

IMPROVED METHOD OF DIRECT MICROSCOPY FOR DETECTION OF ACID-FAST BACILLI IN SPUTUM

Wah Wah Aung¹, Mar Mar Nyein¹, Ti Ti² and Win Maung²

¹Bacteriology Research Division, Department of Medical Research (Lower Myanmar), Yangon; ²Union Tuberculosis Institute, Department of Health, Yangon, Myanmar

Abstract. Microscopy of direct smears for acid-fast bacilli (AFB) is the most commonly used method for diagnosis of tuberculosis. However, direct microscopy of sputum, though rapid, has low sensitivity and there is a need for improved methods. Sputum samples were collected from patients attending the Union Tuberculosis Institute, Yangon. The microscopy of smears made directly from sputum were compared with the microscopy after liquefaction of sputum with household bleach (NaOCl) and concentration of bacteria by centrifugation. Out of 948 samples, 248 samples (26.2%) were positive for acid-fast bacilli by direct microscopy and 293 samples (30.9%) were positive for acid-fast bacilli by the household bleach method. There was a significant increase in the number of acid-fast bacilli positive samples by the house-hold bleach method ($p < 0.05$). The method is simple and cheap. As a disinfectant, household bleach has the advantage of lowering the risk of laboratory infection.

INTRODUCTION

Tuberculosis is a major health problem in most developing countries. Approximately, one third of the world's population has been infected with *Mycobacterium tuberculosis* and there are six to eight million new cases of disease and two to three million deaths each year. The spread of human immunodeficiency virus (HIV) has further aggravated the situation. The number of patients infected with both HIV and tuberculosis is estimated to be 3.8 million (Porter, 1995).

The diagnosis of tuberculosis is hampered by the slow growth of *M. tuberculosis*. Culture of mycobacteria is the reference method for detection of tubercle bacilli but it is slow and needs special safety procedures in the laboratory. Serologic techniques may be useful in some clinical situations but both their sensitivity and specificity are unsatisfactory (Daniel, 1989). Nucleic acid amplification methods are most promising, but the technology is not applicable to control programs in developing countries. So microscopy of direct smears for acid-fast bacilli (AFB) remains "the gold standard" for most laboratories. The

specificity of positive acid fast smear, is high (99.3% to 99.9%) but the sensitivity is low, ranging from 22% to 78% (Pfaller, 1994).

Therefore, there is a need to detect methods for improvement of diagnosis of pulmonary tuberculosis by techniques that are appropriate for control programs in developing countries.

Digestion of sputum with household bleach (sodium hypochlorite, NaOCl) gave the best recovery of AFB (Coper and Nelson, 1949) and concentration of bacilli by centrifugation of sputum increased the recovery of mycobacteria (Ratnam and March, 1986).

The NaOCl technique for preparation of smears was described in 1942 and the method is included in most Laboratory Manuals (Vestal, 1978; Ebersole, 1992). However the method is not widely used to our knowledge in Myanmar. So in this study we have compared the standard Ziehl-Neelsen (ZN) direct staining of sputum smear with household bleach (NaOCl) digestion of sputum, concentration of bacteria by centrifugation and ZN staining of the sediments (Gebre *et al*, 1995; Habeenzu *et al*, 1998).

MATERIALS AND METHODS

Collection of specimens

A total of 948 sputum specimens were collected from patients attending Union Tuberculosis Institute, Zone 1, Yangon from February 2000 to September 2000. Both new cases and retreatment cases, irrespective of age and sex were included in this study.

The sputum was examined by two methods, direct smear microscopy and household bleach concentration method at the Department of Medical Research, Yangon.

Direct smear preparation

Slides for direct smears were prepared from purulent part of sputum. Then the smears were stained by Ziehl-Neelsen method.

Digestion of sputum with household bleach (NaOCl) and concentration by centrifugation

The remaining sputum (1-2 ml) was transferred to 10 ml screw capped tube and mixed with equal volume of commercially available household bleach (sodium hypochloride, NaOCl). The mixture was incubated at room temperature for 10-15 minutes and shaken at regular intervals.

Then 8 ml of distilled water were added and the samples were centrifuged at 3000g for 15-20 minutes. The supernatant was discarded and the pellets was suspended in few drops of remaining fluids. Slides were prepared from the suspended sediment, air-dried, heat fixed and stained by Ziehl Neelsen method.

Microscopic examination and interpretation of results

The sputum smears were examined under oil immersion lens of ordinary light microscope. The number of acid-fast bacilli seen on the smears were recorded according to the recommendation by WHO (Table 1). The AFB positive slides of both methods were counterchecked by two experienced micros-

copists. The data of clinical diagnosis and management were collected to compare with the microscopic diagnosis.

RESULTS

Nine hundred and forty-eight patients were examined, 248 (26.2%) had AFB positive smears using the direct Ziehl-Neelsen (ZN) staining. After household bleach (NaOCl) treatment and centrifugation, the number of smear positive patients was increased to 293 (30.9%).

All AFB positive smears by direct ZN method were also positive by household bleach concentration method. Forty-five AFB negative samples by direct ZN method were positive by household bleach concentration method. The increase in numbers of AFB positive samples by household bleach method was significant ($p=0.02$, $\chi^2=5.24$) (Table 2).

Table 1
Reporting on AFB microscopy.

Number of bacilli seen	Result reported
None per 100 oil immersion fields	Negative
1-9 per 100 oil immersion fields	Scanty, report exact number
10-99 per 100 oil immersion fields	1+
1-10 per oil immersion field	2+
>10 per oil immersion field	3+

Table 2

Results of Ziehl Neelsen staining of smears prepared directly from sputum and smears prepared after digestion of sputum with household bleach and concentration by centrifugation.

Direct ZN staining	Smear after household bleach treatment and centrifugation		
	Positive	Negative	Total
Positive	248	0	248 (26.2%)
Negative	45	655	700
Total	293 (30.9%)	655	948

($p=0.02$, $\chi^2=5.24$)

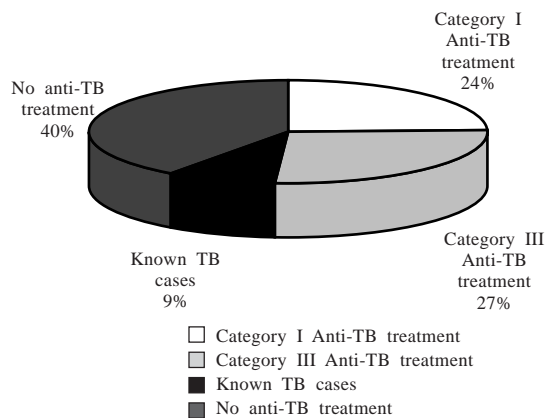


Fig 1—Clinical management of 45 samples which were acid fast bacilli positive only by household bleach-concentration method.

There was also a marked increase in the average number of AFB seen per microscope field in the smears prepared after digestion with household bleach and concentration by centrifugation. Smears which were graded 1+= by direct method increased to 2+= or 3+= after concentration and household bleach treatment. The microscope background field appeared more clear as debris was reduced.

Clinical management of 45 samples which were positive only after household bleach method indicates that 11 (24.4%) samples were given new smear positive pulmonary tuberculosis treatment (Category I) as they were AFB positive in 2nd and 3rd time direct ZN examination. Twelve (26.6%) cases were treated as new smear negative pulmonary tuberculosis (Category III) and 4 (8.9%) cases were known pulmonary tuberculosis in continuation phase of antituberculosis drug treatment (Fig 1).

DISCUSSION

In developing countries, microscopy of sputum is by far the fastest, cheapest and most reliable method for the diagnosis of pulmonary tuberculosis. While a positive smear will alert the physician to a probable AFB infection, a false negative smear may lead to

a false sense of security. Thus although simple, rapid and economical, the estimated detection limit of microscopy is 10^4 bacilli/ml of sputum. Smear sensitivity may be influenced by a variety of factors including type of specimen, efficiency of decontamination and concentration procedures, the type of staining procedures and experience of microscopists. So the range of acid fast smear sensitivity is quite wide from 22% to 78% (Pfaller, 1994).

Digestion of sputum and concentration of bacilli by centrifugation increases the recovery rate of mycobacteria (Ratnam and March, 1986). Improved recovery of mycobacteria after treatment with NaOCl might be attributable to changes in surface properties of the mycobacteria (*ie* charge and hydrophobicity), and for denaturing of sputum constituents leading to flocculation and subsequent increased sedimentation rate of mycobacteria (Gebre *et al*, 1995).

Gebre *et al* (1995) also reported that the use of NaOCl method increased the numbers of samples positive for acid fast bacilli by more than 100%. In the study by Habeenzu *et al* (1998), the use of NaOCl was found to increase the smear sensitivity from 43.4% to 76.3% with the specificity of 100%.

In our study, we could not calculate the sensitivity and specificity as mycobacterial culture cannot be used as a standard method. But the increase in the numbers of AFB positive samples by household bleach treatment and concentration method was significant ($p < 0.05$, $\chi^2 = 5.24$).

By analysing the clinical management of 45 cases which were AFB positive only after household bleach method, 27 cases (60%) were given antituberculosis drug treatment. Out of these 27 cases, 11 cases were treated as new smear positive pulmonary tuberculosis (Category I) as they were positive on 2nd and 3rd time sputum examination by direct ZN method.

This indicates that three sputum specimens are necessary to confirm the diagnosis of tuberculosis. WHO described that if only

a single specimen is taken, nearly 20% of smear positive patients will be missed. The second specimen will identify most of the remaining patients. A third specimen which is obtained at the same time the second specimen is submitted helps to confirm the diagnosis.

Twelve cases were treated as new smear negative pulmonary tuberculosis treatment (Category III) as they were diagnosed by chest X-ray. So the role of chest X-ray is important also.

The major advantage of NaOCl method is the higher density of bacilli per microscope field obtained after concentration of the sample and the reduction of debris present in the sputum leaving a free field for bacteria detection. This facilitates the examination of the slides and reduced the time required for microscopy.

NaOCl is cheap and available almost anywhere as household bleach. Moreover this method has the advantage that 5% NaOCl makes the bacilli nonviable yet they remain acid fast (Vestal, 1978). So the use of NaOCl lowers the risk of laboratory infection. The fact to be noted is that NaOCl will sterilize the sputum within 15 minutes and subsequently will cause disintegration of the bacilli if allowed to act too long, therefore smears should be made, stained and examined promptly.

The major disadvantage is that it requires access to a centrifuge. Gebre *et al* (1995) described that relative centrifugal force needed for sedimentation of mycobacteria was lower after digestion with NaOCl and the necessary relative centrifugal force can easily be achieved by low cost table top centrifuge or even by hand driven model.

Since NaOCl kills mycobacteria, this technique cannot be used for samples intended for culture, but the method can be used in laboratories that perform microscopy only. Introduction of this method is feasible and could make a positive impact on the effectiveness of TB control programs.

ACKNOWLEDGEMENTS

We are grateful to U Mya Than Tun, Daw Naw Sai Khu Yin, all the doctors and staff of Union Tuberculosis Institute, TB Zone 1, Yangon, for their support. We would like to thank Dr Maung Maung Toe, Dr Kyu Kyu Than and Daw Aye Aye Thein, Department of Medical Research (Lower Myanmar) for their advice.

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