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Improved microscopical diagnosis of pulmonary tuberculosis in developing countries

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Abstract

The diagnosis of pulmonary tuberculosis (TB) relies on the bacteriological examination of sputum. However, microscopy of smears made directly from sputum has a low sensitivity and there is an urgent need for improved methods. We have compared microscopy of smears made directly from sputum with microscopy after liquefaction of sputum with household bleach (NaOCl) and concentration of bacteria by centrifugation. In 3 studies performed in Ethiopia and India, the use of the NaOCl method increased the number of samples positive for acid-fast bacilli by more than 100%. The technique is appropriate for developing countries and its application would increase the efficiency of TB control programmes. As a potent disinfectant, NaOCl also has the advantage of lowering the risk of laboratory infection.

Keywords: tuberculosis, diagnosis by microscopy

Introduction

Tuberculosis (TB) is a major health problem in most developing countries. According to the World Health Organization, roughly one-third of the world population is infected with *Mycobacterium tuberculosis* and 20 million have active disease (KOCHI, 1991). The mortality due to TB is close to 3 million per year, making it the leading cause of death from a single pathogen (KOCHI, 1991). The spread of the human immunodeficiency virus (HIV) has further aggravated the situation (HARRIES, 1990). The number of patients infected with both HIV and TB is estimated to be 3·8 million and most of these patients are found in sub-Saharan Africa.

The major objective of TB control programmes is to identify and treat patients with infectious pulmonary TB, the diagnosis of which relies on bacteriological examination of sputum. Culture of mycobacteria is the reference method for detection of tubercle bacilli but it is prohibitively slow and requires special safety procedures in laboratories. Serological techniques are not useful in control programmes due to lack of sensitivity and specificity (DANIEL, 1989). Among the new approaches for rapid diagnosis of TB, the nucleic acid amplification methods are the most promising (EISENACH *et al.*, 1991; SAVIC *et al.*, 1992), but the technology is not applicable to control programmes in developing countries.

Microscopy of direct smears for acid-fast bacilli (AFB) is the most commonly used method for diagnosis of TB but its major disadvantage is discouragingly low sensitivity when used in control programmes. In one study by ABER *et al.* (1980) in several African laboratories, the sensitivity of direct microscopy ranged from 8·8% to 46·4%. Consequently, there is an urgent need for improved methods for the diagnosis of pulmonary TB (L'HERMINEZ, 1993). In this study, we have evaluated different methods for digestion of sputum samples and measured the recovery of radiolabelled mycobacteria after centrifugation. Digestion of sputum with household bleach (sodium hypochlorite) (OLIVER & REUSSER, 1942; CAMERON & CASTLES, 1946; CORPER & NELSON, 1949) gave the best recovery of AFB, and this technique for the preparation of slides for direct microscopy was then evaluated for the microbiological diagnosis of pulmonary TB at 3 diagnostic centres in Ethiopia and India.

Materials and Methods

Culture and radiolabelling of M. bovis

M. bovis (bacillus Calmette–Guérin [BCG]; Institut Pasteur, Paris) were harvested from Löwenstein–Jensen (LJ) medium and suspended in phosphate-buffered saline (pH 7·4) with 0·05% Tween 20® (PBST). The suspension was vortexed to break up large clumps and the remaining clumps were allowed to settle for 10 min. The upper bacterial suspension was removed to a second tube and sonicated at 35 kHz in a water bath (Transonic 460/H®; Elma, Germany) for 5 min. The optical density at 620 nm was measured, and the bacterial concentration was adjusted to 2×10^9 organisms per mL. Bacteria were then radiolabelled with ^{51}Cr (Amersham, UK) by adding 600 μCi of ^{51}Cr to 6×10^8 organisms. The mixture was incubated overnight at room temperature and the bacteria were washed 4–6 times in PBST. Radioactivity in the last supernatant never exceeded 5% of the total activity.

Digestion of sputa and measurement of sedimentation efficiency

Sputum samples were collected from non-TB patients at the Department of Pulmonary Diseases, Lund University Hospital, Lund, Sweden. The sputa were pooled, incubated at 37°C for 1 h to decrease viscosity, and mixed thoroughly. Radiolabelled BCG (3×10^3 cpm) were added to 1·5 mL of sputum in 10 mL glass tubes. The mixtures of sputa and radiolabelled BCG were then liquefied by one of 3 different methods: (i) 1·5 mL of 4·4% NaOCl (pH 12·8) were mixed with the sputum and, after 15 min at room temperature with shaking by hand at regular intervals, 6 mL of distilled water were added; (ii) 3 mL of 1 M NaOH were mixed with the sputum and the mixture was incubated for 15 min at room temperature with shaking by hand at regular intervals and then neutralized with 1 M HCl; (iii) 2·25 mL of dithiothreitol (Sputolysin®; Behring Diagnostics, La Jolla, California, USA) were mixed with the sputum and shaken on a mechanical shaker (DSG 340®; Heidolf, Germany) for 15 min at room temperature. After digestion, each sample was centrifuged for 5 min at varying relative centrifugal forces (RCF). The supernatant was transferred to another test tube and the radioactivity in the pellet and the corresponding supernatant was measured in a γ counter. All experiments were done in triplicate.

Clinical samples

Seven hundred and three sputum samples were collected from suspected pulmonary TB patients: 100 at the Addis Ababa Tuberculosis Demonstration and Training Centre, Addis Ababa, Ethiopia, 500 at Gondar College of Medical Sciences, Gondar, Ethiopia, and 103 at St John's Medical College, Bangalore, India.

Direct microscopy

Slides for direct sputum smears were prepared using a sterile pipette to apply a drop of the purulent part of the sputum. The smears were dried in air, heat-fixed and stained by the Ziehl–Neelsen technique. The remaining

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sputum (1–2 mL) was transferred to a 10 mL screw-capped tube and mixed with an equal volume of commercially available household bleach (4–5% NaOCl). The mixture was incubated at room temperature for 10–15 min and shaken at regular intervals. Then, 8 mL of distilled H_2O were added, and the sample was centrifuged. The RCFs applied varied in different studies between 800 ***g*** for 20 min and 3000 ***g*** for 15 min. The supernatant was discarded and the pellet was suspended in a few drops of the remaining fluid. Slides were prepared using one drop of the dissolved pellet, air-dried, heat-fixed, stained by the Ziehl–Neelsen method, and examined by bright-field microscopy (1000×magnification) independently by different experienced microscopists; the results were compared at the end of the study. The microscopists were instructed to examine all slides according to the established routine at each centre.

Comparison with culture

Culture was included as the reference method in one of the studies. Smears were made directly from sputum as described above. The remaining sputum was mixed by vortexing and divided into 2 equal parts. One part was treated with NaOCl and the slides were prepared, fixed, stained and examined as described above. The number of AFB seen on the smears was recorded according to the recommendation of the American Lung Association (VESTAL, 1975). The remaining half of the sputum was decontaminated with 3% sodium lauryl sulphate and 1% NaOH (Sigma, St Louis, Missouri, USA), neutralized with 0·45% H_2SO_4–bromcresol purple solution, and cultured in triplicate tubes containing LJ medium. Cultures were incubated at 37°C in 5% CO_2 for one week, thereafter at 37°C in air for 8 weeks, and were checked once a week for mycobacterial growth. Mycobacterial species were identified using standard biochemical tests (VESTAL, 1975) and deoxyribonucleic acid–ribonucleic acid hybridization (Accu Probe®, Genprobe Inc., San Diego, California, USA).

Statistical analysis

Values of χ^2 were calculated using Yates's correction.

Results

Sedimentation efficiency of radiolabelled BCG in sputum samples

Radiolabelled BCG was added to sputum from non-TB patients and the samples were digested with NaOCl, NaOH, or dithiothreitol. The recovery rates of radiolabelled BCG in the sediment after centrifugation for 5 min at different RCFs are shown in the Figure. The highest recovery rates were obtained after digestion with NaOCl.

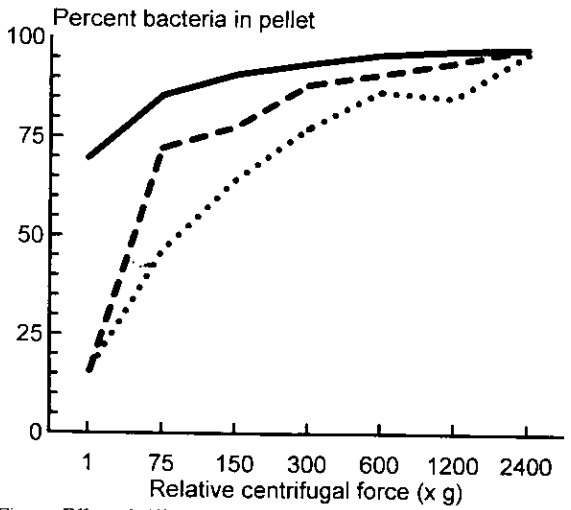

Figure. Effect of different relative centrifugal forces on sedimentation of radiolabelled mycobacteria in sputum after digestion with NaOCl (——), NaOH (----), and dithiothreitol (.....) (mean values of 3 determinations). Centrifugation time was 5 min.

Comparison of results obtained by Ziehl–Neelsen staining

Smears made directly from sputum and smears prepared after liquefaction of sputum with NaOCl and concentration of bacilli by centrifugation were examined microscopically. NaOCl treatment and centrifugation increased the number of positive samples by 108–125% (average 114%; Table 1).

Table 1. Comparison of results of Ziehl–Neelsen staining of smears prepared directly from sputum, of smears prepared after liquefaction of sputum with sodium hypochlorite, and concentration of bacilli by centrifugation

	Addis Ababa (n=100)		Gondar (n=500)		Bangalore (n=103)	
	Positive	Negative	Positive	Negative	Positive	Negative
Direct smear	16	84	39	461	16	87
After NaOCl	36	64	71[a]	429	35	68
P (χ^2)	0·0022		0·0017		0·0037	

[a]Two specimens were positive on direct smear but negative after NaOCl treatment.

Comparison of Ziehl–Neelsen staining with culture

Ziehl–Neelsen staining of smears was compared with culture on LJ medium. Growth of *M. tuberculosis* was obtained from 52 of the 100 samples (Table 2). *Mycobacteria* spp. other than *M. tuberculosis* were cultured from one sputum sample, but this sample was negative by microscopy. In the 52 sputum samples with growth of *M. tuberculosis*, AFB were detected in 16 samples prepared directly from sputum and in 36 samples prepared after digestion with NaOCl and centrifugation (Table 2). Microscopy was negative with all culture-negative samples. The sensitivity of smears for AFB compared with culture was 30·8% when smears were prepared directly from sputum and 69·2% when smears were prepared after NaOCl treatment and centrifugation. There was a more than 10-fold increase in the average number of AFB seen per microscope field in the smears prepared after NaOCl treatment compared to smears made directly from sputum. Specificity was 100% for both methods.

Table 2. Results of Ziehl–Neelsen staining of smears prepared directly from sputum and of smears prepared after liquefication of sputum with sodium hypochlorite and centrifugation compared with culture of *M. tuberculosis* on Löwenstein–Jensen medium

Culture	Direct smear		Smear after NaOCl treatment and centrifugation	
	Positive	Negative	Positive	Negative
Positive	16	36	36	16
Negative	0	48	0	48
Total	16	84	36	64

Discussion

In developing countries, microscopy of sputum is by far the fastest, cheapest and most reliable method for the diagnosis of pulmonary TB. However, a large work-load will adversely affect the alertness and persistence of technicians and the sensitivity of smears made directly from sputum is often not more than 20–30%, as was the case in this study. Meticulous preparation and examination of smears made directly from sputum could increase the sensitivity but experience shows that it is often very difficult to maintain a high level of performance in overburdened control programmes. We made no effort to improve the technical performance of the microscopists before initiating this study and only instructed the technicians to examine all slides according to the established routines at each centre. Digestion of sputum and concentration of bacilli by centrifugation increases the recovery rate of mycobacteria (RATNAM & MARCH, 1986). We evaluated 3 different methods for digestion of sputum and found that the RCF needed to pellet mycobacteria was lower

after digestion with NaOCl than with other methods (Figure). Improved recovery of mycobacteria after treatment with NaOCl might be attributable to changes in surface properties of the mycobacteria (i.e. charge and hydrophobicity), and/or denaturation of sputum constituents leading to flocculation and subsequent increased sedimentation rate of the mycobacteria.

Introduction of new methods for the microbiological diagnosis of TB has to take into account the limited resources of most national TB programmes and logistic problems of procurement. NaOCl is ideal in these respects since it is cheap and available almost anywhere as household bleach. The NaOCl technique for preparation of smears was described in 1942 and the method is included in most laboratory manuals (VESTAL, 1975; SMITHWICK, 1976; EBERSOLE, 1992). However, the method is not widely used and to our knowledge has not been evaluated in developing countries. The results from the 3 diagnostic centres included in this study were very similar and showed a significant increase in the sensitivity of Ziehl–Neelsen staining after digestion of sputum (Tables 1 and 2). The major advantage of the NaOCl method is the higher density of bacilli per microscope field obtained after concentration of the sample and the reduction of debris present in sputum, leaving a free field for bacterial detection. This facilitates the examination of the slides and reduces the time required for microscopy. The major disadvantage is that sample preparation is more laborious and requires access to a centrifuge. Many diagnostic centres are already equipped with centrifuges and our results indicate that the necessary RCF can easily be achieved by a low-cost table-top centrifuge or even by a hand-driven model.

Acid-fast smear examination does not discriminate between tubercle bacilli and other mycobacteria. However, this is not a major problem in developing countries: firstly, because the vast majority of patients with AFB in sputum have TB and, secondly, because other mycobacteria are usually not present in sufficient concentration in sputum to be detected by direct microscopy (LIPSKY *et al.*, 1984). In our study we cultured only one strain of mycobacteria other than *M. tuberculosis*, and this sputum sample was negative by direct microscopy.

With the occurrence of multi-drug resistant TB, the risk of laboratory infection has become a major concern. Few laboratories in developing countries have adequate safety cabinets and therefore many technicians are increasingly reluctant to work with TB samples. Use of the NaOCl method would definitely lower the risk of laboratory infection.

Since NaOCl kills the mycobacteria, this technique cannot be used on samples intended for culture, but the method is strongly recommended for all laboratories that perform direct microscopy only. A two-fold increase in the number of positive samples would improve the efficiency of TB control programmes and make the NaOCl method cost-effective. Introduction of this method does not limit the need for good training, supervision and careful quality control of all laboratory procedures.

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