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A Comparative Study on Ziehl-Neelsen Staining (Light Microscopy), Auramine O Staining (Iled- Fluorescent Microscopy) and Culture on LJ Media of Sputum Samples for the Diagnosis of Pulmonary Tuberculosis

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ABSTRACT

Background: Tuberculosis remains a major public health threat in Nepal and one of the leading causes of death from communicable diseases among adults. The majority of tuberculosis is pulmonary tuberculosis infecting lungs and sputum examination is important for this as it is the major way of transmission of disease. The diagnostic procedures rely on simple and inexpensive methods mostly of microscopy and culture examination. Therefore the evaluation of these diagnostic approaches has great importance.
Objectives: To evaluate and compare the efficacy of Ziehl-Neelsen staining, Auramine O staining and culture of sputum samples for the diagnosis of pulmonary tuberculosis.
Materials and methods: Total 299 sputum samples (170 samples from 78 Group I suspected cases with no treatment; 42 samples from 22 Group II DOTS follow-up cases; and 87 samples from 87 Group III MDR follow-up cases) were subjected to direct smear preparation each for ZN and AO staining for 1000x light microscopy and 400x fluorescent microscopy examination respectively and the remaining sample were further processed with NALC-NaOH method for culture on modified Lowenstein-Jensen Media for detection of *Mycobacterium tuberculosis*. Positive smears were graded according to IUATLD/WHO guideline.
Result: Out of total 299 sputum samples of all types of cases, 19.06%, 29.1% and 24.41% were found pulmonary tuberculosis positive by ZN, AO and culture respectively. The case detection rates for suspected patients with no treatment were 20%, 25.88% & 28.24%; for DOTS follow-up patients were 30.95%, 57.14% & 19.05%; and for MDR follow-up patients were 11.49%, 21.84% & 19.54% for ZN, AO and culture respectively. The difference in their case detection rates was statistically significant ($p < 0.01$). No AO negative result with ZN positive samples was found. More number of paucibacillary cases was detected by AO method than ZN. There were 25 cultural contaminated samples. Removing contaminated cultural samples and taking culture as gold standard, the sensitivity and specificity of direct microscopic examination were found to be 60.03% and 98.51% for ZN method; and 83.56% and 94.53% for fluorescent AO method respectively. The percentage of false negative by AO staining was only 16.44% which was in sharp contrast to that of ZN (39.73%).
Conclusion: This comparative study proves that AO staining (Fluorescent microscopy) is superior to ZN staining (Light microscopy) in several aspects as efficacy, sensitivity, false negativity. Thus the AO staining aided with culture can prove to be important tool for the effective and reliable diagnosis and screening of pulmonary tuberculosis.
Keywords: Mycobacterium tuberculosis, suspected case, follow-up case, MDR, sputum sample

BACKGROUND

Tuberculosis remains a major public health problem in Nepal and one of the leading causes of death from communicable diseases among adults despite the fact that the causative organism was discovered more than 100 years ago, and highly sensitive diagnostic tools aided by effective drugs and vaccines are available making it easily identifiable, preventable and curable disease [1-3].

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Tuberculosis is a particular infectious disease caused by *Mycobacterium tuberculosis* [1]. The disease primarily affects the lungs and cause pulmonary tuberculosis diagnosed with smear positive [4]. Over 80,000 people in Nepal have the disease. Nearly 20,000 people develop infectious sputum positive TB every year and about 45,000 new cases of all forms of TB occur in the same period. It is estimated that about 5,000-7,000 people die from TB every year [5]. Therefore proper and efficient early screening and diagnosis of cases, and good treatment strategies are needed for successful TB control.

The highly accurate diagnosis of pulmonary tuberculosis in developing countries mostly relies on light microscopy with ZN staining in most of the laboratory settings and in very few settings with Fluorescent microscopy and culture method of sputum sample [5-6]. Though culture is regarded as gold standard highly sensitive method for diagnosis allowing susceptibility testing of the isolates, poor availability of culturing facilities, the long span of time (6-9 weeks) required and chances of contamination make it difficult to follow [3]. Studies have shown that the rapid diagnosis with smear microscopy is highly specific in areas of high TB prevalence. The speed and feasibility and cost effectiveness of microscopy aided with high sensitivity make it valuable tool for TB diagnosis but for this technique the yield requirement is between 5,000 to 10,000 organisms per ml sample [7-8]. The use of fluorescent microscopy enhances the visualization by providing contrast coloration to the bacilli present in the sample [9]. There are also other methods like ELISA, Mycodot, X-ray, which are not confirmatory tests and similarly the advanced techniques like BACTEC, gene probe, PCR, gas liquid chromatography are costly and only available in very few laboratories and lack in specificity [10-11].

Therefore, this study only focuses on the comparison of the commonly used diagnostic methods in developing countries as microscopy, culture, which is very useful to give idea about the effectiveness of diagnosis.

METHODOLOGY

The prospective case-control study was conducted in the Nepal Anti-Tuberculosis Association (NATA), GENETUP lab, Kalimati, Kathmandu from July 2010 to October 2010, on sputum samples from patient visiting at the GENETUP lab with suspected pulmonary tuberculosis cases without treatment and follow-up cases after DOTS and MDR treatment. A total of 299 sputum samples (170 from 78 suspected cases, 42 from 22 follow-up cases with DOTS treatment and 87 from Follow-up cases with MDR) were collected aseptically.

Sample Collection and Transportation

Individual were advised to rinse mouth with water, cough forcibly, and collect sputum in mouth and spit carefully and aseptically in to a sterile, wide mouth, unbreakable container and close the lid tightly and transport immediately to the lab. Three sputum specimens were collected i.e. one spot, one early morning and one spot when patient returns next day for suspected out-patients visiting GENETUP. For in patients of GENETUP, two early morning samples were collected. Similarly, one early morning sputum sample was collected for MDR patients and maximum two samples were collected for follow-up patients visiting GENETUP. The samples were transported in the tray for processing within the day of collection. Early morning sputum samples collected at patient's home were transported by patients or their relative to the laboratory. Samples from remote areas were transported through shipping in multilayered plastic packs. The minimum amount of sputum sample was 2 ml, the optimum is 5 ml. At the time of sample collection, data was collected with written consent from individuals taking part in this study.

Sample inclusion and exclusion criteria

Suspected pulmonary tuberculosis individuals, Follow-up patients with DOTS treatment and MDR treatment referred from hospitals were taken for sampling. Those with leakage sample and less than 2 ml of mucopurulent sputum sample were excluded.

Sample processing and smear preparation

The processing of sample was carried out in a biosafety cabinet (BSL-2) and subjected to two direct smear preparations from each sample with the help of sterile wooden stick in clean, dry, thin (1mm) glass slides free from scratches; as scratches may retain flurochrome and appear as fluorescent artifacts. The smear was air dried and fixed by flaming. The remaining sample was further processed with NALC-NaOH method for culture preparation on modified Lowenstein-Jensen media.

Staining and microscopic examination

From each sample, Ziehl-Neelsen staining and Auramine O staining was done on two prepared smear slides from each sample. Both slides were blinded by the reader and were read by two independent readers. For the ZN staining, solutions were prepared and staining was done as per the technique described in standard operative procedure [5]. Acid Fast Bacilli (AFB) was demonstrated by oil immersion lens using 1000x magnification in light microscopy. The AFB appeared as red rods against blue background (Fig. 1).

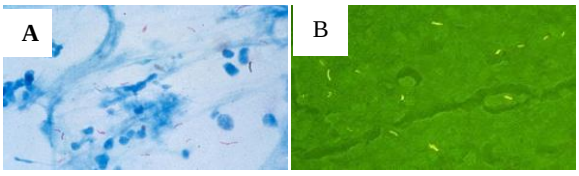

Fig. 1: (A) ZN stained sputum showing red TB bacilli in blue background as 1000x. (B) AO fluorescence stained sputum showing yellow luminous TB bacilli in green background as 400x.

For the Fluorescent AO staining, solutions were prepared and staining was done as per the technique recommended by WHO [1,7]. The fluorescence microscope used is the iLED Primostar Zeiss with an attached illuminator manufactured by Karl Zeiss and is fitted in a dark room. The film is examined at 400x magnification. The tubercle bacilli are seen as yellow luminous slight curved rods in a green background (Figure 1) which was further cross examined by another examiner. Artifacts are distinguished from bacilli by their hazy yellow or grey coloration lacking reddish tinge and were poorly delineated [3].

Smear reporting

Evaluation of smear was done by standardized procedure of scanning by grid pattern proposed by National Tuberculosis Institute with the following reporting standards criteria proposed by IUATLD/WHO [8]. In this study, 2+, and 3+ were classified as multibacillary and scanty, and 1+ as paucibacillary.

Table 1: ZN and Fluorescence smear reporting according to IUATLD/WHO

IUATLD/WHO scale (1000x field = HPF) Result	Microscopy system used		
	Bright-field (1000x magnification: 1 length = 2 cm = 100 HPF)	**Fluorescence (200–250x** magnification: 1 length = 30 fields = 300 HPF)	**Fluorescence (400x** magnification: 1 length = 40 fields = 200 HPF)
No AFB seen	**Zero** AFB / 1 length	**Zero** AFB / 1 length	**Zero** AFB / 1 length
Scanty	**1–9** AFB / 1 length or 100 HPF	**1–29** AFB / 1 length	**1–19** AFB / 1 length
1+	**10–99** AFB / 1 length or 100 HPF	**30–299** AFB / 1 length	**20–199** AFB / 1length
2+	**1–10** AFB / 1 HPF on average	**10–100** AFB / 1 field on average	**5–50** AFB / 1 field on average
3+	**>10** AFB / 1 HPF on average	**>100** AFB / 1 field on average	**>50 AFB** / 1 field on average

Culture

Modified Lowenstein-Jensen media were prepared in-house. After processing of sputum sample by NALC-NaOH method, 0.5 ml of sediment was inoculated on the surface of LJ media using sterile dropper and spread over the surface. The tubes were incubated at 37°C and left in slanted position for a week to allow even distribution of the inoculums over the entire surface of the medium. The caps were tightened and tubes were placed upright and continued incubation for eight weeks. The tubes were examined once a week for eight weeks. Rough, tough and non-pigmented buff colonies with raised, nodular or wrinkled surface and irregular thin margin (Figure 2) indicate the growth of *Mycobacterium* spp. Rapid growers were excluded for further study as *Mycobacterium tuberculosis* is slow grower. The reporting of culture was done according to the bacteriological index (Table 2).

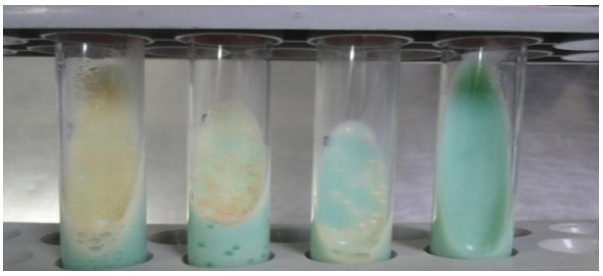

Fig. 2: Growth of Acid Fast Bacilli in LJ Culture media with 3+, 2+, 1+, and no growth.

Table 2: Culture reporting according to the IUATLD/WHO.

Culture Reading	Report
No growth	Negative
1-19 colonies	Scanty (Record exact number of colonies)
20-100 colonies	1+
Innumerable discrete colonies	2+
Confluent growth	3+
Contaminated growth	C

Quality control

Mycobacterium fortuitum was inoculated in modified LJ media in two tubes and incubated for 48 hours. Normally it should show growth after 48 hours. If there is no growth after 48 hours, the media should not be used for culture. The sterility of media was also checked after incubation of 48 hours and any contaminated tubes must be removed from the media box.

Data entry and statistical analysis

All reports from sputum examination request form are taken into Microsoft Office Excel Worksheet, 2010 version for Windows and test accuracy results were calculated as sensitivity, specificity using mycobacterium culture as a reference standard. Test characteristic were compared using the Chi-square test with p-values <0.05 considered statistically significant. The study protocol was approved by the Ethical Review Committee of the institution.

RESULTS

Table 3 shows that, out of total 299 sputum samples of pulmonary tuberculosis including general suspected cases and with follow-up cases having treatment, 19.06%, 29.1% and 24.41% were found pulmonary tuberculosis positive by ZN, AO and culture respectively. In respective category of cases, the case detection rates for general suspected patients without treatment were 20%, 25.88% & 28.24%; for follow up patients of DOTS treatment were 30.95%, 57.14% & 19.05%; and for MDR follow-up patients were 11.49%, 21.84% & 19.54% for ZN, AO and culture respectively. The rate of case detection is significantly higher with culture in case of general suspected cases without treatment followed by AO and ZN. But the follow-up cases showed decrease in case detection rate by cultural methods and in these AO staining proved to be more sensitive than other two. The difference in their case detection rates was statistically significant ($p < 0.01$).

Table 3: Comparison of ZN microscopy and Fluorescent microscopy with Culture

		Culture			
		Positive	**Negative**	**Contaminated**	
		A+B+C	**A+B+C**	**A+B+C**	**Total**
ZN Microscopy	**Positive**	31+6+7 = 44	1+1+1 = 3	2+6+2 = 10	**57**
	Negative	17+2+10 = 29	108+23+67 = 198	11+4+0 = 15	**242**
	Total	**48+8+17 = 73**	**109+24+70 = 201**	**13+10+2 = 25**	**299**
Fluorescent Microscopy	**Positive**	38+8+15 = 61	3+7+2 = 12	3+9+2 = 14	**87**
	Negative	10+0+2 = 12	106+17+66 = 189	10+1+0 = 11	**212**
	Total	**48+8+17 = 73**	**109+24+70 = 201**	**13+10+2 = 25**	**299**
	A = Group I = **170** (General suspected cases without treatment) **B** = Group II = **42** (DOTS Follow-up patients) **C**= Group III = **87** (MDR Follow-up patients)				

Out of total 299 samples 73 (48+8+17) were culture positive and 25 were contaminated culture. Removing contaminated cultural samples and taking culture as gold standard, the sensitivity and specificity of direct microscopic examination were found to be 60.03% and 98.51% for ZN method; and 83.56% and 94.53% for Fluorescent AO method respectively. The percentage of False negative by AO staining was only 16.44% which was in sharp contrast to that of ZN (39.73%)

The sensitivity and specificity, false positive and false negative of ZN and AO in respective types of cases are depicted in Figure 3.

The comparison between ZN and AO methods with respect to culture and their types of cases (Figure 4) points out the sensitivity was the highest in case of follow-up patients with DOTS treatment in both ZN and AO but there was decrease in specificity and increase in false positivity in AO in the same. The false negativity was highest with ZN microscopy in MDR cases.

Table 4: Comparison between ZN and Fluorescent AO microscopy in total cases.

		Fluorescent Microscopy		
		Positive	**Negative**	**Total**
ZN Microscopy	**Positive**	57	0	**57**
	Negative	30	212	**242**
	total	**87**	**212**	**299**

The grade wise reporting with standard IUATLD/WHO pointed out that the ZN stained smears were able to detect 35 (31+4) multibacillary and 22 (5+17) paucibacillary cases whereas the AO stained smears were able to detect 36 (29+7) multibacillary and 49 (2+47) paucibacillary cases. More number of paucibacillary cases was detected by AO stained fluorescent microscopy in which the ZN was not sensitive enough as shown in the table 5.

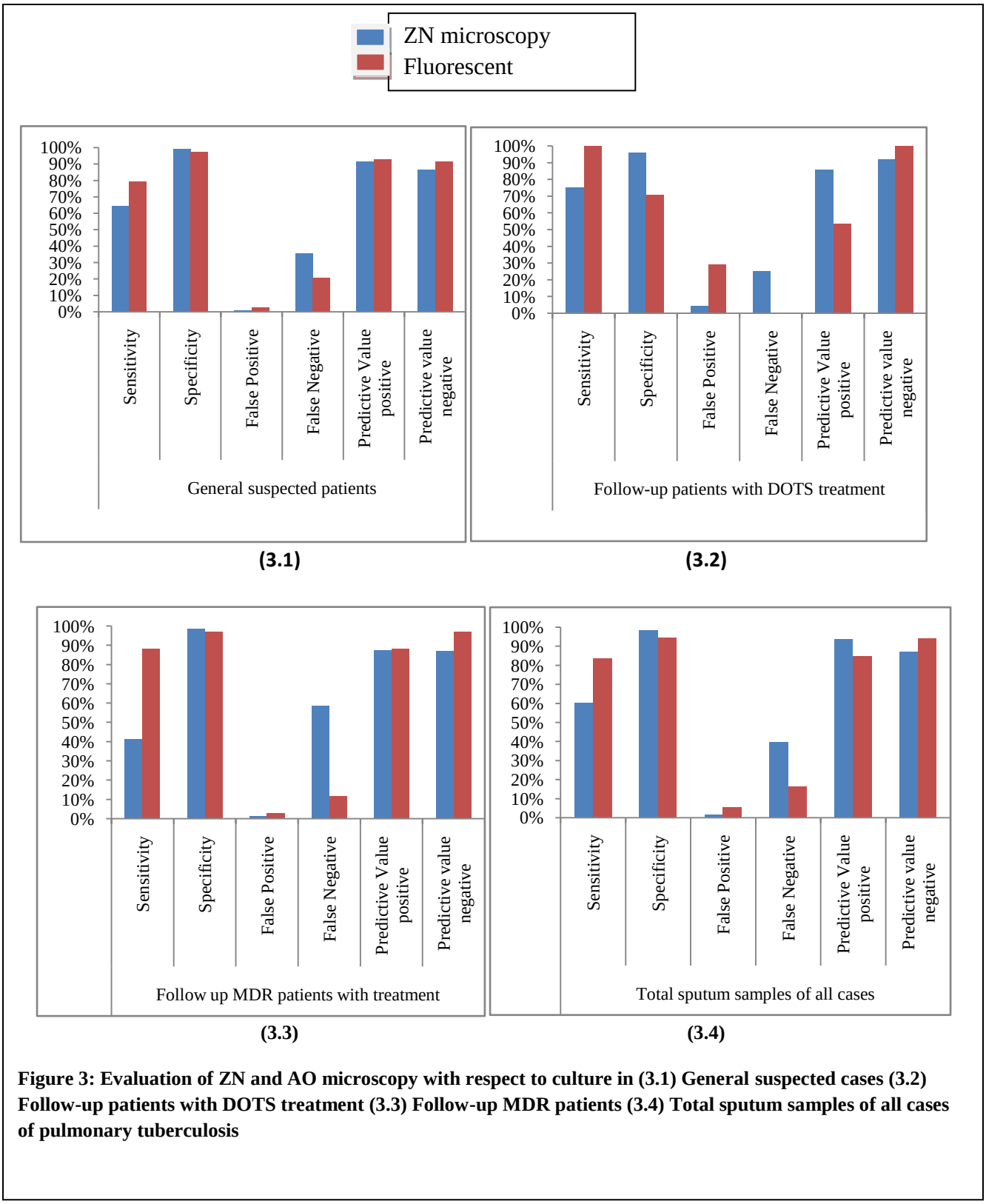

Figure 3: Evaluation of ZN and AO microscopy with respect to culture in (3.1) General suspected cases (3.2) Follow-up patients with DOTS treatment (3.3) Follow-up MDR patients (3.4) Total sputum samples of all cases of pulmonary tuberculosis

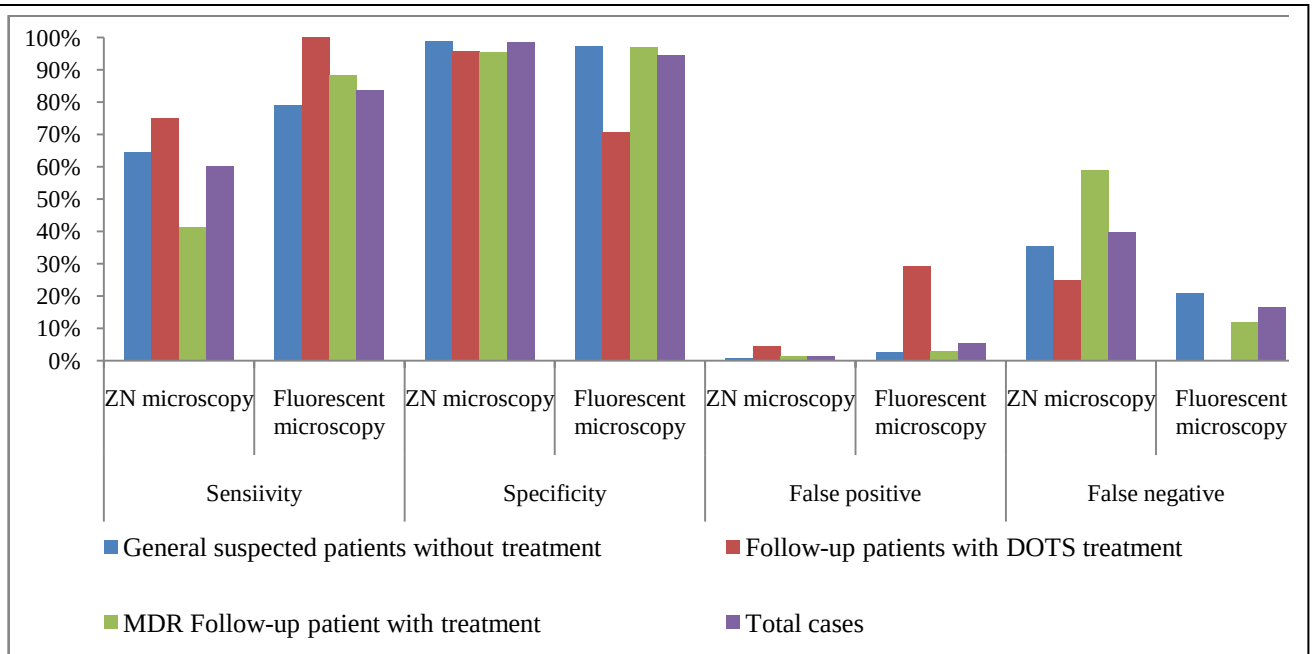

Fig. 4: Evaluation of ZN and AO microscopy with respect to culture in different types of cases.

Table 5: Grade-wise correlation of Ziehl-Neelsen and Fluorescent staining techniques (NS = No AFB seen; S = Scanty; PB = Paucibacillary; MB = Multibacillary)

Ziehl-Neelsen Microscopy	Fluorescent Microscopy					Total	
	Grading	NS	S	1+	2+	3+	
	NS	212	2	25	3	0	242
	S	0	0	5	0	0	5
	1+	0	0	15	2	0	17
	2+	0	0	3	25	3	31
	3+	0	0	0	1	3	4
Total		212	2	47	29	7	299

PB = 22

MB = 35

PB = 49

MB = 36

The comparison between ZN and AO methods with respect to culture and their types of cases (Figure 4) points out the sensitivity was the highest in case of follow-up patients with DOTS treatment in both ZN and AO but there was decrease in specificity and increase in false positivity in AO in the same. The false negativity was highest with ZN microscopy in MDR cases.

DISCUSSION

Early diagnosis of tuberculosis with aided sensitive and reliability is very much important for reducing transmission, morbidity and mortality especially in developing countries where resources lack in advanced methods of diagnosis. Because the clinical signs and symptoms of pulmonary tuberculosis are not specific, the fundamental principal for the diagnosis of tuberculosis is the accurate demonstration of *M. tuberculosis* in a suitable specimen from the suspected cases. The detection of smear positive cases is the highest priority in any TB control

program, as these cases are infectious and contribute to transmission of disease. Therefore the comparisons among the currently used methods in diagnosis are looked for in this study.

Smear microscopy using the conventional ZN stain and the recent AO stain are highly specific, rapid and cheapest method used for the early diagnosis of *Mycobacterium tuberculosis.* Though the culture method requires longer time, it is regarded as gold standard for diagnosis and useful for Drug Susceptibility Test (DST). ZN stain can detect bacilli when they are in the order of 10^5/ml of the sputum whereas a more sensitive AO stain can detect in the order of $10^{4/}$ml of sputum (Forbes BA, Bailey and Scott). Several studies by various authors have been shown the different case detection rate as with Jain et.al. ZN 32.7%, AO 41.6%, Githui et. al ZN 65%, AO 80%, Laifangbam et. al. ZN 44.1%, AO 71.6%, culture 70%, Prasanthi et. al. ZN 50%, AO 69% [9,11-13] In most of the study, the case detection rates were higher with AO staining than ZN staining as was the case in this study where 19.06%, 29.1% and 24.41% were found pulmonary tuberculosis positive by ZN, AO and culture respectively. The difference in the case detection rates was found to be statistically significant with better method of microscopy by AO method than ZN method ($p < 0.01$). In respective category of cases, the case detection rates for general suspected patients with no treatment were 20%, 25.88%, and 28.24%; for follow up patients of DOTS treatment were 30.95%, 57.14% and 19.05%; and for MDR follow-up patients were 11.49%, 21.84% and 19.54% for ZN, AO and culture respectively. This showed the decrease in culture detection in case of follow up cases with treatment where AO is most effective approach.

The range of sensitivity and specificity of ZN staining reported by various authors have been found in between 61% to 86.4% ; and 96.2% to 100% respectively ; whereas the same with AO staining (fluorescent microscopy) reports were 59.7% to 83% ; and 85.5% to 99% respectively[9,12-16]. Taking culture as gold standard and removing culture contaminated cases the overall sensitivity and specificity in the present study in all cases were found to be 60.03 % and 98.51% for ZN method; and 83.56% and 94.53% for fluorescent AO method respectively. The sensitivity of AO method is significantly much higher than that of ZN method. The specificity of AO method (94.53%) was quite lower than that of ZN method (98.51%). In the present study, the percentage of false negative by AO staining was only 16.44 % whereas the ZN method showed marked high false negative, 37.73% as also noted by ZN et. al. that around 40-50% of active pulmonary TB (culture positive) cannot be detected by ZN microscopy. No AO negative result with ZN positive samples was found, which makes the use of only AO staining fluorescent microscopy method is reliable microscopic method for diagnosis. There are also 25 cultural contaminated samples thus the sensitivity of these microscopic techniques lies in only those with culture positive and negative samples.

In a study conducted by Kumar et. al., 85% of culture positive cases could be diagnosed by microscopy alone. In this study, the diagnosis by ZN light microscopy alone was achieved in 60.03% (44/73) of the culture positive cases which is remarkably increased with AO fluorescent microscopy alone, 83.56% (61/73) of the culture positive cases. All cases of ZN positive were found to be positive by AO fluorescent method. Hence, it is apparent that the number of cases missed by ZN stain is higher which can be detected by AO fluorescent method.

CONCLUSION

The most commonly and routinely used method of diagnosis of pulmonary tuberculosis is the sputum examination by microscopy and culture method. The cost effectiveness and easy to perform with limited resources

made these methods most prominent approached in resource poor countries where the detection by molecular techniques are very limited and not usually done. In microscopy, there are conventional light microscopy (ZN staining) and fluorescent microscopy (AO staining). These two methods differ with each other in several aspects as efficacy, cost of processing and instrumentation. Similarly the culture method is prerequisite for standard diagnosis and testing of drug.

The study clearly indicated that the case detection rate (efficacy) of fluorescent microscopy (AO stain) is remarkably higher than that of ZN (light microscopy) with aided advantages of less eye strain, easy visualization, less time consuming and even detection of low number of bacteria (paucibacillary cases) in comparison to ZN method. As the screening was done under lower power magnification (400x) i.e. larger area per field than ZN method (1000x), less time is consumed for examination of same area by AO method. Taking culture as gold standard, there is high agreement between AO staining and culture than that of ZN staining and culture. The use of AO staining alone could be reliable microscopic method as there were no cases of ZN positive where AO was negative. But ZN staining alone missed most of the positive pulmonary cases with respect to AO and culture. In case of Fluorescent microscopy, though the capital cost is higher for expensive instrumentation, the overall cost with large number of sample processing by limited manpower makes it no difference in cost. Thus, it has been recommended as very effective method of choice in high risk areas where a large number of sputum samples are to be examined.

Similarly, culture examination is very efficient, most reliable and gold standard technique which is most prerequisite for determining strength of bacteria to antibiotics and differentiating from other non-pathogenic mycobacterium by growth rate and biochemical test. But the requirement of longer time duration (6-8 weeks), high cost, well trained manpower and chances of contamination are making it difficult to implement in developing countries. Thus culture along with fluorescent microscopy should be method of diagnosis of pulmonary tuberculosis.

Hence the correct diagnosis of pulmonary tuberculosis requires combination of AO (fluorescent microscopy), culture and biochemical analysis.

COMPETING INTERESTS

We declare that we don't have competing of interest.

AUTHORS' CONTRIBUTIONS

BT, BK, RP performed the sample collection, laboratory experiments and data recording. BM supervised and guided in the research. BT performed statistical analysis, conceived part of this study and prepared the manuscript. All authors read and approved the final manuscript.

Ethical Consideration

This study was approved by Institutional Review Committee of Nobel College, Sinamangal and GENETUP, Kalimati. Informed verbal agreement was taken from Patients. The intent and the importance of the study were.

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