Comparison of Methods for Tuberculosis Bacteriology

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To improve efficiency of isolation of tubercle bacilli from clinical specimens, the following recommendations are presented. (i) Employ multiple specimens consisting of a combination of morning sputums for the early detection of positives, along with 24-hr sputum pools for the greatest total yield of positives. (ii) When timing is rigorously controlled, Zephiran-trisodium phosphate and sodium hydroxide-acetylcysteine are comparable, but if timing cannot rigorously be controlled, employ the Zephiran-trisodium phosphate digestion procedure to allow the greatest freedom in exposure time with the lowest kill rate to tubercle bacilli. (iii) Employ both an agar medium incubated in 5% CO₂, for the early detection of positives as well as positives in the presence of contaminants, and an egg medium, preferably with CO₂, to increase the yield of positives.

Among patients with minimal tuberculosis, it is frequently difficult to isolate tubercle bacilli, and the cultures usually yield few colonies. Thus, 80% of the positive cultures from our patients with far advanced disease had yielded more than 50 colonies, but 84% of the positive cultures from patients with minimal disease had fewer than 50 colonies. It is obviously important that the techniques employed be sensitive enough to detect small numbers of organisms.

Our research in this field has been directed toward improvement in the areas of sensitivity of techniques and simplicity of performance. In earlier papers, we reported a simple method for performance of tubercle bacillus drug susceptibility studies (7) and an evaluation of killing rates of tubercle bacilli in various digestion systems (3). In this paper, we describe a combined evaluation of three specimen sampling schedules, three digestion techniques, and two types of culture media.

MATERIALS AND METHODS

Sampling. Upon admission, each patient was assigned one of six possible regimen sequences by means of a table of random numbers. Each regimen sequence employed three consecutive early morning (AM) sputums submitted to the laboratory to be processed for recovery of tubercle bacilli. The order of application of digestion methods employed on a given patient's series of specimens was determined by the assigned regimen sequence. Each patient had one specimen digested by each of the three methods. In addition, as part of our admission routine, three or more 24-hr sputum specimens were submitted on each patient at approximately the same time. All of the 24-hr specimens were digested by our standard Zephiran-trisodium phosphate (Z-TSP) method only.

Digestion methods. Sodium hydroxide-acetylcysteine (NaAC) was employed as described by Kubica et al. (4). Zephiran-acetylcysteine (ZAC; Winthrop Laboratories, New York, N.Y.) digestion was employed, as described in an earlier paper (3), with the exposure time set at 90 min. Sediments from the first centrifugation were suspended in pH 6.6 buffer to dilute residual Zephiran and were centrifuged once again. Z-TSP was used as described in earlier papers (2, 3), again employing the buffer wash.

Media. All sediments were inoculated into one plate of Middlebrook 7H10 agar and into two bottles of Lowenstein-Jensen (L-J) egg medium. The 7H10 agar plates were incubated at 37°C under 5% CO₂ tension. Bottles of L-J were incubated in an ordinary incubator at 37°C. Cultures were examined weekly.

Statistical method. Unless otherwise stated the Mann-Whitney U test (6) was employed to determine the significance of observed differences in results.

RESULTS AND DISCUSSION

Sampling. A review of 261 patients, from whom multiple 24-hr specimens were received and digested by the Z-TSP method, showed that only 30% were positive on the first admission specimen; the cumulative percentage of positive patients increased to 34.5% with two specimens, to 37.5% with three specimens, and some additional increase was seen through the fifth specimen. In an earlier review, we were able to examine 10 serial admission specimens submitted by each patient, and only 1 in 300 (0.3%) showed the first positive culture after the fifth specimen. Thus, a series of five sputum specimens appears to offer optimal probability of finding tubercle bacilli.
Contrary to an earlier report by Kestle and Kubica (1), our study does not show the clear superiority of the AM specimen over a sputum pool. Since first-morning specimens are being used in a number of laboratories, we compared the recovery of tubercle bacilli from 668 single AM sputum specimens with a like number of 24-hr sputum pools from the same patients. Growth appeared earlier with AM specimens, which yielded 91 positive cultures in 3 weeks, as compared to only 56 from the 24-hr pools. The difference in numbers of positives at this point is highly significant ($P < 0.01$). The total numbers of positives on prolonged incubation, however, are 17% greater (173 positives from pools against 143 positives from AM) in the 24-hr sputum pools. The difference here is also significant ($P = 0.05$). Contamination with either type of specimen was less than 5%. Thus, each type of specimen has its advantage, and it is recommended that a combination of both AM and 24-hr pools be employed for diagnosis of tuberculosis.

**Digestion methods and culture media.** The comparative results of the study of the digestion methods are shown in Fig. 1. The Z-TSP and NaAC methods are comparable. It should be pointed out that great care was taken to avoid overexposure of the sputum specimens to the digesting agents. It was reported previously that prolongation of digestion time with sodium hydroxide causes a logarithmic reduction in the number of viable bacilli to a much greater degree than is seen with TSP methods. Thus, the NaAC system requires a rigid adherence to a timing schedule, whereas the Z-TSP permits greater flexibility and a smaller chance of excessive killing of tubercle bacilli by overexposure. The earlier studies with a mixture of acetyl-cysteine and Zephiran, without NaOH, demonstrated negligible lethal effects upon tubercle bacilli; use of this mixture, however, resulted in a very high contamination rate, and further studies must be done to see whether a practical modification of this mixture can be developed.

Figure 2 shows the differences in recovery of tubercle bacilli from 208 specimens, by each of three methods, on L-J egg and on 7H10 agar medium. It should be pointed out that the 7H10 agar was incubated under 5% CO$_2$ tension, as recommended by Schaeffer et al. (6), whereas the egg medium was incubated in a normal atmos-
ultimately, medium; growth appeared in cultures method. Whether the CO2 and egg media were not. In one study, a series of 372 specimens was plated, after NaAC digestion, to one agar plate and two bottles of egg medium. One L-J culture was incubated in CO2 and the other was not. In this limited series, the agar yielded 58 positive cultures, the L-J without CO2 yielded 56, and the L-J with CO2 yielded 64 positive cultures (P = > 0.10 in all cases by the Chi-square method). When rate of growth was calculated in terms of distribution of numbers of cultures exhibiting first growth after different periods of incubation, the growth on L-J with CO2 occurred significantly earlier than the growth without CO2 (P = 0.03), but on agar with CO2 cultures still appeared significantly earlier than those on Lowenstein-Jensen with CO2 (P = < 0.001).

The marked discrepancy between total yields of positive cultures on the egg and agar medium with the Z-TSP digestant is probably related to carry-over of traces of Zephiran in the inoculum. Although Zephiran is not bacteriocidal for tubercle bacilli, it can be bacteriostatic in agar medium. The lecithin in egg medium, on the other hand, inactivates any Zephiran carried over. We have since learned that use of a neutralizing agent such as Tamol (Rohm and Haas, Philadelphia, Pa.) in suitable concentration in the wash of the sediment can prevent this carry-over effect. In the case of the Z-AC digestion, contamination greatly reduced the number of positive cultures on egg medium, whereas the number of positive cultures on agaris comparable to the number obtained with the other two methods. In this study, it was often possible to detect colonies of *Mycobacterium tuberculosis* on contaminated agar, whereas contaminated egg medium was useless.

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LITERATURE CITED