

Evaluation of Different Staining Techniques (Ziehl Neelsen Stain, Kinyoun Stain, Modified Cold Stain, Fluorochrome Stain) for The Diagnosis of Pulmonary Tuberculosis

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Abstract

Introduction	Bacteriological examination of sputum is the cornerstone in the diagnosis of pulmonary tuberculosis in the developing world. The risk of spread of infection and emergence of drug resistant strain has created the need of rapid, sensitive and specific diagnostic test.
Objectives	This prospective study was performed in Tuberculosis research laboratory, Department of Microbiology, BPKIHS, Dharan with an objective to evaluate different staining techniques (Ziehl-Neelsen (ZN), Kinyoun, Modified cold (MC), Fluorochrome) for detection of acid fast bacilli (AFB) for diagnosis of pulmonary tuberculosis.
Methods	The study was cross-sectional study. All the samples were processed by modified Petroff's method. From each sample four smears were prepared and were stained with four different staining techniques: ZN, Kinyoun, MC, and Fluorochrome respectively. Culture was employed as gold standard for tuberculosis diagnosis.
Results	A total of 1365 specimens from 500 patients were analyzed. One hundred nine patients (21.8%) were diagnosed as having TB by culture. The positive yield found with the staining techniques were 14.2 percent by ZN, 13.8 percent by Kinyoun, 14.4 percent by MC and 17.6 percent by Fluorochrome staining methods. With reference to culture, sensitivity of ZN, Kinyoun, MC and Fluorochrome were found to be 57.8 percent, 56 percent, 59.6 percent, and 71.6 percent respectively. The specificity in ZN, Kinyoun and Fluorochrome methods was 98 percent and that in MC as 98.2 percent. The positive predictive value of ZN, Kinyoun, MC, Fluorochrome was found to be 88.7 percent, 88.4 percent, 90.3 percent and 90.7 percent and the negative predictive value of ZN, Kinyoun, MC, Fluorochrome was found to be 89.3 percent, 88.9 percent, 89.7 percent and 92.5 percent respectively.
Conclusion	The fluorescent staining method was found most reliable out of the other staining techniques. The MC method also could be viable alternative to ZN and Kinyoun for primary diagnosis of tuberculosis.
Keywords	M. tuberculosis, Ziehl-Neelsen, Kinyoun, Modified cold, Fluorochrome

Introduction

Tuberculosis (TB) is humanity's greatest killer which is out of control in many parts of the world. The disease is preventable and treatable but it has been grossly neglected and no country is immune to it. The diagnosis of TB infection is vital both clinically and epidemiologically. The history of sputum examination dates back to March 24, 1882 when Robert Koch discovered the tubercle bacillus and confirmed the bacterial etiology of TB. Acid-fast bacilli (AFB) microscopy which is a means of detecting and screening of pulmonary TB, has been used worldwide and it remains as a mainstay of case finding^{1,2}. The finding of

AFB in sputum establishes a presumptive diagnosis of TB and is crucial to guide treatment, to limit person to person spread of the disease and to assess the degree of the activity of the disease. The highest priority for TB control is given to the identification of patients with sputum smear positive (SS+) PTB and cure of the infectious cases because this helps in the reduction of the mode of transmission thereby reducing overall mortality and morbidity^{3,4}.

Presently, two types of acid-fast stains are used in clinical mycobacteriology laboratories. One type is carbol

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fuchsin (Ziehl Neelsen [ZN] or Kinyoun methods), and the other is Fluorochrome either auramine phenol (AP) or auramine-rhodamine (AR). The present ZN method has evolved from Koch's original alkaline methylene blue-based method following significant modifications. Kinyoun cold staining is a modification of the classical ZN method that excludes the heating step during staining and therefore uses a higher concentration of carbol fuchsin⁵. There are several other modified techniques for staining acid fast bacilli and one of these is Modified cold (MC) stain. MC stain is two steps cold staining method which requires concentrated carbol fuchsin as a primary stain and Gabbet methylene blue as counter stain. This simple staining has added advantage of non requirement of heat as well as a separate step for decolourisation and counter stain. Simple Modified cold stain is a new, cheaper, safer, and easier cold staining method which is suitable for laboratories with minimum facilities and in setting where work load is heavy^{6,7}. Methods applying fluorochrome have been used for acid-fast staining since the introduction of an AP and an AR based methods. ZN stain is commonly used throughout the world and still remains the standard method against which new tests must be measured⁵. All of the techniques are based on the relatively unique property of Mycobacterium to retain the primary stain even after exposure to strong mineral acid or acid-alcohol, hence the term, acid-fast bacilli. The smears stained by ZN method can detect bacilli when they are at the order of 10^5 /milliliter (ml) of sputum, whereas a more sensitive staining technique like fluorescent stain detects the bacilli when they are at the order of 10^4 /mL of sputum^{8,9}.

The definitive diagnosis of tuberculosis depends on the isolation and identification of *M. tuberculosis*. The inoculation of concentrated bacilli from processed clinical specimens on solid media is a standard approach for confirmation of tuberculosis. Culture methods are more sensitive than microscopy as it can detect 10-100 mycobacteria per ml of sample and give positive result. Therefore culture is deemed to be the gold standard for diagnosis of TB. Despite its enhanced sensitivity and specificity, culture is of impractical clinical use, because it is costly, time consuming and requires specialized safety laboratories, which is usually not performed in most low income countries^{10,11,12}.

Key factors in TB control are rapid detection, adequate therapy and contact tracing to arrest further transmission. The traditional diagnostic tools, apart from a thorough clinical examination, are chest X-ray, which is sensitive but not specific and use of serological tests and PCR are of unproven value in TB control. Progress in molecular methods is restricted to only few sophisticated laboratories^{7,13}. In developing countries like Nepal, TB

laboratories services are able to conduct sputum smear microscopy at 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. Culture and sensitivity testing are available only at the central level. Therefore, under the present circumstances sputum microscopy is nevertheless a rapid way of detecting the most contagious patients, and its specificity is high. If the sensitivity could be increased it would be even more useful diagnostic tool in the developing world¹⁴.

Hence, for developing countries with a large number of cases and financial constraints, evaluation of rapid and inexpensive diagnostic methods like demonstration of acid fast bacilli in smears is great importance¹⁵. No other diagnostic tool offers the affordable as well as efficiency in diagnosis of tuberculosis in public health setup, as sputum microscopy does. In sputum smear microscopy, ZN is the most commonly used technique, because of its simplicity and low cost. There are also other staining techniques for detection of acid fast bacilli, which are simpler, rapid and more sensitive than ZN. The present study evaluates the four different staining techniques used in the detection of AFB. The comparative evaluation of different staining procedures used in sputum smear microscopy will help to know appropriate staining method for demonstration of AFB in laboratory and public set-up on the basis of sensitivity and specificity obtained in each staining technique with the reference to sputum culture.

Methods

Settings

This study was conducted in Tuberculosis Research Laboratory at B. P. Koirala Institute of Health Science, Dharan, Nepal. The ethical clearance was taken from the BPKIHS ethical committee as per the institute guidelines.

Sputum samples

Routine fresh clinically suspected Pulmonary TB patients referred from out patient department (OPD) to Tuberculosis Research Laboratory were included. Patients suspected of Pulmonary TB from Inpatient Department like: medical wards, tropical wards, patient with extra pulmonary tuberculosis and patient undergoing antituberculous treatment (ATT) were not selected. Three sputum samples from each patient, one "on spot" and two early morning samples were collected on the consecutive days. The sample size in the study is 500 clinically suspected PTB patients.

Study design

For staining and culture, sputum samples were first decontaminated and concentrated by centrifugation

using 4 percent NaOH, according to Modified petroff method. Smears on four individual slides were prepared for four different staining methods i.e. ZN, Kinyoun, MC and Fluorochrome stains. Remaining sediments were inoculated in the Lowenstein-Jensen (LJ) media. With culture deemed, the gold standard for diagnosis, it was compared with the respective staining techniques.

Smear preparation

Concentrated sputum sample was smeared evenly with an uneven end of broom stick on the respective four slides; the smear size being 2 cm x 3 cm and it was not too thick. The smear was air dried. Then was methanol fixed. Then the slides were then placed in serial order on the staining rack with the smeared slides facing upward ensuring slides do not touch each other.

Staining procedure

Ziehl-Neelsen Stain

The procedure was that described by WHO laboratory guidelines.¹⁶ The fixed smears were flooded with the solution of 1 percent carbol fuchsin prepared by dissolving 1g of basic fuchsin in 10 ml of ethanol; this solution was diluted to 100ml with aqueous 5 percent phenol. The smears were heated underneath until vapour start rising and were allowed to stand for 5 minutes. The smears were then rinsed with water and decolourised with 3 percent acid alcohol for 3 minutes. The slides were rinsed thoroughly with water and counterstained 0.1 percent malachite green solution and were let to stand for 1 minute. The slides were flooded with water and were allowed to air dry. The slides were then examined under microscope in x100 oil immersion.

Kinyoun stain

The procedure was that described by CDC guidelines¹⁷. The fixed smears were flooded with the solution of concentrated carbol fuchsin prepared by dissolving 4g of basic fuchsin in 20 ml of ethanol; this solution was diluted to 100ml with aqueous 8 percent phenol. The smears were allowed to stand for 5 minutes. The smears were then rinsed with water and decolourised with 3 percent acid alcohol for 3 minutes. The slides were rinsed thoroughly with water and counterstained 0.1 percent malachite green solution and were let to stand for 1 minute. The slides were flooded with water and were allowed to air dry. The slides were then examined under microscope in x100 oil immersion.

Modified cold stain

The fixed smears were flooded with the solution of concentrated carbol fuchsin. The smears were allowed to stand for 10 minutes. The smears were then thoroughly rinsed with water and decolourised and counterstained with Gabbet methylene blue solution prepared by dissolving 1g methylene blue in 20 ml of sulphuric acid, 30 ml absolute alcohol and 50 ml distilled water were let to stand for 2 minutes. The slides

were flooded with water and were allowed to air dry. The slides were then examined under microscope in x100 oil immersion.

Fluorochrome staining

The procedure was that described by WHO laboratory guidelines¹⁶. The fixed smears were flooded with auramine-phenol and allowed to stand for 15 minutes. The smears were then rinsed with water and decolourised with 1 percent acid alcohol for 2 minutes. The slides were rinsed thoroughly with water and counterstained 0.1 percent potassium permanganate and allowed to counter stain for 2 minutes. The slides were flooded with water and were allowed to air dry. The slides were then examined by fluorescence microscope in x400 oil immersion.

Microscopy reports

In recording and reporting of microscopic results, the following reporting scale was used for ZN stain, Kinyoun stain and MC stain. (As per the guidelines given by WHO)¹⁶.

Number of Bacilli Seen in a Smear	Results Reported
<i>No AFB per 300 oil immersion fields</i>	<i>negative</i>
<i>1-9 AFB per 100 oil immersion fields</i>	<i>record the exact number</i>
<i>10-99 AFB per 1 00 oil immersion fields</i>	<i>1+</i>
<i>1-10 AFB per 10 oil immersion field</i>	<i>2+</i>
<i>>10 AFB per oil immersion field</i>	<i>3+</i>

The number of AFB found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis.

In order to equate the number of bacilli observed with fluorescence microscope (400x magnification) to the number of bacilli observed with immersion microscopy (1000x magnification), the values were divided by 4.

Culture and identification

Single slope slant per specimen were inoculated each with one 4 mm loopful of the centrifuged sediment, distributed over the surface. All cultures were incubated at 37^o C until growth was observed and those tubes in which growth was not observed after 8 weeks were regarded as negative were discarded.

All cultures were examined after 48-72 hours after inoculation to detect any contaminants. Thereafter cultures were examined on 7th day for rapid growers once weekly thereafter, up to 8 weeks, for slow growers after which a definitive result was obtained.

Typical colonies of *M. tuberculosis* were rough, tough, crumbly, waxy, non-pigmented (buff colored) and

slow-growers (growth appeared after 2-3 weeks after inoculation). Growth of mycobacteria was confirmed by typical colony morphology and microscopy for AFB.

Reporting of TB cases

A patient was defined as a "TB-positive case" if one of the three sputum specimens had a positive culture and as a "non-TB case" if none of the three sputum specimens showed growth.

Statistical analysis

The sensitivity, specificity, positive predictive value and negative predictive value were calculated using statistical product and service solution (SPSS) software version 11.5.

Results

The present study evaluated the different staining techniques used in the diagnosis of pulmonary tuberculosis. The different staining techniques were ZN stain, Kinyoun stain, MC stain, and Fluorochrome stain methods. The staining methods were evaluated against culture on LJ medium, employed as 'gold standard'. In the study group 59.60 percent (n=298) were males and 40.4 percent (n=202) were females, among whom 24.5 percent male and 17.8 percent female were suffered from TB. Maximum numbers of TB cases were observed in the

economically most productive age group of 15-24 years (26.1%). Tuberculosis was not diagnosed in suspected cases below 15 years. 1365 specimens were collected from 500 suspected pulmonary patients, among whom One hundred nine (21.8%) patients were diagnosed as having *M. tuberculosis* by the isolation of the organism from cultures of sputum. 1.2 percent of PTB suspects were identified with all four staining methods but were not verified by culture.

The validities of the four staining methods using culture as the gold standard for tuberculosis diagnosis are shown in Table 1. The sensitivity and specificity of ZN, Kinyoun, MC and Fluorochrome staining methods were found to be 57.8 percent/ 98 percent, 56 percent/ 98 percent, 59.6 percent/ 98.2 percent, and 71.6 percent/ 98 percent respectively. The positive predictive value (PPV) and negative predictive value (NPV) of ZN, Kinyoun, MC and Fluorochrome staining methods were found to be 88.7 percent/ 89.3 percent, 88.4 percent/ 88.9 percent, 90.3 percent/ 89.7 percent and 90.7 percent and 92.5 percent respectively. The false positive rate of ZN, Kinyoun, MC and Fluorochrome staining methods were found to be 2 percent, 2 percent, 1.8 percent and 2 percent respectively. The false negative rate of ZN, Kinyoun, MC and Fluorochrome staining methods were found to be 42.2 percent, 44 percent, 40.4 percent, and 28.4 percent respectively.

Table2: Validities of the Ziehl-Neelsen stain, Kinyoun stain, Modified cold stain and Fluorochrome stain for the primary diagnosis of pulmonary tuberculosis using culture results as the gold standard.

Staining methods	Culture result		total	Sensitivity (%)	Specificity (%)	Predictive value (%)	
	positive	negative				positive	negative
Ziehl-Neelsen							
positive	63	8	71	57.8	98	88.7	89.3
negative	46	383	429				
total	109	391	500				
Kinyoun							
positive	61	8	69	56.0	98	88.4	88.9
negative	48	383	431				
total	109	391	500				
Modified cold							
positive	65	7	72	59.6	98.2	90.3	89.7
negative	44	384	428				
total	109	391	500				
Fluorochrome							
positive	78	13	91	71.6	98	90.7	92.5
negative	31	378	409				
total	109	391	500				

In this present study, 500 sputum samples were examined in parallel. Table 2, shows the correlation of Kinyoun stain, MC stain and Fluorochrome stain with ZN stain. The total yield of positive results was slightly higher by ZN: 71(14.2%) positive compared with 69(13.8%) positive by the Kinyoun stain. The positive agreement in between both stains was 91.5 percent and ZN could detect 8.5 percent

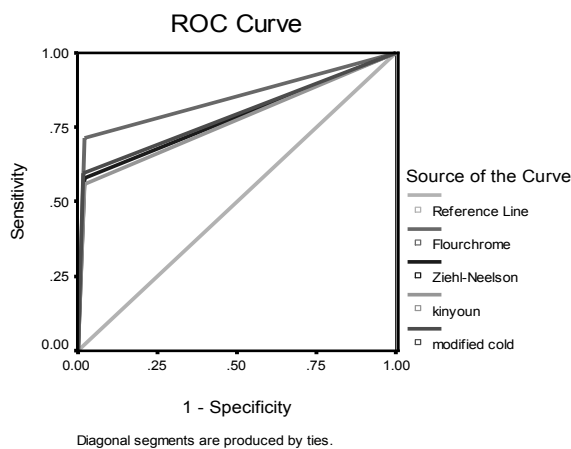
more than Kinyoun method. The total yield of positive results was slightly higher by MC staining method: 72 (14.4%) positive as opposed to 71 positive by ZN (14.2%). Positive agreement in between both stains was 98.6 percent. The total yield of positive result by Fluorochrome was 86 (17.2%) as compared to 71 (14.2%) by ZN method. The positive yield is much greater in fluorescent method.

Table 3: Correlation between the Ziehl-Neelsen and the Kinyoun staining techniques in slide reading of AFB- smear positive and negative.

Staining methods	Ziehl- Neelsen stain % (n)		Total % (n)
	Positive	negative	
Kinyoun stain			
Positive	91.5 (65)	0.9 (4)	13.8 (69)
Negative	8.5 (6)	99.1 (425)	86.2 (431)
total	100 (71)	100 (429)	100 (500)
Modified cold stain			
Positive	98.6 (70)	0.5 (2)	14.4 (72)
Negative	1.4 (1)	99.5 (427)	85.6 (428)
total	100 (71)	100 (429)	100 (500)
Fluorochrome stain			
Positive	98.6 (70)	3.7 (16)	17.2 (86)
Negative	1.4 (1)	96.3 (413)	82.8 (414)
total	100 (71)	100 (429)	100 (500)

Figure 1 shows the graphical relationship between sensitivity and specificity for respective staining techniques by the Receiver Operator Characteristic curve (ROC) for which confidence interval was 95 percent.

Figure 1: ROC curve in different staining techniques



Discussion

According to the WHO guidelines for tuberculosis control, patient with more than three weeks history of cough should be screened for PTB with smear microscopy for AFB.

Because the clinical signs and symptoms of PTB are not specific, the accurate performance of acid-fast microscopy is vital for the early recognition of PTB patients for the adequate treatment, respiratory isolation, and contact investigation. Although acid-fast microscopy is more than 100 years old, it still remains the initial and most rapid step in the diagnosis of tuberculosis. Acid-fast microscopy is simple to perform and therefore could be applied successfully at any laboratory⁵. The added advantage of Sputum smear microscopy is that it has very close relation with infectiousness: patient who are sputum smear positive and culture positive are far more likely to be infectious than culture positive but smear negative¹⁸.

The usual staining laboratory technique for staining AFB used worldwide has been the ZN method, which has also accepted as the conventional method. However the method requires controlled heating for its success, and there are certain disadvantages, e.g. multistage staining, a cumbersome heating procedure and the discomfort caused by aerosols of phenol. There are several modified staining techniques for detection of AFB in sputum. Kinyoun is a well known method which requires high concentration of basic fuchsin and phenol or the addition of a detergent (tergitol no 7) there by avoiding the need for heat. In this study, an improved acid fast staining technique for

staining of sputum, the MC staining method was described. This procedure used familiar Kinyoun solution, but the stages of staining were reduced required no heating and combined counterstaining stages using Gabbet methylene blue solution ultimately making the processing step faster, cheaper and safer. The method makes economical use of the laboratory and materials and would be useful in large scale case finding programmes and laboratories with minimum facilities^{6,7,13}. Another staining method for detection of AFB is the fluorescent microscopy that used AR or AP. The success of staining techniques depends on the ability of the dye to penetrate uniformly the cell wall of tubercle bacilli through their surface coating of waxy substance¹⁹. This present study evaluates these four staining techniques used for preliminary diagnosis of TB.

In the present study, maximum numbers of TB cases were observed in the economically most productive age group of 15-24 years (26.1%), and more males were detected (24.2%), as compared to females (17.8%). Considering the gender in TB positive cases more males were found to be suffered from the disease than females (data not shown). This does not however reflect an increase in the occurrence of disease in males, since the attendance of females to OPD is lower than males. TB was not diagnosed in the PTB suspects below 15 years. This data shows that the diagnosis of TB in childhood is surrounded by considerable uncertainty. The reason behind this may be that purulent sputum is not available from children. This finding is in accordance to the previous report²⁰.

In the present study, the total specimens studied were 1365 specimens from 500 clinically suspected PTB patients. During sample collection all the patients were advised to submit three sputum samples from each patient. But probably due to their personal reasons, 47 (9.4%) patients submitted only two samples and 44 (8.8%) patients submitted only single sample. 109 (21.8%) of the 500 patients were diagnosed as having PTB by the isolation of the organism from culture of sputum. The growth of organism in culture as *M. tuberculosis* was identified by cultural characteristics and acid fast staining. However positive culture confirmed by microscopy could not be tested with biochemical test and other nucleic acid probes for further identification.

In the study, the validity of four staining techniques was found by using culture as gold standard, for the diagnosis of TB. The sensitivity of ZN, Kinyoun, MC, and Fluorochrome were found to be 57.8 percent, 56 percent, 59.3 percent and 71.6 percent (Table 1). The sensitivity determination of ZN and MC stain was in accordance to those of previous reports^{6,7,13} who reported that no significant differences were found between the ZN and MC and that of Kinyoun method was reported to be slightly inferior to that of other three techniques and the finding was in agreement with the study of Somoskövi *et al*⁵.

In the present study, the sensitivity of fluorescent staining technique (71.6%) was found significantly higher than that of other carbol fuchsin stained smear, this findings contradict with the findings of Tanuphasiri and Kladphuang¹³, however this finding was strongly supported by the several other reports^{5,15,21,22,23,24}.

The specificities of the ZN, Kinyoun, MC, and Fluorochrome were found to be 98 percent, 98 percent, 98.2 percent, and 98 percent. The true negative rate was very high in all four staining methods. These results are in agreement with the other findings^{5,13,21}. However the specificity of smear examination methods should be interpreted with caution because it does not allow differentiation of *M. tuberculosis* from mycobacteria other than tubercle bacilli (MOTT).

Comparing to the culture results, the false positive results obtained by four staining techniques were 2 percent in ZN and Kinyoun methods; 1.8 percent in MC method and 2 percent in Fluorochrome method. These results are comparable with the several other findings^{5,13,21,23}. The false positive results suggest that occasionally, a sputum specimen or a smear may contain particles that are acid fast: these particles may some time resemble tubercle bacilli, *i.e.* MOTT or the precipitate of staining, which hampers reading. Because of false positive results patient have to suffer from unnecessary therapy or prolonged hospital stay and further delays in the correct diagnosis and proper treatment of other diseases. In the study, false positive rate was found similar in Fluorochrome method with other three staining techniques. But in several other studies claimed that higher false positive rate might occur in Fluorochrome staining technique²¹. Therefore, it is a good laboratory practice to confirm any smear-positive or doubtful result in newly diagnosed patients. Since, false positive noted with the staining techniques was lower; a positive smear could be reliable as a good diagnostic indicator with these staining techniques.

In the study, it was also observed that very small percentage (1.2%) of smear positive specimen by all four staining techniques, were not able to be isolated on LJ medium, this may be due to the presence of non viable bacilli in sputum specimen received. Acid fast smear examination does not discriminate between viable and non viable bacilli, also tubercle bacilli and other mycobacteria²⁵. This study is in accordance to the result obtained by Jain *et al*¹⁵. who reported that 2-3 percent of AFB specimens could not be confirmed by growth on LJ medium.

The false negative rate by ZN, Kinyoun, MC and Fluorochrome methods in the study was found to be 42.2 percent, 44 percent, 40.4 percent, and 28.4 percent respectively. Of the staining techniques, Kinyoun had higher false negative rate while that of Fluorochrome result was lowest. False negative results in the staining methods

were commonly due to deficiencies in the preparation of the smear such as too little materials spread on the slide or too thin / thick smears. In the present study, concentrated sputum was divided into 5 aliquots, due to the fact, concentration of bacilli have may get varied. These data suggested that, a negative smear should be interpreted with caution because it does not rule out the active tuberculosis. The results are in accordance with the results of other reports^{5,13,21}.

In the present study, higher percentages of patient were found to miss diagnosis when tested with sputum smear microscopy because of low sensitivity. These data supports that culture is the only definitive diagnosis of tuberculosis that depends on the isolation and identification of *M. tuberculosis*. Culture remained the gold standard diagnostic method for tuberculosis. Culture methods are highly sensitive and specific than microscopy for detection of bacilli, since approximately 10-100 mycobacteria per milliliter of sample is required for positive result while approximately 10⁴ organism per ml of sputum is required to be seen by microscopic examination. But even when culture facilities are available, microbiological treatment is started on the basis of arbitrary clinical criteria and lack of response to other treatments. It has been shown that the proportion of positive sputum smear cases in the PTB-AIDS complex is even lower²⁶. So to provide the accurate diagnosis of pulmonary TB; a culture should always be requested concomitantly with AFB smear where the culture facilities are available. Culture requires at least a moderately well-equipped laboratory and necessarily lengthy time for its isolation and identification. So the cost and complexity associated with culture restricted its use only in major centers.

In the present study, the positive predictive values and negative predictive values were high for all four staining techniques (Table 1). The PPV and NPV for ZN, Kinyoun, MC, Fluorochrome staining methods were 88.7, 89.3, 88.4, 88.9, 90.3, 89.7, 90.7 and 92.5 percent respectively. These data suggested that all these four methods have sufficient validity to predict the presence or absence of the disease in TB prevalence population.

The accuracy of all four staining techniques results is expressed by ROC curve in figure 1. The ROC curve shows the relationship between sensitivity and specificity for respective staining techniques. Tests that perform less well have curves that fall closer to the diagonal running from lower left to upper right. ROC curves are particularly valuable way of comparing alternative tests for the same diagnosis. The overall accuracy of a test can be described as the area under the ROC curve; larger the area, better the test. In the present study the Fluorescent method has the largest area under the ROC curve of the four staining techniques and then the MC, ZN and Kinyoun respectively.

In the study, positive yield detected by Fluorochrome method (17.2%) was nearly close to that of culture (21.8%) than other staining methods. From the result obtained in the present study, it can be said that Fluorochrome method was more sensitive and more reliable than the remaining other three techniques for demonstration of AFB. Fluorochrome method has an added advantage of allowing a large number of sputum specimens to be examined in a given time as low power is used for examination. More over definite advantages of Fluorochrome method was that it enabled the detection of positive smears, which were over-looked with the carbol fuchsin stained smears containing low- density bacilli. So, the use of Fluorochrome method significantly increases the diagnostic value of the smear, particularly where there were low density bacilli (paucibacillary cases). Fluorochrome acid-fast microscopy is not only easy to perform and cost effective, but is currently the most rapid procedure for detecting acid-fast bacilli in clinical specimens and to screen for the most infectious cases of presumed tuberculosis. The fluorochrome-stained organisms can be seen at a lower magnification without the use of oil immersion. When lower magnifications are used, less microscopic viewing time is required, which creates the potential for decreasing the turnaround time needed to report microscopy results. Fluorochrome method is quite economical in terms of both time and expenses in large laboratories handling large number of sputum specimens. Although the advantages of fluorescence microscopy are easiness in application, speed, and better contrast due to the dark background and higher sensitivity, it is not economical technique in rural areas of developing countries because of its associated cost and equipment maintenance.

In the present study, among the carbol fuchsin stained smear, the MC stain has increased sensitivity (59.6%) than ZN (57.8%) and Kinyoun method (56.0%). The specificity of MC method (98.2%) was also higher than that of ZN (98%) and Kinyoun method (98%) and fluorescent method (96.4%). The PPV and NPV of MC were comparable with that of ZN, Kinyoun and Fluorochrome methods (Table1). According to the present study and previous reports^{6,7,13}, it can be said that two step MC can be suitable and valuable alternative to that of ZN and Kinyoun because it has added advantage of ease, speed and is more economic than ZN and Kinyoun and Fluorochrome methods. Modified cold method can be very important diagnostic tool for the demonstration of AFB in developing countries with a large number of cases but have limited resources.

The present study concludes that Fluorochrome method is reliable among four techniques and economical both in terms of time and expenses and it can be recommended for laboratories handling large number of cases and the laboratories which can afford fluorescence microscope. But

in laboratories with minimum resources, facilities and heavy workload, two step simple Modified cold staining technique is found to be more valuable for the preliminary diagnosis of tuberculosis, as this technique appeared to be more practical, rapid and effective than Ziehl-Neelsen or Kinyoun method.

To the best of our knowledge, this is the first study to evaluate different staining techniques used in the primary diagnosis of pulmonary TB in Nepal. Today, attention has turned to nucleic acid technology: the 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 TB. Detection of AFB by sputum smear microscopy is the only feasible method recommended for the tuberculosis control program in Nepal and many other developing countries in detecting infectious pulmonary tuberculosis cases and for monitoring the progress of patients during treatment.

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