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Improved microscopic diagnosis of smear positive tubercle bacilli among patients suspected of Pulmonary Tuberculosis in Western Region of Nepal

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ABSTRACT

Tuberculosis, caused by a bacterium called *Mycobacterium tuberculosis*, is the largest cause of death from a single infectious disease and is still a major health problem worldwide. The infection spreads by inhaling droplets of mucus that have been expelled by an infected person. Factors associated with infectivity include bacillary load, severity of coughing, proximity to the patient and the duration of anti-TB therapy. A cross-sectional study was carried out where two dependent variables (direct and concentration method) were compared with other independent variables. A total of 177 samples were collected from patients visiting OPD of western regional tuberculosis centre, Pokhara. The sputum was collected in sterile container and transferred to the laboratory of SHAS, Pokhara University and those samples that were delayed for processing were stored at 2-8°C in refrigerator. The detection of tubercle bacilli was done by direct microscopy and sedimentation method. Data were analyzed by using SPSS v 17.0. Concentration method increases the detection rate of tubercle bacilli (14.1%) as compared with direct microscopy (12.4%). Concentration method is more effective in TB diagnosis in resource limited areas as it increases the positivity rate than direct microscopy. There were no significant relation between tuberculosis and other independent variables except smoking and drinking (p=0.0001). Despite being rapid, cost effective and specific for tuberculosis in terms of sensitivity as well as in positivity rate for smear positive cases, AFB staining is less effective compared to the Concentration by using hypochlorite. Bleach sedimentation microscopy is an effective, simple method to improve the yield of smear microscopy

INTRODUCTION

Tuberculosis is the largest cause of death from a single infectious disease and is still a major health problem worldwide [1]. It is caused by a bacterium called *Mycobacterium tuberculosis* ('*M. tuberculosis*' or '*M.Tb*'). First discovered in 1882 by Robert Koch, *M. tuberculosis* has an unusual, waxy coating on its cell surface, which makes the cells impervious to Gram staining. The Ziehl-Neelsen stain, or acid-fast stain, is used instead. The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system, it infects the lungs. Mycobacteria are slender bacilli and sometimes exhibit filamentous forms resembling fungal mycelium and they are so named. They are better stained by hot carbol fuschin, and once stained they resist decolourization by dilute mineral acids and are, therefore referred as Acid fast bacilli. Even though TB could be transmitted by ingesting infected meat and milk, mostly the disease is spread by aerosols created by coughing or sneezing. Transmission can only occur from people with active-not latent-TB *[2]*. The infection spreads by inhaling droplets of mucus that have been expelled by an infected person. Factors associated with infectivity include bacillary load, severity of coughing, proximity

to the patient and the duration of anti-TB therapy[3]. The classic clinical features of pulmonary tuberculosis include chronic cough, sputum production, appetite loss, weight loss, fever, night sweats and hemoptysis [4].

Tuberculosis (TB) is one of the most ancient diseases of mankind, with molecular evidence going back to over 17,000 years. In spite of newer modalities for diagnosis and treatment of TB, unfortunately, people are still suffering, and worldwide it is among the top 10 killer infectious diseases, second only to HIV. According to World Health Organization (WHO), TB is a worldwide pandemic. It is a leading cause of death among HIV-infected people [5]. World Health Organization estimates the prevalence of all types of tuberculosis case for Nepal at 66,000 while the number of all forms of incidence cases is estimated around 45,000. Nepal adopted Direct Observed Treatment Short course (DOTS) in 1996 and nationwide coverage was achieved in 2001.

Pulmonary TB is the most important type of TB from the public health point of view and can be diagnosed by its symptoms, chest radiography, sputum smear microscopy, and by cultivation of M. tuberculosis[6]. Sputum smear microscopy has been the primary method for detecting TB and monitoring treatment response in most resource-constrained countries for decades. While inexpensive and requiring minimal biosafety standards, microscopy is not a sensitive test, particularly in people living with HIV and in children[7]. Demonstration of Acid Alcohol Fast Bacillus (AFB) in the sputum is the easiest, quickest and a reliable tool for diagnosis of bacillary pulmonary tuberculosis. Bleach (sodium hypochlorite) is an ideal chemical processing agent for use in low-income countries. It is widely available and inexpensive, and its disinfectant properties could improve infection control in laboratories lacking adequate biosafety facilities. Bleach has been reported to increase the sensitivity of smear microscopy primarily through digestion of the mucus and debris in sputum, resulting in a clearer microscopy field [8]. For a 50% probability of finding a single acid fast bacillus in 100 microscopy fields, approximately 5, 000 acid-fast bacilli must be present per ml of sputum. Thus, reliance on smear microscopy may cause missed or delayed tuberculosis diagnosis, potentially increasing morbidity, mortality and tuberculosis transmission. Diagnostic sensitivity increases if acid-fast bacilli are concentrated into the small volume that can be visualized by microscopy. Sputum concentration by homogenization and microscopic examination of the sediment can increase the detection rate of AFB. Several concentration techniques using sedimentation or centrifugation have been reported. Bleach homogenization followed by overnight sedimentation can significantly increase detection of AFB compared with direct microscopy. as centrifugation requires electricity as well as equipment that might not be present in peripheral laboratories in resource-limited settings [9]. The concentrations of 2-5% of NaoCl digest the sputum products and they inactivate the mycobacteria without altering their structures, so that even when they are killed, they can still be stained and observed. This provides a greater safety for laboratory use [10].

MATERIALS AND METHODS

A cross-sectional study was carried out on total 177 patient visiting OPD of Western Regional Tuberculosis Centre, Pokhara between 23rd November 2014 to 25th December 2014. The study was carried out between two dependent variables (AFB and concentration) are compared with other independent variables. Participants were verbally informed about the study and oral consents were taken from eligible patients. Then the question were asked as per our questionnaire format, then sputum samples were collected which were labeled with unique lab number.

For sputum sample collection, sputum were collected on the basis of chest X ray examination, clinical symptoms including chest pain, coughing period, weight loss, loss of appetite and family history as well as their habits i.e. smoking and drinking. While those patients on medication were excluded. In sputum collection priority is given to mucopurulent bloody samples while saliva as well as slivery samples was not taken for the study because of inappropriate in nature for processing. Morning specimen was collected in wide mouth sterile container for the study. The sputum was collected in sterile container and transferred to the laboratory of SHAS, Pokhara University in ice box for processing and those samples that were delayed for processing were stored at 2-8°C in refrigerator.

For direct microscopy smear was prepared in clean grease free slide .The smear was then stained with AFB stain where acid fast bacilli took the AFB stain and appear red rod shape slightly curve bacilli in blue background whereas non-acid fast bacilli do not take the stain.

Concentration technique is based on the principle of sedimentation. Hypochloride inactivates the bacilli as well as when the load of bacilli is low in specimen then sedimentation help to settle down those bacilli or concentrate bacilli in sediments, this help in better diagnosis in case of smear positive tuberculosis.

The ZN technique was used to stain *Mycobacterium* species. The stain (carbol fuschin) binds to mycolic acid in the mycobacterial cell wall. Acid decolorization removes the red dye from the background cells, tissue fibers and any organisms in the smear except mycobacteria which retain (hold fast to) the dye and are therefore referred to as acid fast bacilli. Then the smear is counterstained with methylene blue which stains the background material, providing a contrast color against which the red AFB can be seen(11). Data were analyzed by using SPSS v 17.0.

RESULTS

A total of 177 patients were enrolled in our study. Morning sputum sample from each patient were collected. The mean age was 42.66(range 5-91) as shown in Table 1.Out of 177 patients, 58 were female and 119 were male. The female/male ratio was 0.49. Figure 1.

Direct microscopy vs. concentration method:

Overall, AFB were detected on 22 smears prepared by the direct method (12.4%) and 25 smears prepared by the bleach method (14.1%) (Table 3). The number of positive cases increased by 1.7% using concentration method. All samples (22) positive by direct method were also positive by concentration method. 3(1.7%) samples negative by direct method were positive by concentration method as shown in table 2.

Relation with age and gender:

No significant relation with age was found. No significant relationship was found with gender with direct microscopy and concentration method as shown in table 4.

Relation with alcoholism:

Out of 177 patients, 128(72.3%) were non-alcoholic and

49(27.7%) were alcoholic. 121(68.4%) non-alcoholics were AFB negative and 7(4.0%) non-alcoholics were AFB positive by direct method. 34(19.2%) alcoholics were AFB negative and 15(8.5%) alcoholics were AFB positive by direct method. There is significant relation between alcoholism and direct method for detection of AFB (p=0.001) (table4). 118(66.7%) non-alcoholic were AFB negative and 10(5.6%) non-alcoholic were AFB positive by concentration method. 34(19.2%) alcoholic were AFB negative and 15(8.5%) alcoholic were AFB positive by concentration method. There is significant relation between alcoholism and concentration method for detection of AFB (p=0.001) (table 4)

Table 1. : Age wise distribution of population

Age group	Frequency
5-15	4 (2.3%)
15-25	40(22.6%)
25-35	27(15.3%)
35-45	17(9.6%)
45-55	40(22.6%)
55-65	23(13.0%)
65-75	18(10.2%)
75-85	5(2.8%)
85-95	3(1.7%)
Total	177(100%)
Mean age	42.66
Range	5-91
Minimum	5
Maximum	91

Relation with smoking:

Out of 177 patients, 120(67.8%) were non-smoker and 57(32.2%) were smoker (figure 15). 113(63.8%) non-smoker were AFB negative and 7(4.0%) non-smoker were AFB positive by direct method. 42(23.7%) smoker were AFB negative and 15(8.5%) smoker were AFB positive by direct method. There is significant relation between smoking and direct method for detection of AFB (p=0.001) (table 5).

111(62.7%) non-smoker were AFB negative and 9(5.1%) non-smoker were AFB positive by concentration method. 41(23.2%) smoker were AFB negative and 16(9.0%) smoker were AFB positive by concentration method. There is significant relation between smoking and concentration method for detection

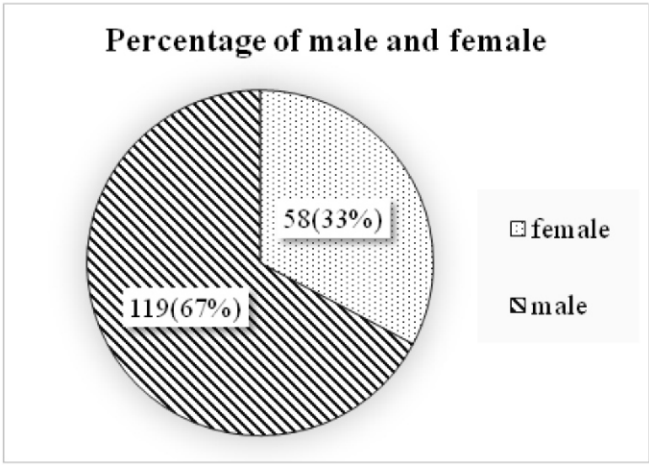

Fig 1. : Gender in percentage

Table 2. : No. of positive and negative cases by direct microscopy and concentration method

	Direct microscopy	Concentration
Negative	155(87.6%)	152 (85.9%)
Positive	22(12.4%)	25 (14.1%)
Total	177(100%)	177(100%)

Table 3. : Direct microscopy vs. concentration method

		Concentration method		Total
		Negative	Positive	
Direct microscopy	Negative	152(85.89%)	3(1.7%)	155(87.59%)
	Positive	0(0%)	22(12.41%)	22(12.41%)
	Total	152(85.89%)	25(14.11%)	177(100%)

of AFB (p=0.001) (table 5)

Relation with ethnic group:

Out of 177 patients, 51(29%) were Brahmin, 13(8%) were Chetri, 48(27%) were Dalit, 64(36%) were janajati and 1 was others (table 6). 5 Brahmin, 11 Dalit and 6 Janajati were AFB positive by direct microscopy (table 6). 5 Brahmin, 12 Dalit and 8 Janajati were AFB positive by concentration method (table 6). There is no significant relationship between AFB positive and ethnic group. (P=0.095)

DISCUSSION

This study was conducted to compare microscopic diagnosis of TB in suspected tuberculosis patients visiting tuberculosis center by direct microscopy and concentration technique. Direct microscopy was performed on provided specimen and similarly the same specimens were processed by concentrated technique. There are various concentration techniques like sedimentation and centrifugation but we preferred sedimentation technique with hypochlorite because centrifugation requires electricity, expert resources as well as the concern for biosafety although the process is rapid (12). All these problems can be overcome when using sedimentation technique although it has also some limitation.

This study shows that use of concentrated technique for preparing smear of AFB microscopy increases positivity of tuberculosis diagnosis as compared to direct microscopy. The specimen that was negative by direct microscopy was found positive by concentration technique. This study could be even better if compared with gold standard technique i.e. culture where the actual sensitivity can be determined but is not performed due to limited resources and infrastructure. Three specimens were found positive by concentration technique which was negative by direct microscopy.

In most low-income countries, the only practically available bacteriological method for diagnosing pulmonary tuberculosis is direct sputum smear microscopy for acid-fast bacilli (AFB). Enhanced smear microscopy is likely the only diagnostic test or

Table 4. : Gender vs. direct microscopy and concentration method

Gender	Direct microscopy		Total	Concentration method		Total
	Negative	Positive		Negative	Positive	
Female	52(29.4%)	6(3.4%)	58(32.8%)	52(29.4%)	6(3.4%)	58(32.8%)
Male	103(58.2%)	16(9.0%)	119(67.2%)	100(56.5%)	19(10.7%)	119(67.2%)
Total	155(87.6%)	22(12.4%)	177(100%)	152(85.9%)	25(14.1%)	177(100%)

Table 5. : Alcoholism vs. direct microcopy and concentration method

	Direct microscopy		Total	Concentration method		Total
	Negative	Positive		Negative	Positive	
Non-alcoholic	121(68.4%)	7(4.0%)	128(72.3%)	118(66.7%)	10(5.6%)	128(72.3%)
Alcoholic	34(19.2)	15(8.5%)	49(27.7%)	34(19.2%)	15(8.5%)	49(27.7%)
Total	155(87.6%)	22(12.4%)	177(100%)	152(85.9%)	25(14.1%)	177(100%)

Table 6. : Smoking vs. direct microscopy and concentration method

	Direct microscopy		Total	concentration method		Total
	Negative	Positive		Negative	Positive	
Non-smoker	113(63.8%)	7(4.0%)	120(67.8%)	111(62.7%)	9(5.1%)	120(67.8%)
Smoker	42(23.7%)	15(8.5%)	57(32.2%)	41(23.2%)	16(9.0%)	57(32.2%)
Total	155(87.6%)	22(12.4%)	177(100.0%)	152(85.9%)	25(14.1%)	177(100.0%)

Table 7. : Ethnic group vs. direct microscopy and concentration method

Ethnic group	Direct microscopy		Total	concentration method		Total
	Negative	Positive		Negative	Positive	
Brahmin	46	5	51	46	5	51
Chhetri	13	0	13	13	0	13
Dalit	37	11	48	36	12	48
Janajati	58	6	64	56	8	64
Others	1	0	1	1	0	1
Total	155	22	177	152	25	177

strategy that can be widely implemented in the short term to improve tuberculosis case finding. Several approaches have been proposed to optimize smear microscopy, including fluorescence microscopy, same-day sputum collection strategies, and sputum processing methods, such as bleach processing. Microscopy to detect acid-fast bacilli can be improved by sputum liquefaction and concentration by centrifugation and gravity sedimentation. Liquefaction of sputum with sodium hypochlorite and concentration by either centrifugation or sedimentation is the most widely studied procedure. Studies using bleach and overnight sedimentation showed a 6% mean increase in incremental yield. Specificity ranged from 96% to 100% with the bleach method alone and from 95% to 100% with the Ziehl-Neelsen method alone. Sodium hypochlorite is mycobactericidal and also kills HIV and thus improves safety and acceptability in laboratories (13). In our study also we found that bleached processed smear microscopy increases the positivity rate of TB diagnosis than direct microscopy.

A study conducted by Barez et al.,(14) showed that the sensitivity was almost similar in both methods as described 81.6% for direct method and 82.7% for the concentrated method. In another study, Cattamanchi et al.,(15) failed to find a difference in sensitivity between direct and concentrated sputum smear microscopy, the calculated sensitivity of direct and concentrated smear microscopy was not significantly different (51% vs. 52%). In a similar study conducted by Peterson et al.,(16) in two different laboratory settings (a tertiary-care laboratory and several local outpatients clinics) found that in a tertiary-care hospital the direct smear was significantly less sensitive than the concentrated smear (28% and 51%, respectively) and in the samples from outpatients of the Pacific islands the direct smear was less sensitive than that made from the concentrated specimen (82 versus 93%, respectively)(17). Similarly in our study also sputum concentration prior to AFB staining increases the positivity rate in smear positive patients.

A study conducted by Apers et al.,(18) showed that the sensitivity of direct microscopy was 67.5% and the sensitivity of the concentration method 87.1%, an increase of 29%.A study conducted by Angeby et al.,(19) showed that the number of positive samples increased by 33% using hypochlorite technique. A study by Mindolli et al.,(17) showed that there was a significant increase in the sensitivity with the use of 5% NaOCl . The increase in the 23.14% smear positivity with the use of 5% NaOCl with the centrifugation method was very encouraging as compared to that of the direct smears. In our study also there is increase in smear positivity with 5% hypochlorite by sedimentation as compared to that of direct smear. This is due to that NaOCl digests the sputum, which when followed by the concentration of bacilli by either centrifugation or sedimentation, greatly increases the number of bacilli per microscopic field, which explains the increase in the positivity(17).

A study by Kaore et al.,(20) showed that there is rise of 7.11% in sputum positivity over direct microscopy by concentration method. A study by Ongkhzmmy et al., reported that the implementation of the bleach method yields an overall increase in positivity of 33.5% which support our study, in our study also sputum positivity is raised up by bleach (concentration) method as compare to direct method.

Our study shows that there is significant relation between alcoholism and direct method for detection of AFB (p=0.001) and also between alcoholism and concentration method for detection of AFB (p=0.001) which means there is a significant relation between alcoholism and pulmonary tuberculosis. In the study conducted by *N. Gyawali et al.,* alcohol consumption, was found to be associated with the prevalence of TB(21). Abioye IA et al., identified alcohol consumption as significant socio-demographic determinants of stigma among TB patients(22). K. Lanoroth et al., conducted a systematic review in which they established that alcohol use was independently associated with TB(23). The reason for risk of active TB in people who drink alcohol is due to both increased risk of infection related to specific social mixing patterns associated with alcohol use, as well as influence on the immune system of alcohol itself and of its related conditions(21).

Our study shows that there is significant relation between smoking and direct method for detection of AFB (p=0.001) and also between smoking and concentration method for detection of AFB (p=0.001) which means there is significant relation between smoking and pulmonary tuberculosis. In the study conducted by. B Tulu et al., concluded that active smoking was significantly associated with smear positive TB(24). Gyawali N et al., found greater prevalence of TB among cigarette smoker in their study

than nonsmokers(21). C Kolappan et al., in their study concluded that there is an association between tobacco smoking and the development of pulmonary tuberculosis which is dose dependent(25). N. Ariyotha et al., found that the number of cigarettes/ day and duration of smoking are strongly associated with active pulmonary TB in active smokers(26). Shanmuganathan et al., concluded that Smoking appears to be the most important risk factor for contracting TB among Malaysians. Impaired clearance of mucosal secretion and reduced phagocytic activities of the alveolar macrophages are some of the reasons for smokers being at high risk for TB infection(27). In contrast, G.B. Ploubidis et al., did not observe any significant association between smoking with either incidence or prevalence of TB(28).

CONCLUSION

The main diagnostic test for *Mycobacterium Tuberculosis* in most of developing countries is by AFB staining. Despite it is rapid, cost effective and specific for tuberculosis in terms of sensitivity as well as in positivity rate for smear positive cases it is less effective. Several other serological test, molecular and genetic test can also be performed but they are more expensive and require more infrastructures and culture of TB is time consuming and are not easily available in resource limited areas. Concentration by using hypochlorite followed by overnight sedimentation has several advantages. It increases the sensitivity and positivity rate of smear positive cases. It also provides safety to laboratory personnel. It is more rapid than other methods in tuberculosis diagnosis, also require less and easily available reagent and materials.

Bleach sedimentation microscopy improves the diagnosis of tuberculosis as well as provide important information related to timely diagnosis of tuberculosis. Therefore, it is an effective, simple method to improve the yield of smear microscopy. So this method should be implied in resource limited areas where culture facility is not available.

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