DECONTAMINATION OF SPUTUM AND PRESERVATION OF *M. TUBERCULOSIS* BY PANCREATIN-DESOGEN

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Chemotherapy of tuberculosis is a strictly antimicrobial treatment and the main criterion by which such treatment should be judged is the result of bacteriological investigation. Drug resistance is one of the foremost problems in the management of tuberculosis, and this can only be established in the laboratory. The isolation of the causative organism on culture media has other main advantages over the microscopical examination. It gives an accurate confirmation of the diagnosis. It gives a measure of response of the patient to treatment. It can establish the nature of the infective agent and its resistance to drugs before therapy has started. It serves to distinguish between typical *M. tuberculosis* and atypical mycobacteria. It is much more accurate than microscopy. Fifty thousand to 100,000 organisms/ml. of sputum are necessary for a positive smear and 50 - 100 viable organisms for a positive culture. A single proper culturing of a sputum specimen is of more value than 3 successive microscopical examinations of a patient.

Most classical methods of pre-treatment of sputum for culturing are time-consuming, cumbersome, and refrigeration of sputum for preservation in transit is expensive. These are the main reasons why relatively little culturing is done in South Africa.

This paper describes a method of preserving and decontaminating tuberculous sputum which allows the use of only one container and eliminates all centrifugation, washing and decanting of sputum.

Laboratories in Sweden, France, Denmark, Germany and the USA have experimented with the use of surface-active detergents combined either with enzymes or alkaline solutions. Saxholm[10] described the pre-treatment of sputum with the Pancreatin-Desogen (PD) solution in 1954. Tacquet and Tison[18,19] used Teepol plus 1% NaOH and later lauryl sulphate for decontamination and homogenization of sputum. Engbaek[5] examined the same agents for their usefulness in the isolation of atypical mycobacteria. Meissner and Tuncman[8] perfected the method of Saxholm to a stage where it can be used in a routine laboratory. Nelles and Möbius[9] introduced Mersolat plus 2% NaOH, and Kubica *et al.*[7] recommended the use of a N-acetyl-L-cysteine digestion procedure, incorporating 1% NaOH. All authors found an improvement over conventional methods in the culturability of *M. tuberculosis*, but the methods which incorporate alkali into the solution still necessitate adherence to strict timing. Only the PD method allows for longer time intervals between addition of solution and inoculation. Schröder[17] studied the rate of survival of the tubercle bacillus and the different groups of atypical mycobacteria, and found that the detergents were superior to 6% H_2SO_4, in all types of mycobacteria except *M. kansasii*.

MATERIAL AND METHODS

For the preparation of the Pancreatin-Desogen solution, the method of Saxholm and Meissner was used: but exposure times and concentrations were changed. The solution consisted of a phosphate buffer of pH 8·0, usually 1 l., which is divided into 2 parts of 500 ml. The right amount of Desogen is added to get a final solution of 1½%. Desogen is a quarternary ammonium compound available unperfumed in a concentration of 70%, and produced by Geigy, Basle.* The pancreatin solution was prepared by dissolving 5 gr. in 500 ml. buffer to give a final solution of ½%.† This solution was then filtered. The solution is self-sterilizing and is kept in a refrigerator where it remains stable for weeks. When mixing of sputum with PD solution was done only 5 hours before culturing, a shaking apparatus was used for quarter of an hour for better homogenization. Cultures on a Loewenstein-Jensen (LJ) medium were prepared by transferring 2 drops of the mixture by pipette onto the medium, and left horizontally till the next morning to allow fixation to the surface of the medium.

Test 1. The standard strain *H37RV* was suspended in 0·01% Tween water. One part of the suspension was mixed with one part PD solution, to give the same concentration as used in sputum. Another test series was done in the same way using *B. subtilis* as a representative spore-forming contaminant. The suspensions were kept at room-temperature for different periods up to 5 days. The suspension and the controls were then diluted to give actual colony counts of about 200 colonies and were inoculated onto LJ media using wide-mouthed 1 oz. McCartney bottles.

Test 2. Sputum free of tubercle bacilli was mixed in equal parts with PD and artificially infected with fully drug-sensitive human wild strains and INH-resistant strains of *M. tuberculosis*. Ten-fold dilutions were made with a mixture of equal parts of negative sputum and PD solution to have an unchanged ratio of sputum and PD. LJ media were inoculated as in Test 1. An inoculum of 10^{-4} mg. bacteria per slope produced an average of 150 colonies after 5 hours of exposure and the results with this dilution are shown in Fig. 2 (see below).

Test 3. Ten positive sputa of patients for whom treatment had not yet commenced, were mixed with PD and cultures were prepared in the above manner, after 5 hours and every day for 6 days. The same was done with sputa from 10 chronically open cases excreting INH-resistant tubercle bacilli.

Test 4. Sputa of 500 patients were mixed in the hospital with equal amounts of PD solution, and then transported to the

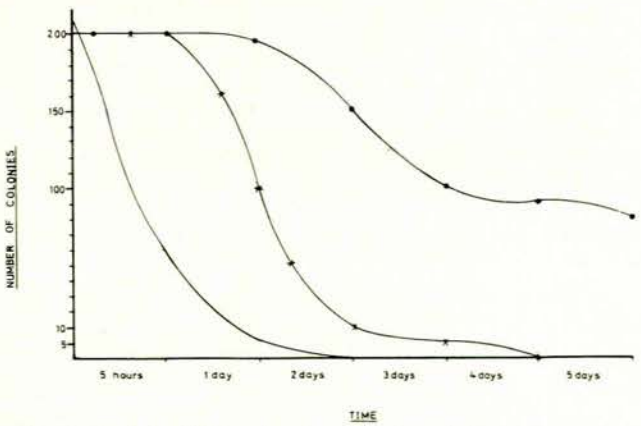

Fig. 1. Survival of *M. tuberculosis* and *B. subtilis* in Pancreatin-Desogen solution (culturability on Löwenstein-Jensen medium). ●——● H37RV in Tween (0·01%). x——x H37RV in Pancreatin-Desogen. —— *B. subtilis* in Pancreatin-Desogen.

*Obtainable from Pharmakers Limited, Johannesburg.
†For sputum in transit, ¼% pancreatin is sufficient.

laboratory over periods of 2 - 5 days, where this mixture was directly inoculated as above, without any further treatment. Results were compared with 500 untreated sputa which arrived after 12 hours transportation, and where the PD solution was added in the laboratory, and kept for 5 hours to allow for homogenization and decontamination.

Test 5. A batch of 40 sputa which arrived 24 hours after admixture of PD solution in the hospital were inoculated onto cultures immediately, and again 7 days later. They were kept at an average room temperature of 20°C.

RESULTS

Results of Test 1 are given in Fig. 1. It is shown that *B. subtilis* is killed by PD solution within 2 days. The number of viable *M. tuberculosis* decreased in PD solution to 50% after 1 day, and to 5% after 2 days. After 4 days, tubercle bacilli were no longer cultivable, while the control in Tween had decreased to 50%.

Results of Tests 2 and 3 are shown in Fig. 2. The decrease in culturable bacilli was much steeper when the organisms were artificially added to negative sputum than when the organisms were excreted with the sputum. There was a loss of about 30% in the original sputa after 5 or 6 days, but when the organisms were added later, this loss occurred within 1 day, and after 5 days three-quarters of the INH-sensitive tubercle bacilli were dead as were all of the INH-resistant bacilli. This marked difference can be explained by the fact that in original sputum, most of the bacilli are intracellular and would be set free only after full action of pancreatin, while in the sputum made positive artificially, all bacilli are extracellular.

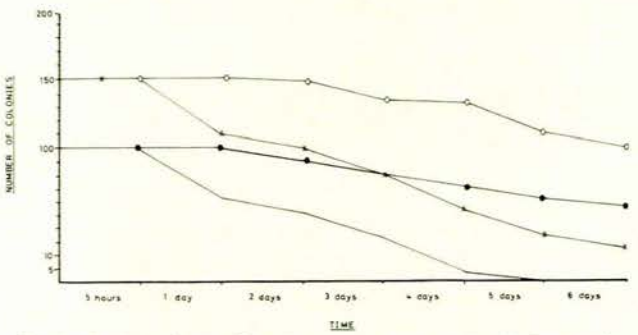

Fig. 2. Survival of *M. tuberculosis* in sputum treated with Pancreatin-Desogen solution (culturability on Löwenstein-Jensen medium). 0——0 INH-sensitive *M. tuberculosis* in original sputum. ●——● INH-resistant *M. tuberculosis* in original sputum. x——x INH-sensitive *M. tuberculosis* artificially added to neg. sputum. —— INH-resistant *M. tuberculosis* artificially added to neg. sputum.

The result of Test 4 was as follows. Forty-eight percent of the specimens that were transported over periods of 2 - 5 days were positive, and 51% of specimens were positive that were kept under the influence of PD solution for 5 hours only. There was also no significant difference in the number of colonies per sputum, between the 2 groups.

Table I gives the result of Test 5, i.e. the comparison of culturing 40 sputa kept under the influence of PD solution for 1 day and 1 week. A loss of 40% of colonies is shown and only 2 of the 28 positive sputa were positive after 24 hours and negative after 7 days.

TABLE I. SURVIVAL OF *M. tuberculosis* IN 40 SPUTA UNDER ACTION OF PANCREATIN-DESOGEN

Time in days	*No. of cultures after 8 weeks*			*No. of colonies per slope*
	Pos.	*Neg.*	*Contam.*	*(mean)*
1	28	10	2	100
7	26	12	2	60

The rate of contamination of culture-media of over 2,500 sputa, I treated with the PD solution at this laboratory and the Knight's Bacteriological Laboratory, Germiston was under 1%. Out of 1,170 sputa which were cultured from 1 January 1965 to 21 April 1965 at the Knight's Laboratory on 3 tubes of LJ media each after treatment with PD solution for 5 hours, 42% were positive. One hundred and ninety-two were found to be microscopically negative on thorough examination, but positive on culture. This is a rate of 24% of culture-positive specimens with a negative microscopical result.

DISCUSSION

Surface active quarternary ammonium compounds have a bactericidal or bacteriostatic action. A complex compound of the detergent with the cell surface is formed and precipitation of albumin occurs.[20] Quarternary ammonium compounds are known to be ineffective as disinfectants for tubercle bacilli in the usual concentration. They seem to be an ideal selector of mycobacteria in sputum. Superiority of the PD method over the sodium hydroxide method was shown to exist regardless of the nature of the specimen.[10-14] It can be used for centrifuged or filtered urine and gastric lavage specimens as well. The advantages of the PD method over the treatment with acid was shown by Meissner and Tuncman.[5]

They found 10·8% more positive sputum samples and 34% more positive gastric content samples after PD than after 6% H_2SO_4 treatment. Pure culture of *M. tuberculosis* survived PD treatment to 26% of organism, NaOH treatment to 9·4%, and H_2SO_4 treatment to 3·5%. Mention is also made of the speedier growth of bacilli after PD treatment. Anz and Meissner[21] mechanically homogenized positive PD-treated sputum and kept it at 31°C for 5 days. The survival rate of 0 - 1% indicates that high-speed homogenization is detrimental to the tubercle bacilli. When comparing a solution of 1% trypsin plus 1½% Desogen with 4% NaOH, Lind[22] obtained somewhat better results with the latter treatment.

It is well known that sputum samples which are transported without preservation for up to a week at subtropical temperatures are too contaminated for successful isolation. Decontamination by acids or alkali must be so severe that the tubercle bacilli are also killed. Working in temperate zones Engbaek and Bentzon[4,5] found that if a temperature is sufficiently high to promote growth, the bacterial flora cannot always be killed by treatment with 4% NaOH. At 22°C, deterioration takes place during the course of the first week. For the survival of tubercle bacilli temperatures from +22°C to +4°C offer equal conditions.[5]

Our summer temperatures exceed such temperatures by far, especially during transportation, therefore the addition of PD solution before despatch is a great advantage. The contaminating bacteria are killed within 5 hours, if not less, while the tubercle bacilli are only gradually damaged. Only *M. kansasii* are likely to be killed earlier by PD than by conventional pre-treatment.[1,17] However, to our knowledge, this organism has not yet been isolated from South African patients.

It might be argued that the PD method means dilution of specimens while conventional methods use the concentration by centrifugation. However, centrifugation at the usual speed in the routine centrifuge is actually a very ineffective way of concentrating tubercle bacilli in the sediment.[2,15,16] Further, the action of pancreatin and Desogen results in an even dispersion of the bacilli. This can

be observed microscopically and later on the slope, where the colonies are equal in size and well dispersed. The lack of any cell structure as a guide in microscopical examination is a disadvantage. Smears must be made before mixing with PD solution to avoid the washing off of the material. Precipitation of PD-sputum mixtures is an alternative method.

Another important advantage of the PD method is the improvement in safety for the laboratory workers. Conventional methods entail the decanting of sputum into another tube, the repeated centrifugation which in itself is one of the most hazardous procedures in a tuberculosis laboratory, and more decanting after washing off the sediment. The PD method eliminates all such handling and the entire process is done in the one container in which the sample arrives. Only one pipette is needed to put 2 drops onto the slopes. We found that 1 technician can usually do the culturing onto 2 media each of 100 - 150 specimens per day. Another advantage is that there is no rigid time schedule imposed on the technician. The culturing can be done the same day or the next day. There is a considerable saving in glassware as only culture media bottles or tubes, the specimen bottles and pipettes are needed, and centrifuges are unnecessary.

SUMMARY

A method of decontamination, homogenization and preservation of tuberculous sputum using a solution of an enzyme and a quarternary ammonium compound is described. The mixture consists of pancreatin in a ½% solution and Desogen in a 1½% solution. Experiments are reported with pure culture of *B. subtilis* and *M. tuberculosis*, and with sputum which was either naturally or artificially infected with tubercle bacilli. It is shown that contaminants are killed more rapidly than *M. tuberculosis*, and that about 60% of tubercle bacilli in tuberculous sputum survive this treatment for 5 days. After 1 week of exposure 90% of specimens were still positive on culture. The rate of contamination was found to be under 1%. The rate of positive cultures is as high as with the use of conventional pre-treatment methods. The method offers a greater degree of safety in the laboratory. There is a considerable saving in time, glassware and equipment, it allows for the transportation of sputum over long distances up to one week, and mission hospitals in remote areas can thus be served. A much greater number of sputum specimens can also be cultured by the same technical staff.

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