	1
From east Texas town survey	5	2	1	1	0	0	2	0
From central Texas lake survey	10	1	0	0	1	0	8	0

[a] Any *Mycobacterium* species, including *M. kansasii, M. gordonae, M. avium* complex, and *M. fortuitum*.
[b] High-catalase strains.
[c] One isolate was identified as "most closely resembles *M. kansasii*; nitrate reduction test negative."

TABLE 4. *Water samples from a central Texas town*

Water sample no.	Total chlorine level (mg/liter)	Positive smear results	Isolation results				
			Negative	*M. gordonae*	*M. kansasii*[a]	*M. avium* complex	Contaminated
1	2.6		+				
2	1.4			+			
3	1.4			+			
4	0.8	+		+	+		
5	1.4		+				
6	0.0					+	
7	1.6	+					+
8	1.2		+				
10	2.2		+				
11	0.9	+		+			
13	0.2		+				
16	0.2			+	+		
17	0.2	+		+	+		
19	1.2		+				

[a] High-catalase strains.

TABLE 5. *Water samples from an east Texas town*

Water sample no.	Total chlorine level (mg/liter)	Positive smear results	Isolation results			
			Negative	*M. gordonae*	*M. kansasii*[a]	*M. avium* complex
1	2.5		+			
2	2.0	+		+		+
3	0.0[b]			+		
4	0.8	+		+	+	
5	0.0[c]	+	+			

[a] High-catalase strain.
[b] Nonchlorinated water prior to entry into the water distribution system.
[c] Lake water; partial source of town's water supply.

high-catalase strains of *M. kansasii* from water. Further studies are aimed at determining the source of the lost organisms so that a more sensitive test can be developed which will result in an even higher recovery rate of *M. kansasii* from water sources.

In the search for a more selective isolation medium, an attempt was made to take advantage of the nitrate-reducing capability of *M. kansasii* by using yeast carbon base (Difco) with nitrate as the nitrogen source; although *M. gordonae* was inhibited on this medium and *M. kansasii* grew, colonies of the latter were too small (microscopic) to be helpful. Several enrichment-deficient formulations of Middlebrook 7H-10 medium also were examined. One-liter amounts of 7H-10 basal medium were supplemented by the addition of 0, 5, 10, 25, 50, 75, or 100 ml of oleic acid-albumin-dextrose-catalase enrichment (Difco). Results revealed that only the addition of 5 ml of this enrichment (DE 7H-10) provided sufficient differences in the colonies of *M. kansasii* and *M. gordonae* to be of differential value (see Fig. 1).

The Texas Department of Health, Bureau of Laboratories, daily receives water samples for chemical and bacteriological analyses from different areas of the state of Texas. These water samples provided the opportunity to evaluate the newly developed procedure and medium and in addition to gather data on the presence of high-catalase strains of *M. kansasii* in specific water supplies. The initial phase of the survey was simply to determine the feasibility of testing large numbers of water samples. The water samples for chemical analysis were readily available. Even though these samples were not collected aseptically and were not processed immediately after collection, the 163 samples did allow volume testing with only minor difficulties. Many of these samples were of natural water, and yet only 22 out of 163, or 13.5%, were contaminated with non-acid-fast bacteria and fungi. In view of the source and the age of the samples, this confirmed that the decontamination process was adequate. Additionally, the 63 positive smears from 163 samples, or 39%, was indicative of the effectiveness of the smear technique. Table 3

shows the significance of the positive smear correlation to positive cultures. *Mycobacterium* species were isolated from 58 of the 63 samples (92%) with positive smears, and the two isolations of *M. kansasii* were from positive smear samples. A possible explanation for the low percentage of *M. kansasii* isolations (1.2%) may be the fact that a large number of the water samples taken for chemical analysis were natural waters. The probability of isolation is possibly decreased because of the dilution of the organisms in the lakes or streams. Another possible explanation may be that the organism died during the storage time. Two different types of studies demonstrate the effects of time on survival of *M. kansasii*. Joynson reported isolation of a constant number of colony-forming units from seeded waters after 12 months (6), yet the studies of Kubica and Kim indicate an 82% loss in viability in growth medium after 19 days (9).

In the next phase, water samples taken for fecal coliform and for coliform testing were surveyed. Even though there was poor correlation between positive smears and positive cultures for *Mycobacterium* species, all three of the isolations of *M. kansasii* were made from samples with positive smears. The single isolation of *M. kansasii* from the water study for fecal coliforms was from a river in central Texas. This is the first recorded isolation of a high-catalase strain of *M. kansasii* from a natural water supply. Data were not sufficient to establish any correlation between the number of coliform organisms in the water samples and the single isolation of *M. kansasii*.

Sufficient data had been generated from these preliminary studies to adequately document the value of this procedure and the isolation medium. A survey was conducted with stringent controls on the collection, transportation, and rapid testing of samples. Consideration was given to locations for survey. Since a central Texas town had yielded isolations of *M. kansasii* (14), it was selected as a primary site. Studies of water samples submitted from an east Texas town for chemical analysis resulted in the isolation of *M. kansasii*, so a limited survey was made using water samples from representative sites in the community. Finally, in an effort to learn more about this organism's habitat, studies of water from a central Texas lake were included. This is a man-made lake on the river from which an isolation of *M. kansasii* was made during the survey of water for fecal coliforms.

Table 4 contains the results of the repeat survey of the central Texas town. Only 14 samples were processed, but each was from a site which had been tested earlier (14). All chlorine levels were higher than in the previous study, with the exception of site no. 6, which remained at 0.0 mg/liter. The number of samples from which mycobacteria could be cultured decreased from 13 to 7. Two possible explanations could account for the reduction in positive cultures: (i) the increased chlorine levels and (ii) the different seasons of the year in which the surveys were made. No data are available on chlorine sensitivity for *M. kansasii*. Samples 4, 16, and 17, which had been positive for high-catalase strains of *M. kansasii* in the first survey, were again positive. All three sites were dead-end mains, thus adding support to the theory that dead-end mains may serve as reservoirs for *M. kansasii* (14). Engel et al. from an independent study have proposed a similar hypothesis, that *M. kansasii* colonizes in stagnant water lines (3). Indications are that samples with chlorine levels less than 1.0 mg/liter may have a higher probability of mycobacterial isolation, especially *M. kansasii*.

Results from the survey of the east Texas town, seen in Table 5, are very similar. Sample 4 was collected from a dead-end main, had a chlorine level less than 1.0 mg/liter, and contained high-catalase strains of *M. kansasii*. Only *M. gordonae* was isolated from sample 3, even though it contained no chlorine. This sample was tested to determine the possible existence of *M. kansasii* in the water from a deep well before it entered the water distribution system. The last sample, no. 5, was included because the lake is a source of water for the town.

The data from the survey of the central Texas lake are contained only in Table 3. A separate tabulation was not included because chlorine levels were not determined on natural waters and no *M. kansasii* was found. It may be concluded from this survey of the lake that *M. kansasii* may exist and multiply in natural waters, but the dilution factor of the lakes and streams greatly decreases the probability of recovery of the organism. Additional studies during other seasons of the year are to be performed which will generate data on the effects of temperature on the multiplication of mycobacteria in water supplies.

The investigations during the various phases of this survey have shown that large numbers of water samples, both potable and natural, can be properly processed by the method described in this paper, with successful isolation of high-catalase strains of *M. kansasii*. Also, DE 7H-10 medium, when combined with the spread-plate technique, is a valuable tool in the isolation process. Finally, the repeat isolations of *M. kansasii* from previously positive sites plus the ad-

ditional isolation from another town continue to add support to the dead-end main theory. Continued studies over an extended period of time from additional locations will be necessary to document these findings further.

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