Effect of Relative Centrifugal Force and Centrifugation Time on Sedimentation of Mycobacteria in Clinical Specimens

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Optimum relative centrifugal force (RCF) and centrifugation time to concentrate mycobacteria in clinical specimens were determined by processing split samples of sputa and urines containing mycobacteria with combinations of different RCFs and centrifugation times. Although individual test results showed considerable variation in the recovery rates of mycobacteria in the sediment, the data indicated that higher recovery rates occurred as centrifugation speed and time were increased. With a 15- to 20-min centrifugation time, on the average, 67 to 71% of mycobacteria were recovered at an RCF of 2,074 × g, and 76 to 80% were recovered at 3,005 or 3,895 × g at maximum radius. The remainder of mycobacteria was mostly recovered from the supernatant, but culturing of supernatant was not profitable. Increasing RCF had a negligible effect on acid-fast bacillus smear sensitivity. The smear sensitivity for about 25,000 clinical specimens processed with an RCF of 3,800 × g for 20 min was 71% compared with 69% as determined for over 30,000 specimens processed in a similar manner but with an RCF of 2,000 × g. An RCF of 3,000 × g applied for 15 min, or an RCF of about 2,000 to 2,500 × g applied for 20 min, is considered adequate to concentrate mycobacteria in clinical specimens.

Concentration of acid-fast bacilli (AFB) in clinical specimens is an important step in the laboratory diagnosis of tuberculosis and other mycobacterial diseases. A relative centrifugal force (RCF) of 1,800 to 2,400 × g and a centrifugation time of 15 to 30 min have been recommended for recovering mycobacteria (5, 7, 11). A recent report concluded that AFB smear culture correlation could be increased from 25 to 82% as the RCF used for processing specimens increased from 1,260 to 3,800 × g; the use of increased RCF also resulted in an increase in the number of positive cultures (4). This report has attracted considerable attention (7, 8) and also raised concern, because most diagnostic laboratories are not equipped with centrifuges that provide a force of 3,800 × g. In contrast, an AFB smear sensitivity of 60 to 78% has been reported for an RCF not exceeding 2,400 × g for concentrating specimens (3, 6, 9, 10).

Mycobacteria have a low specific gravity and may remain buoyant during centrifugation (7). The importance of examining the supernatant fluid for mycobacteria has been stressed in the past (1, 7). However, the advantage of this procedure in routine practice has not been established (7).

This report describes a study which was carried out to determine the effects of RCF and centrifugation time on the concentration of mycobacteria in clinical specimens, the value of culturing supernatant, and the effect of increasing RCF on AFB culture-smear correlations.

MATERIALS AND METHODS

Test organisms. Strains of *Mycobacterium tuberculosis* (six strains), *M. fortuitum*, *M. chelonae*, and *M. avium* complex (three strains each) were obtained from the Canadian National Reference Centre for Tuberculosis, Ottawa, Canada, or were primary isolates from clinical specimens received at the Provincial Public Health Laboratories, St. John's, Canada. Cultures were maintained on Lowenstein-Jensen (LJ) slants (Difco Laboratories, Detroit, Mich.) at

35°C under 5 to 10% CO_2. Growth was harvested during the exponential growth phase and ground in Dubos broth containing Tween 80 (BBL Microbiology Systems, Cockeysville, Md.) with a tissue homogenizer. The suspension was centrifuged at 800 × g for 10 min to remove clumps. The supernatant was mixed with fresh Dubos broth and recentrifuged at 1,200 × g for 10 min. The density of the supernatant was adjusted to a 0.5 McFarland standard. The suspension contained approximately 90% single cells. Whenever possible, the inoculum was initially adjusted to yield about 30 to 100 CFU when 100 μl of seeded specimens was cultured following various treatment procedures.

Specimens. Sputa and urines containing *M. tuberculosis* were obtained from known cases of tuberculosis. For experiments in which seeded specimens were used, AFB smear-negative sputa and urines were randomly picked from routine submissions. Sputa were liquefied by mixing on the vortex with a small volume of N-acetyl-L-cysteine (NALC) digestant (Sigma Chemical Co., St. Louis, Mo.) without NaOH; the pHs of sputum and urine specimens were adjusted to 7.0. These were used either as such or after autoclaving to seed the test organisms.

Digestion-decontamination procedure. Where indicated, the NALC-NaOH digestion-decontamination procedure (2, 11) was used. The final concentration of NaOH in all instances was 1%. NALC digestant without NaOH was prepared by dissolving 0.5 g of NALC in 50 ml of 2.9% sodium citrate and 50 ml of M/15 phosphate buffer (PB), and the pH was adjusted to 7.0.

Centrifugation. A Beckman Instruments, Inc. (Fullerton, Calif.), J-6B refrigerated centrifuge fitted with a Beckman rotor model JS4.2 with a 24-place adapter configuration was used throughout the study. Falcon 50-ml polypropylene conical tubes (Becton Dickinson Labware, Oxnard, Calif.) were used for all centrifugations. RCFs were determined at maximum radius of 254 mm.

Experimental design and test procedures. All reagents and supplies were brought to room temperature (24 to 25°C), and all procedures, including centrifugation, were performed at

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this temperature. Split samples of sterilized sputa and urines were distributed into sets of nine centrifuge tubes (9 ml per tube), and each tube was seeded with 1.0 ml of a mycobacterial suspension adjusted to contain approximately 2×10^3 CFU/ml. NALC digestant without NaOH was added in 10.0-ml quantities and then mixed on the vortex for 15 s. M/15 PB (pH 7.0) was then added to the 40-ml mark, and the contents were thoroughly mixed. Each set of specimens was centrifuged at 2,074, 3,005, or 3,895 × g for 10, 15, or 20 min. The supernatant was poured off, and the sediments were suspended in 2.0 ml of PB (pH 7.0) containing 0.2% bovine serum albumin and thoroughly mixed on the vortex. Four LJ slants were inoculated with 100 μl of the sediment each and incubated at 35°C under 5 to 10% CO_2. Cultures of M. fortuitum and M. chelonae were read after 1 to 2 weeks, and those of the other species were read after 4 to 6 weeks. The recovery rate of mycobacteria was expressed as a percentage of the mean count of colonies obtained on LJ slants cultured directly with the seed inoculum. Previously unsterilized sputum and urine specimens seeded with strains of M. tuberculosis (~4 × 10^3 CFU) were processed as before but by the regular NALC-NaOH method (11) for digestion and decontamination. To determine the loss of mycobacteria due to NaOH toxicity during the treatment, samples of specimens were processed under identical conditions without centrifugation. The recovery rates of mycobacteria were determined as before; loss of test organisms owing to NaOH toxicity was calculated and taken into account in computing recovery rates following centrifugation.

The effect of RCF on the buoyancy of mycobacteria during centrifugation was determined by using seeded specimens. Split samples of sterilized sputa and urine were distributed into sets of three tubes (9 ml per tube) and seeded with 1.0 ml of mycobacterial suspensions adjusted to contain ~3 × 10^5 CFU, processed by the NALC method without NaOH, and centrifuged at RCFs of 2,074, 3,005, or 3,895 × g for 15 min. Prior to centrifugation, 100 μl of suspension was withdrawn from throughly mixed specimen-digestant mixtures, appropriately diluted, and cultured. Following centrifugation, an equal volume of the supernatant was carefully aspirated from the top layer and upper half of the suspension and cultured. The sediment was suspended and cultured as before. The recovery rate of mycobacteria in the supernatant was expressed as a percentage of colony counts obtained in specimen suspensions before centrifugation. The recovery rate in the sediment was determined as described earlier.

The effect of increasing RCF on AFB smear sensitivity was determined with clinical specimens that contained AFB obtained from patients with tuberculosis. These were diluted to contain various numbers of AFB within the lower ranges. All sputum specimens were initially liquefied by mixing on the vortex with a small volume of NALC digestant without NaOH. Each specimen was thoroughly mixed, distributed in equal volumes into a set of three tubes, treated in accordance with the standard NALC-NaOH method (11), and centrifuged at an RCF of 2,074, 3,005, or 3,895 × g for 15 min. Smears were prepared by spreading 25 μl of the sediment on microscope slides into a circle 1.4 cm in diameter; 100-μl samples of the sediments were cultured on each of two LJ slants. Smears were stained by the auramine fluorochrome method (11) and scanned for AFB at ×250, with positive smears being confirmed at ×630. Thirty fields per smear were searched independently by two workers, and smears were graded by an established method (5, 7). Presence of even a single mycobacterial colony constituted a

positive culture. The smear findings were correlated with culture results and the RCF used.

The effect of increasing RCF on AFB smear sensitivity was also determined by comparing culture-smear correlations of clinical specimens processed with an RCF of 3,800 × g during a 3-year period with those processed in a preceding 3-year period, when an RCF of 2,000 × g was used to concentrate specimens. An identical procedure was followed during these 6 years, with the only exception being the RCF. Specimens were treated by the standard NALC-NaOH method (11) and concentrated by centrifugation at the specified force for 20 min. Smears were prepared with a drop of the centrifugate without spreading, stained by the auramine fluorochrome method (11), and then scanned for AFB at ×250. Positive smears were confirmed at ×630 and, occasionally by additional smears stained by the Ziehl-Neelsen method. A reading of ≥3 AFB constituted a positive smear. Cultures were obtained on LJ slants (2 to 4 slants per specimen) as per standard procedure (11).

RESULTS

In experiments designed to determine the rate of recovery of mycobacteria in urines and sputa, individual test results showed considerable variations and overlap between RCFs and time of centrifugation. Recovery rates in the sediment ranged from under 50 to greater than 80% (Table 1). The cumulated data indicated a trend of a higher recovery rate as RCF and and time were increased. The increased recovery was, nevertheless, not proportional to the increase in RCF and time. The differences in individual recovery rates were minimal between 15 and 20 min for 2,074, 3,005, and 3,895 × g. When centrifugation time was constant, the differences in recovery rate were relatively less between the RCFs of 3,005 and 3,895 × g than those between 2,074 and 3,005 × g.

Recovery rates were similar for M. tuberculosis, M. fortuitum, M. chelonae, and M. avium, with no strain differences within each of the species. No significant differences were observed between recovery rates from specimens that were presterilized and then seeded with mycobacteria and those from specimens that were not sterilized prior to seeding, once the data obtained for the latter were corrected for NaOH toxicity. Recovery rates were essentially the same for sputa and urines.

The recovery rates of mycobacteria from the supernatant fluid following centrifugation were inconsistent, ranging from 0 to 30% with means of 12, 7, and 4% at RCFs of 2,074, 3,005, and 3,895 × g, respectively. Although there was a reduction in the number of bacteria occurring in the supernatant as RCF was increased, approximately 10% of mycobacteria were frequently recovered from the superna-

TABLE 1. Effect of RCF and centrifugation time on recovery of mycobacteria from sputa[a]

Time (min)	% Recovery of colonies with an RCF of:[b]		
	2,074 × g	3,005 × g	3,895 × g
10	54.4 ± 13.0	61.8 ± 9.8	66.6 ± 12.3
15	66.8 ± 10.5	75.8 ± 10.9	79.3 ± 10.9
20	71.0 ± 9.5	78.8 ± 8.2	80.2 ± 8.2

[a] Sterilized sputa were seeded with M. tuberculosis and treated with NALC without NaOH and then centrifuged. The recovery rate in the sediment is expressed as the percentage of the mean count of colonies on LJ slants cultured directly with the seed inoculum.

[b] Mean percent recovery of M. tuberculosis based on five individual determinations ± the standard deviation.

TABLE 2. Effect of RCF on AFB smear sensitivity[a]

RCF	No. of specimens with a smear grade of:[b]				Smear sensitivity (%)[c]
	−	±	1+	2+	
2,074 × g	33	17	29	17	47.9
3,005 × g	29	20	24	23	49.0
3,895 × g	26	22	25	23	50.0

[a] Split samples of sputum and urine from patients with tuberculosis processed at different RCFs for 15 min.

[b] Smear examination at ×250, 30 fields per smear. −, No AFB; ±, 1 to 2 AFB per smear; 1+, 3 to 29 AFB per smear; 2+, ≥30 AFB per smear.

[c] A smear reading of ≥1+ was considered positive. Smear sensitivity was based on 96 specimens yielding positive cultures.

tant. Recovery of mycobacteria from the supernatant and the sediment was not always proportional to the initial inoculum size. At times as much as 15% of the inoculum could not be accounted for.

A total of 96 sputa and urines were processed experimentally to determine the effect of increasing RCF on culture-smear correlations. AFB were demonstrated in smears of a total of 50 specimens having a reading of ≥3 AFB per smear (30 fields). All 96 specimens grew mycobacteria in cultures, with smear-positive specimens mostly yielding 50 to >100 colonies per slant, while smear-negative specimens yielded no more than 30 colonies per slant. Overall smear sensitivity was 52%. Increasing RCF showed a slight increase in the number of colonies recovered in culture and occasionally in the number of AFB in smears. However, the increase was only marginal and, although it occasionally had some effect on the degree of positivity of positive smears, it mostly failed to increase smear sensitivity significantly (Table 2).

Culture-smear correlations obtained for a total of 25,071 clinical specimens processed for mycobacteria with an RCF of 3,800 × g during a 3-year period between 1982 and 1984 were compared with those of a total of 30,180 specimens similarly processed with an RCF of 2,000 × g during the preceding 3 years. The overall smear sensitivity was 65 to 72%. There were no significant differences in culture-smear correlations of specimens that could be attributed to the increased RCF used (Table 3).

DISCUSSION

The highest rates of recovery of mycobacteria were generally obtained with RCFs of 3,005 and 3,895 × g applied for

15 to 20 min. Recovery rates were not significantly lower when using an RCF of 2,074 × g for 20 min, which is within the range of the normally recommended speed (5, 7, 11). This force applied for 10 min yielded a low recovery of organisms which is not adequate for concentrating mycobacteria in clinical specimens. Over 75% of seeded mycobacteria can be recovered in the sediment with an RCF of 3,000 to 4,000 × g for 15 to 20 min. Since our data were corrected for NaOH toxicity, it should be pointed out that the actual rate of recovery of viable mycobacteria from clinical specimens would depend on the method of treatment and individual species and strain differences of mycobacteria for alkali or acid tolerance. Extended centrifugation time may have adverse effects on the viability of mycobacteria in partially neutralized specimens, such as those treated by the NALC-NaOH method. A centrifugation time of 15 min appears to be adequate to concentrate mycobacteria in clinical specimens, especially when using an RCF of 3,000 × g or greater. When using an RCF of approximately 2,000 × g, a 20-min centrifugation may be desirable. The additional benefit of using higher RCFs and longer centrifugation times is likely to be minimal. Moreover, centrifugation at high speed may result in the generation of heat, which could injure or kill mycobacteria; hence, refrigerated centrifuges are required when RCFs of ≥3,000 × g are used (7). On the whole, an RCF of about 2,500 × g applied for 15 to 20 min appeared to be ideal to concentrate mycobacteria in clinical specimens both technically and from the practical standpoint of most diagnostic laboratories.

Our data indicate that not all of the mycobacteria are concentrated in the sediment. Some remain in the supernatant in an uneven distribution throughout the fluid column. It may not be uncommon to recover as much as 30% of the AFB from the supernatant following centrifugation at RCFs of 2,000 to 4,000 × g. However, the supernatant is an inappropriate source of inoculum for culture because of the large volume of the supernatant and the relatively small number of AFB. The loss of mycobacteria in the supernatant is not likely to significantly affect the culture results obtained from centrifugates of respiratory specimens in routine practice. However, centrifugation may not be an appropriate method to concentrate specimens which are likely to contain small numbers of organisms, especially when used in conjunction with a decontamination procedure. The membrane filtration technique may be more useful to process such specimens (6, 12).

The NALC-NaOH procedure requires the addition of PB or water to digested specimens prior to centrifugation to

TABLE 3. Effect of increased RCF on AFB-positive culture-smear correlation: analysis of laboratory data of routinely processed clinical specimens[a]

RCF[b]	Year	No. of specimens processed	No. of specimens positive by culture	No. of specimens positive by smear[c]	Smear sensitivity (%)
2,000 × g	1979	10,532	369	238	64.5
2,000 × g	1980	10,788	558	403	72.2
2,000 × g	1981	8,860	276	194	70.3
3,800 × g	1982	8,988	332	237	71.4
3,800 × g	1983	7,843	280	201	71.8
3,800 × g	1984	8,240	165	116	70.3

[a] Sputa and other respiratory specimens constituted approximately 55% of the specimens processed. urines were 32%, and other specimens were 13%. A level of ≥3 AFB per smear constituted a positive smear. M. tuberculosis represented 83 to 94% of the positive cultures.

[b] Centrifugation was for 20 min.

[c] Represents positive smears of only those specimens which were positive in culture.

dilute the alkali and to lower the viscosity of specimens to better facilitate sedimentation of mycobacteria during centrifugation (7, 8). Preliminary studies in our laboratory indicated that omission of this step could simplify and shorten the NALC-NaOH treatment procedure without significantly affecting the culture results (S. Ratnam, and F. Stead, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C218, p. 348). This observation suggests that addition of PB or water to digested specimens may not be critical for concentrating mycobacteria by centrifugation by this method, but it requires further study.

A dramatic increase in the AFB smear-culture correlation was reported as the RCF used for processing specimens increased from 1,260 to 3,800 × g (4). Under the experimental conditions used in the present investigation, this was not confirmed. The data from our evaluation of routinely processed clinical specimens during the last six years also do not support the above observation; fluorochrome smear sensitivity remained the same regardless of the RCF (Table 3). Our experimental data showed that the maximum recovery of mycobacteria in the sediment was less than twofold when the RCF was increased from 2,074 to 3,895 × g and this increase did not appear to result in a significant increase in positive smears. Likewise, in routine clinical specimens processed with an RCF of 3,800 × g, the marginal increase in the recovery rates would likely have enhanced the degree of smear positivity and facilitated isolation of a slightly larger number of mycobacteria, but it apparently failed to increase smear sensitivity. It was evident from a review of our laboratory data that smear sensitivity is dependent on the type of patient population, the nature of the specimen, and the numbers and species of mycobacteria present in the specimen, as previously observed (3). The discrepancy between our findings and those of others (4) could probably be explained on this basis. Since the smear sensitivity of mycobacteria other than *M. tuberculosis* is generally much lower than that of *M. tuberculosis*, it should be pointed out that about 90% of our clinical isolates were *M. tuberculosis*. Smear sensitivity of >60% is not uncommon for *M. tuberculosis* in specimens processed by using an RCF of 1,800 × g for 15 min (3). It is concluded that increasing the RCF beyond 2,500 × g or so is not likely to significantly improve smear sensitivity, especially for *M. tuberculosis* from respiratory specimens. As pointed out by Lipsky et al. (3), experience, alertness, and persistence of technologists in detecting small numbers of AFB are important for reliable acid-fast microscopy.

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