EVALUATION OF SPUTUM STAINING BY MODIFIED COLD METHOD AND COMPARISON WITH ZIEHL-NEELSEN AND FLUOROCHROME METHODS FOR THE PRIMARY DIAGNOSIS OF TUBERCULOSIS

Unchalee Tansuphasiri[1] and Booncherd Kladphuang[2]

[1]Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok 10400, Thailand; [2]Tuberculosis Division, Department of Communicable Disease Control, Ministry of Public Health, Bangkok 10400, Thailand

Abstract. An improved acid-fast staining technique for sputum examination for the primary diagnosis of tuberculosis is described. The technique was modified and simplified by the elimination of heating and by combining the stages of counterstaining : making the technique easier and safer, with less risk of phenol aerosols. The efficiency of this method was evaluated by comparison with two conventional methods, Ziehl-Neelsen (ZN) staining and fluorochrome staining; culture was deemed the gold standard for tuberculosis diagnosis. Of the 392 sputum samples examined, 22.7%, 19.4% and 22.9% were positive by the ZN, fluorochrome and modified cold (MC) staining methods respectively. In comparison with culture results, the sensitivities of ZN, fluorochrome and MC methods were 68.9%, 59.7% and 70.6% respectively; the specificities were 97.4%, 98.2% and 97.8% respectively and the efficiencies were 88.8%, 86.5% and 89.5% respectively. The fluorochrome method was statistically less sensitive than the ZN and MC ($p < 0.05$), but no significant differences between the ZN and MC were found ($p > 0.05$). The results of the MC and ZN methods were in close agreement (97.2%); the slides stained by these techniques could be stored for a long time and the staining reagents were stable for several weeks. In conclusion, the MC method proved to be a valuable alternative to ZN staining for the primary diagnosis of tuberculosis.

INTRODUCTION

The importance of tuberculosis (TB) as a global public health concern has been emphasized by the high incidence rates and the recent outbreaks of multidrug-resistant tuberculosis, particularly in HIV-positive individuals (Kochi, 1991). It is the leading infectious cause of death worldwide, being responsible for 3 million deaths a year. The World Health Organization has calculated that the number of annual deaths could rise from 3 to 4 million by the year 2004 (WHO, 1994). We need urgent improvements to the implementation of existing strategies for tuberculosis control, with an emphasis on early diagnosis and effective treatments. The traditional diagnostic tools, apart from a thorough clinical examination, are: chest X-ray, which is sensitive but non-specific; sputum microscopy, which is specific but of limited sensitivity; and culture, which is more sensitive but requires more time and expense.

In Thailand, TB activities at the provincial level are conducted mainly by provincial and district hospitals. Communities and health centers have minor roles in carrying out TB services because health workers have insufficient experience with diagnostic testing. Sputum smear microscopy is the most cost-effective method and is used throughout the country for the primary diagnosis of pulmonary tuberculosis and for case finding and the assessment of treatment: the method is quick and simple. Of the available staining techniques for direct microscopy, the Ziehl-Neelsen (ZN) and the auramine O fluorescence acid-fast stains are the most widely used; however, ZN requires heating to allow the penetration of the dye into

the cell and thus gives rise to an aerosol of phenol; fluorochrome staining is much more sensitive than ZN because the smears are examined with a fluorescence microscope using a low power objective, making the method appropriate for use in central or large laboratories with heavy workloads but less tenable in small laboratories because of the associated cost, equipment maintenance and lower specificity (Toman, 1979).

Several cold staining methods have been described (Deshmukh *et al*, 1966). Kinyoun is a well known cold stain method which requires a high concentration of basic fuchsin and phenol or the addition of a detergent (tergitol No.7) thereby avoiding the need for heat; however, the method has several disadvantages: it is uneconomical, time consuming, and uses an unstable stain, restricting its use to major centers. In this study, a new, cheaper, safer and easier cold staining method is described for the demonstration of acid-fast bacilli (AFB) in sputum samples; the efficiency of this method was evaluated by comparison with two conventional staining methods, ZN and fluorochrome, by using the result of culture as the gold standard for the primary diagnosis of tuberculosis.

MATERIALS AND METHODS

Sputum specimens

Either spot or collection sputum was obtained from 406 newly diagnosed tuberculosis patients of the Central Chest Clinic, Tuberculosis Division, Department of Communicable Disease, Ministry of Public Health, Bangkok. These patients were over 15 years old and had chest symptoms suggestive of tuberculosis. The samples were collected prior to the administration of medication; samples were each of at least 3 ml.

Smear preparation

Three slides were prepared from each sputum specimen by direct smear from the purulent or mucopurulent portion of the sputum; sputum was spread evenly over an area of 1 x 2 cm. The smears were air dried, and heat-fixed on a hot plate for a few minutes; and the fixed smears (3) of each specimen were then stained: one by ZN; one by modified cold (MC); one by fluorochrome.

Staining procedure

Ziehl-Neelsen stain: The procedure was that described previously by Smithwick (1976) and Kent and Kubica (1985). The fixed smears were flooded with a solution prepared by dissolving 0.3 g of basic fuchsin in 10 ml of ethanol; this solution was diluted to 100 ml with aqueous 5% phenol. The smear was gently heated until steaming with a Bunsen burner for 5 minutes. The smear was then rinsed with water and decolorized with 3% acid-alcohol and allowed to stand for 2 minutes. After standing, the smear was rinsed with water and counterstained with 0.1% methylene blue for 10 seconds. The slide was finally rinsed with water and air dried before examination. The stained smears were scanned with a x100 oil immersion lens for the presence of red thin rods or coccobacilli.

Fluorochrome stain: The auramine O fluorescence acid-fast stain procedure followed the method described by Bennedsen and Larsen (1966). Fixed smears were flooded with auramine-phenol and allowed to stand for 10 minutes. Smears were then rinsed with water and decolorized with 1% acid-alcohol and left to stand for 5 minutes. A second rinse with water was followed by counterstaining with 0.1% potassium permanganate for 10 seconds. Slides were finally rinsed with water, air dried, and examined by fluorescence microscopy using a x10 objective lens for the presence of bright yellow-fluorescing bacilli.

Modified cold (MC) stain: Fixed smears were flooded with a solution prepared by dissolving 0.3 g of basic fuchsin in 10 ml of ethanol; this solution was diluted to 100 ml with aqueous 5% phenol. The smears were allowed to stand for 10 minutes and then rinsed with water. Following rinsing, the slide was coun-

terstained with modified methylene blue for 2 minutes (dissolving 1 g of methylene blue in 20 ml of sulfuric acid, 30 ml absolute alcohol and 50 ml distilled water). The slide was finally rinsed with water and dried before examination. The stained smears were scanned with a x100 oil immersion lens for the presence of red thin rods or coccobacilli.

Microscopy reports

Smear examination reports included quantification by expressing the actual number of bacilli seen per field or by giving a 1+ to 3+ rating according to the convention of the American Thoracic Society (1981).

Culture and identification

Sputum samples were decontaminated by the sputum swab culture method of Nassua (1958): 2 swabs were used to stir the sputum and then put into a test tube. Five percent oxalic acid was added to 2/3 of the test tube, which was then left to stand at room temperature for 25 minutes. Both swabs were then transferred to a test tube containing 25 ml of 5% sodium citrate, left to stand for 10 minutes, and then inoculated onto two slants of Lowenstein-Jensen (L-J) medium. The inoculated slants were incubated at 37ºC for 8 weeks and examined weekly for growth. Cultured mycobacteria were identified from the L-J slants by conventional methods (Kent and Kubica, 1985).

RESULTS

A total of 406 sputum specimens from newly suspected tuberculosis cases were examined in this study. Each sputum sample was divided into 4 aliquots: three aliquots were stained by either ZN, fluorochrome or MC technique; one aliquot was cultured on L-J medium. The contamination of 14 samples (3.5%) left 392 samples for study. Most samples were from males (70.4%; female 29.6%) whose mean age was 42.05 ± 15.06 years (females 43.3 ± 17.05 years).

Sputum staining results

Table 1 shows the percentages of sputum staining results obtained by the three methods. The positive results (rated 1+, 2+, 3+) given by ZN were 14.0%, 4.3% and 4.3% respectively; for the fluorochrome stain, the positive results were 5.9%, 5.4% and 8.2% respectively; for the MC stain, the positive results were 11.5%, 6.1% and 5.4% respectively. The negative results by the ZN, fluorochrome and MC methods were 77.3%, 80.6% and 77.0% respectively.

Validities of the three methods for the diagnosis of pulmonary tuberculosis

Table 2 shows the validities of the three staining methods using culture as the gold standard for tuberculosis diagnosis. ZN gave sensitivity, specificity, positive and negative

Table 1
Percentages of sputum staining results by the Ziehl-Neelsen, fluorochrome and modified cold staining methods (392 sputum specimens).

Result by grading	Sputum staining method		
	Ziehl-Neelsen (%)	Fluorochrome (%)	Modified cold (%)
Negative (-ve)	303 (77.3)	316 (80.6)	302 (77.0)
1+	55 (14.0)	23 (5.9)	45 (11.5)
2+	17 (4.3)	21 (5.4)	24 (6.1)
3+	17 (4.3)	32 (8.2)	21 (5.4)
Total	392 (100.0)	392 (100.0)	392 100.0)

Table 2

Validities of the Ziehl-Neelsen, fluorochrome and modified cold staining methods for the primary diagnosis of pulmonary tuberculosis using culture result as the gold standard.

Results by the following staining methods	Culture result			Sensitivity (%)	Specificity (%)	Predictive value (%)		Efficiency (%)	Youden's index
	Positive	Negative	Total			Positive	Negative		
Ziehl-Neelsen staining				68.9	97.4	92.1	87.8	88.8	0.66
Positive	82	7	89						
Negative	37	266	303						
Total	119	273	392						
Fluorochrome staining				59.7	98.2	93.4	84.8	86.5	0.58
Positive	71	5	76						
Negative	48	268	316						
Total	119	273	392						
Modified cold staining				70.6	97.8	93.3	88.4	89.5	0.69
Positive	84	6	90						
Negative	35	267	302						
Total	119	273	392						

Table 3

Comparison of Ziehl-Neelsen with fluorochrome staining by grading of the AFB-smear positivity.

Result by Ziehl-Neelsen staining	Result by fluorochrome staining				Total
	Negative	1+	2+	3+	
Negative	298	3	1	1	303
1+	18	15	14	8	55
2+	-	4	3	10	17
3+	-	1	3	13	17
Total	316	23	21	32	392

K = 0.552, Z = 8.263, $p < 0.01$

predictive values and efficiency of 68.9%, 97.4%, 92.1%, 87.8% and 88.8% respectively. For fluorochrome, these measures were 59.7%, 98.2%, 93.4%, 84.8% and 86.5% respectively; for MC, they were 70.6%, 97.8%, 93.3%, 88.4% and 89.5% respectively. The false positive results produced by the ZN, fluorochrome and MC methods were 2.6%, 1.8% and 2.2% respectively; the false negative results were 31.1%, 40.3% and 29.4% respectively.

Correlation of the three sputum staining methods

In this study, 392 sputum samples were

Table 4

Correlation between the Ziehl-Neelsen and the fluorochrome staining in slide reading of AFB-smear positive and negative.

Ziehl-Neelsen staining	Fluorochrome staining		Total
	Positive	Negative	
Positive	71	18	89
Negative	5	298	303
Total	76	316	392

Mc Nemar's $\chi^2 = 7.35$, d.f. = 1, $p = 0.007$
K = 0.823, Z = 11.502, $p < 0.01$

Table 5
Comparison of the modified cold staining with the fluorochrome staining by grading of the AFB-smear positivity.

Result by modified cold staining	Result by fluorochrome staining				Total
	Negative	1+	2+	3+	
Negative	296	3	2	1	302
1+	19	16	5	5	45
2+	1	2	12	9	24
3+	-	2	2	17	21
Total	316	23	21	32	392

K = 0.635, Z = 9.797, $p < 0.01$

examined in parallel. The total yield of positive results was only slightly higher by ZN: 89 (22.7%) positive compared with 76 (19.4%) positive by the fluorochrome method. The grading result agreement rate was 83.9% (329/392), as shown by the data in Table 3 and data from a simplified version of Table 4, obtained by pooling the data under negative (N) and those under 1+, 2+ and 3+; 369 of 392 pairs of smears gave identical results (94.1% agreement) with a statistically significant difference ($p < 0.05$).

When comparing the fluorochrome method with the MC method, the scores were definitely higher for MC. The total yield of positive results was 90 (23.0%) by the MC method but 76 (19.4%) by the fluorochrome method. The grading result agreement rate was 87.0% (341/392), as shown in Table 5. Disregarding the scores shown in Table 6, 366 of 392 pairs of smears gave identical results (93.4% agreement) with a statistically significant difference ($p < 0.05$).

Comparing the ZN method with the MC method, the total yield of positive results was only slightly higher by MC method: 90 (23.0%) positive as opposed to 89 (22.7%) positive by ZN. The grading result agreement rate was 91.6% (359/392), as shown in Table 7. Disregarding the scores shown in Table 8, 381 of 392 pairs of smears gave identical results (97.2% agreement) with no statistically significant difference ($p > 0.05$).

Table 6
Correlation between the modified cold staining and the fluorochrome staining in slide reading of AFB-smear positive and negative.

Modified cold staining	Fluorochrome staining		Total
	Positive	Negative	
Positive	70	20	90
Negative	6	296	302
Total	76	316	392

Mc Nemar's $\chi^2 = 7.54$, d.f. = 1, $p = 0.006$
K = 0.801, Z = 11.230, $p < 0.01$

DISCUSSION

In Thailand, TB has recently re-emerged as a public health problem after its considerable decline during the past decades. Re-emergent TB is fuelled by the pandemic of HIV and AIDS and by single- and multidrug resistance. HIV renders a person infected by *M. tuberculosis* much more likely to develop overt tuberculosis, the evolution of which is considerably accelerated. At present, 8-10% of all cases of tuberculosis worldwide are related to HIV infection; this association is much more marked in many African countries in which co-infection rates may be 20% or more (Raviglione and Nunn, 1997). The main stratagem used in

Table 7
Comparison of the Ziehl-Neelsen with the modified cold staining by grading of the AFB-smear positivity.

Result by modified cold staining	Result by Ziehl-Neelsen staining				Total
	Negative	1+	2+	3+	
Negative	297	5	-	-	302
1+	6	37	2	-	45
2+	-	12	10	2	24
3+	-	1	5	15	21
Total	303	55	17	17	392

K = 0.780, Z = 12.176, p < 0.01

Table 8
Correlation between the Ziehl-Neelsen and the modified cold staining in slide reading of AFB-smear positive and negative.

Modified cold staining	Ziehl-Neelsen staining		Total
	Positive	Negative	
Positive	84	6	90
Negative	5	297	302
Total	89	303	392

Mc Nemar's χ^2 = 0.09, d.f. = 1, p = 0.763
K = 0.920, Z = 13.441, p < 0.01

tuberculosis control is the reduction of the mode of transmission, thereby reducing mortality and morbidity. Case finding is another essential stratagem used to control tuberculosis: its objective is the identification of the sources of infection in a community – in the case of tuberculosis, people who are discharging tubercle bacilli. By rendering patients noninfectious by using chemotherapy, the chain of patient-to-patient transmission of tubercle bacilli is broken.

The definitive diagnosis of tuberculosis depends on the isolation and identification of *M. tuberculosis*. Culture remains the gold standard diagnostic method for tuberculosis: it is a specific and sensitive process that is necessarily lengthy because of the slow growth of *M. tuberculosis*, which requires weeks before a positive culture can be identified; moreover culture requires at least a moderately well-equipped laboratory. There are several automated systems for the quicker detection of the growth of *M. tuberculosis*: their cost and complexity restrict their use to major centers.

Recently, attention has turned to nucleic acid technology: the polymerase chain reaction (PCR) and related techniques are rapid, specific and sensitive. However, these methods require more sophisticated laboratory methods and are not being used for the routine diagnosis of tuberculosis.

In Thailand, the TB laboratory services, at all levels down to that of district hospitals, is able to conduct smear microscopy; culture examination and sensitivity testing are available only at the central level and in certain TB centers and provincial hospitals. For this reason, microscopic examination for AFB has been the mainstay of the diagnosis of pulmonary tuberculosis while the results of sputum cultures were pending. One advantage of the AFB sputum smear is its close correlation with infectiousness: patients who are sputum smear positive/culture positive are far more likely to be infectious than persons who are culture positive but smear negative (Narain *et al*, 1971).

The usual staining technique has been the ZN method which is the most common laboratory technique for staining AFB and is ac-

cepted as the conventional method. However, the method requires controlled heating for its success, and there are certain disadvantages, *eg* multistage staining, a cumbersome procedure and the discomfort caused by aerosols of phenol. In this study, an improved acid-fast staining technique for the staining of sputum, the modified cold staining method, was described. This procedure used the familiar ZN staining solution, without an increase in the concentration of the basic fuchsin-phenol solution. The stages of staining were reduced: no heating and a combined counterstaining stage – making the method faster and safer than ZN. The method makes economical use of the laboratory and materials and would be useful in large scale case finding programs as an alternative to ZN staining.

When comparing these staining methods with culture and biochemical identification of *M. tuberculosis*, the gold standard for the diagnosis of tuberculosis, it was found that ZN and MC were able to detect tuberculosis by sputum staining with sensitivities as high as 68.9% and 70.6% respectively with no statistically significant difference ($p > 0.05$). The sensitivity determination was in accordance with that of Tan (1962) who showed that the sensitivities of ZN and his cold stain method were 60.1% and 65.1% respectively. The success of any staining technique depends on the ability of the dye to penetrate uniformly the cell wall of tubercle bacilli through their surface coating of waxy substances. With ZN stain, this is achieved by heating the slide during the staining process; the MC method is slightly longer than the conventional ZN because exposure favors the uniform penetration of the dye through the cell wall.

The sensitivity of the fluorochrome stain (59.7%) was slightly less than that of the other methods. Hence only positive yields of the fluorochrome method were compared with those of the ZN and MC method and showed a statistically significant difference ($p < 0.05$). Reading errors in this study might have included interpersonal and intrapersonal variation and may have varied with both the experience and the workload of the laboratory technicians. In fact, fluorochrome staining might give slightly higher sensitivity than, or be no different to the other methods. However, the varied sensitivity of each sputum staining method depended on the probability of finding or not finding AFB in smears prepared from specimens containing bacilli in low, intermediate and high concentrations. In our study we divided the sputum into 4 aliquots due to the fact that the concentration of bacilli in these specimens varied; moreover each sample or loopful of specimen contains the same amount of bacilli spread evenly over the smear. Of the three staining methods, fluorochrome gave the most false negative results (40.2%), compared with 31.1% for ZN and 29.4% for the MC method. False negative results were commonly due to deficiencies in the preparation of the smear, such as too little material spread on the slide or a smear's being too thin or too thick. Scanning and reading may have been subject to observer error, which is mainly due to visual or psychological factors.

When compared with the culture results, the false positive results obtained by all three staining methods were not different (2.6% by ZN; 1.8% by fluorochrome; 2.2% by the MC method). These results suggested that occasionally a sputum specimen or a smear may contain particles that are acid-fast: these particles may sometimes resemble tubercle bacilli, *ie* mycobacteria other than *M. tuberculosis*, or the precipitate of staining, which hampers reading or occasionally misleads an inexperienced microscopist. However, the result was in accordance with those of previous reports (Marraro *et al*, 1975; Burdash *et al*, 1976).

The specificities of the ZN, fluorochrome and MC methods were 97.4%, 98.2% and 97.8% respectively. Although the true negative rate was very high in all three staining methods, false-positive results were comparatively rare and therefore a positive smear could be relied upon as a good diagnostic indicator: a finding similar to those of previous studies (Marraro *et al*, 1975; Boyd and Marr, 1975; Burdash *et*

al, 1976). In this study the positive predictive values and the negative predictive values were high for all three staining methods. The positive predictive values of the ZN, fluorochrome and MC methods were 92.1%, 93.4% and 93.3% respectively and the negative predictive values were 87.8%, 84.8% and 88.4% respectively. These data suggested that these methods have sufficient validity to predict the presence or absence of the disease in a featured population.

The MC method was as reliable as the ZN method in retaining color after storage of the stained slides for 4, 8 and 16 weeks (data not shown). Storage should be for no longer than 16 weeks because the quality and efficiency of stained slides might decrease with longer storage. The staining reagents of both methods were stable for several weeks (data not shown); enabling the stocking of aliquots of staining reagents for more than 16 weeks without the loss of efficiency. The staining reagents remained clear and no precipitation occurred.

In conclusion, we have demonstrated that the efficiency of all sputum staining methods reached the satisfactory level of performance expected: the sensitivity and specificity were high. Of the three staining methods, the fluorochrome was statistically less sensitive than ZN and MC ($p < 0.05$); no significant differences between the ZN and MC ($p > 0.05$) were found. The results for MC and ZN were in close agreement (97.2%); the MC method appears to be a practicable and effective alternative to ZN for smear staining during the primary diagnosis of tuberculosis – the method was easy, safe, and inexpensive.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Vallop Payanandana, formerly the Director of the Tuberculosis Division, Department of Communicable Disease Control, Ministry of Public Health, for his helpful comments, and to his colleagues in the Laboratory Section, Tuberculosis Division, for their kind help with specimen collection, culture and identification.

REFERENCES

American Thoracic Society. Diagnostic standards and classification of tuberculosis and other mycobacterial diseases. American Lung Association Publication. *Am Rev Resp Dis* 1981; 123: 343.

Bennedsen J, Larsen SO. Examination of tubercle bacilli by fluorescence microscopy. *Scand J Respir Dis* 1966; 47: 114-20.

Boyd JC, Marr JJ. Decreasing reliability of acid - fast smear techniques for detection of tuberculosis. *Ann Intern Med* 1975; 95: 489-92.

Burdash NM, Manos JP, Ross D, Bannister ER. Evaluation of the acid - fast smear. *J Clin Microbiol* 1976; 4: 190-1.

Deshmukh SR, Mantri SB, Kendre PB, Nagoba BS. A comparison of sputum examination for acid-fast bacilli by modified Schaeffer and Fulton stain, Ziehl-Neelsen stain and cold stain. *Indian J Med Res* 1966; 103: 294-5.

Kent PT, Kubica GP. Public health mycobacteriology: A guide for the level III laboratory. Centers for Diseases Control, Atlanta. 1985.

Kochi A. The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle* 1991; 72: 1-6.

Marraro RV, Ragers EM, Roberts TH. The acid - fast smear: fact or fiction? *J Am Med Technol* 1975; 37: 277-9.

Narain R, Rao MS, Chandrasekhar P, *et al*. Microscopy positive and microscopy negative cases of pulmonary tuberculosis. *Am Rev Respir Dis* 1971; 103: 761-73.

Nassua E. Sputum swab culture: simple method of isolation tubercle bacilli from sputum. *Tubercle* 1958; 39: 18-23.

Raviglione M, Nunn PP. Epidemiology of tuberculosis. In: Zumla A, Johnson M, Miller RF, eds. AIDS and respiratory medicine. London: Chapman and Hall, 1997: 117-43.

Smithwick RW. Laboratory manual for acid fast microscopy. 2nd ed. Atlanta, Georgia: US Department of Health, Education and Welfare Public Health Service/Center for Disease Control, 1976.

Tan TH. Simple and rapid cold stain method for acid fast bacteria. *Am Rev Respir Dis* 1962; 85: 753-4.

Toman K. Tuberculosis case-finding and chemotherapy: question and answers. Geneva: WHO, 1979.

World Health Organization. TB a global emergency. WHO report on the TB epidemic. Geneva: WHO, 1994.