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Comparison of Ziehl-Neelsen And Auramine Rhodamine Staining For The Detection of Mycobacterium Tuberculosis In Sputum Samples

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ABSTRACT

Introduction: Tuberculosis is a global health problem. A rapid and accurate diagnostic technique is necessary for effective management. The primary method for detection of *Mycobacterium Tuberculosis* (MTB) is sputum smear microscopy, using Ziehl-Neelsen (ZN) stain which has limitations. However, Auramine Rhodamine (AR) staining provides similar specificity but increased sensitivity. This study was conducted for comparison of ZN and AR staining in the detection of *Mycobacterium Tuberculosis* (MTB).

Methods: This study was conducted between March to July, 2025. Ethical approval was taken from Institutional Review Committee (Ref. No. MTH/001/2025). Written consents were taken from patients. Early morning sputum samples were collected and processed for ZN and Auramine staining. Data were analyzed using SPSS software.

Results: Out of 100 samples, 13 (13%) were positive for *Mycobacterium tuberculosis*. Eight of these were positives by AR staining only and 5 were positive by both ZN and AR staining. There were no cases that were negative with AR but positive with ZN method. There was no association between demographic and other factors such as age (χ^2=1.705; p=0.636), sex (χ^2=0.048; p=0.826), smoking (χ^2=0.088; p=0.766) and diabetes (χ^2=1.076; p = 0.300) with the detection of MTB. However, a significant association was observed between family history of TB (χ^2 =13.613; p < 0.001) and patient's occupation (χ^2 = 7.696; p = 0.006).

Conclusions: Auramine rhodamine staining was found superior to ZN for the detection of MTB. Our results suggest that house hold contacts of TB and high-risk groups especially farmers should be investigated on a priority basis.

Key Words: *Mycobacterium Tuberculosis; Ziehl-Neelsen staining; Auramine- Rhodamine staining*

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INTRODUCTION

Mycobacterium tuberculosis is the bacterium responsible for causing tuberculosis. It primarily spreads through airborne droplet nuclei by person-to-person transmission. It continues to remain a public health problem according to global TB report of WHO. In global report 2024, the number of people newly diagnosed with tuberculosis were 8.2 million. Among them 55% of people who developed TB were men, 33% were women and 12% were children and young adolescents. [1] In Nepal, tuberculosis is the leading cause of healthy life years lost among the productive age group. [2] In Nepal 2079/80, TB burden was estimated to be 70,000 total cases and in the fiscal year 2079/80, total of 36,819 cases of TB were notified and registered at NTCC (National Tuberculosis Control Center) /DOHS (Department of health service) Nepal. In the fiscal year 2079/80, 98.3% of reported TB cases were newly diagnosed or relapse cases. Among all TB cases, 72.5% were pulmonary TB, 57.3% of those being bacteriologically confirmed. Among the infected cases, 62.35 % were men, 37.7% were women and 8% were children below 14 years. [3]

The primary method for the detection of MTB is sputum smear microscopy with the help of light microscope. It is simple, rapid and inexpensive technique, but it has some limitations in its performance that it has low sensitivity. It is also valuable for evaluating the effectiveness of treatment and determining whether the patient has been cured by the end of therapy. [4]

Auramine Rhodamine(AR) staining is another microscopic technique which provides similar specificity and increased sensitivity (mean improvement of 10%) and allows more rapid screening of sputum smear compared to ZN – staining. [5,6]

Culture is considered the gold standard technique; however, the traditional culture method requires over two weeks to produce initial results, leading to delays in treatment due to the lack of a confirmed diagnosis. Automated culture system such as BACTEC MGIT are also available but it also has long turnaround time and higher contamination rate. [6] The GeneXpert system is a fully automated, rapid and highly sensitive molecular technique with ability to

detect drug resistance. However, it is expensive and requires specific infrastructures. [7]

This study was conducted for comparison of ZN and fluorochrome (AR) staining techniques in the diagnosis of tuberculosis.

METHODS

In this cross-sectional comparative study, a total of 100 sputum samples were collected and examined in the Department of Microbiology, Manipal Teaching Hospital (MTH), Pokhara during March to July 2025. Patients under anti tubercular therapy, samples with saliva only and samples less than 2 ml were excluded. The purpose of the study was clearly explained and written consent was obtained from each patient.

Processing: Following exclusion criteria, all the samples were collected, recorded into register using their assigned laboratory numbers and processed in a biosafety cabinet. Four slides were labeled for each sample as a ZN and AR. The samples were processed by modified Petroff's method to prepare smear. Smear preparation, staining technique and microscopic reporting was done according to the laboratory service in tuberculosis control guideline. [8]

Specimen collection and transportation: With the help of questionnaire, suspected TB patient were choosen, the sputum samples matching with inclusion criteria were collected from all the suspected patients who reported between the period from March to July 2025 for sputum examination at Manipal Teaching Hospital, Fulbari, Pokhara.

Appropriate collection instruction was given at site and consent was taken from the patients. The deep coughed sputum samples were collected by all the patients with full aseptic precautions in a sterile, dry, wide mouthed, leak proof container. The samples were labeled properly with demographic information of patients such as name, age, sex, hospital number, date and time of collection of specimens. Specimens were immediately transported to the Department of Microbiology of Manipal Teaching Hospital, Pokhara for further processing.

CONCENTRATION OF SPECIMENS BY MODIFIED PETROFF'S METHOD

A sputum sample (3–5 ml) was mixed with an equal volume of 4% NaOH and agitated on a shaker for 15 minutes. The mixture was then centrifuged at 3000 rpm for 15 minutes, after which the supernatant was carefully decanted. The remaining deposit was neutralized with 20 ml of sterile distilled water and centrifuged at 3000 rpm again for 15 minutes, followed by decanting of the supernatant. Finally, a smear was prepared from the resulting deposit. [9]

AR AND ZN STAINING METHODS:

AR and ZN staining procedures were followed in accordance with standard techniques. [10]

Briefly for ZN staining, the smeared slides were heat fixed and placed on a staining rack with the smear side facing upward and flooded with strong carbol fuchsin. The stain was gently heated until vapours appeared (without boiling) and maintained for about 5 minutes, ensuring the smear does not dry. The slides were then rinsed with clean water and decolorized using 20% sulphuric acid until the smear appeared faintly pink, followed by thorough washing. Counter staining was done with methylene blue for about 1 minute, after which the slides were rinsed again, allowed to air dry in a vertical position, and then were ready for microscopic examination. [11]

For AR staining the procedure was as follow:

A thin smear was prepared on a clean glass slide, air-dried, and heat-fixed. The smear was flooded with Auramine–Rhodamine stain for 15 minutes without heating and then rinsed with distilled water. It was decolorized with 3% acid-alcohol for 1–2 minutes, followed by rinsing and draining. The smear was then counter stained with potassium permanganate for about 1 minute, rinsed again, and air-dried without blotting. Finally, the slide was examined under a fluorescent microscope at 200X magnification, with confirmation at higher magnification if required. [11]

ANALYSIS

The generated data were compiled in a data entry form and also stored in Microsoft Office Excel Programme and later, exposed to SPSS 28.0 version software of windows for analysis. McNemar's chi-square test was calculated from SPSS software, to demonstrate any relationship between results obtained.

RESULTS

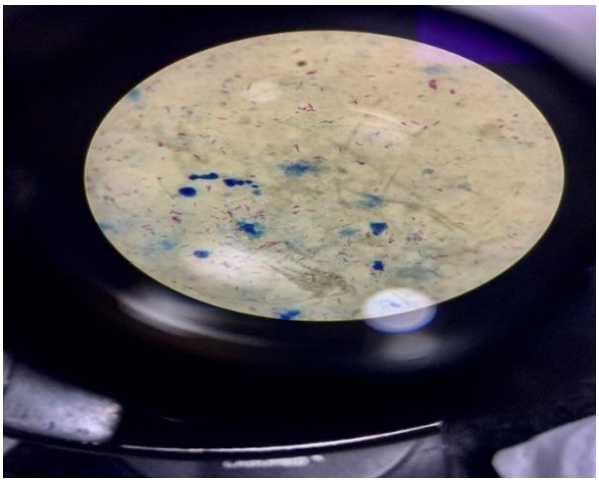

Figure 1: MTB under light microscope (100X).

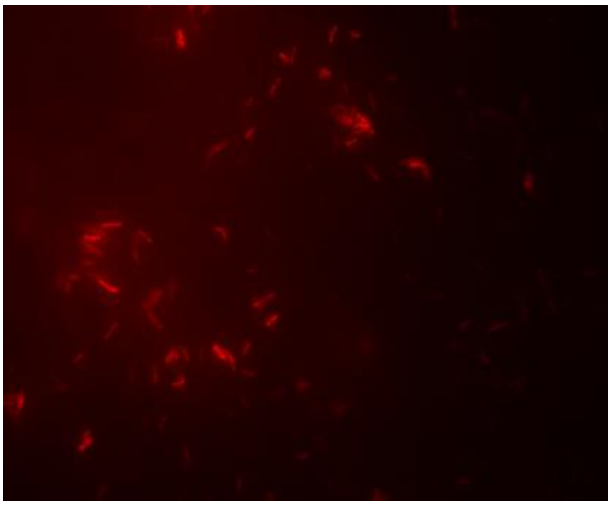

Figure 2: MTB under fluorescent microscope (40X).

In this study, total 100 sputum samples were processed. Representative staining results of ZN and AR methods have been depicted vide figure 1 and 2 respectively which show pink colored acid-fast bacilli and orange fluorescent bacilli.

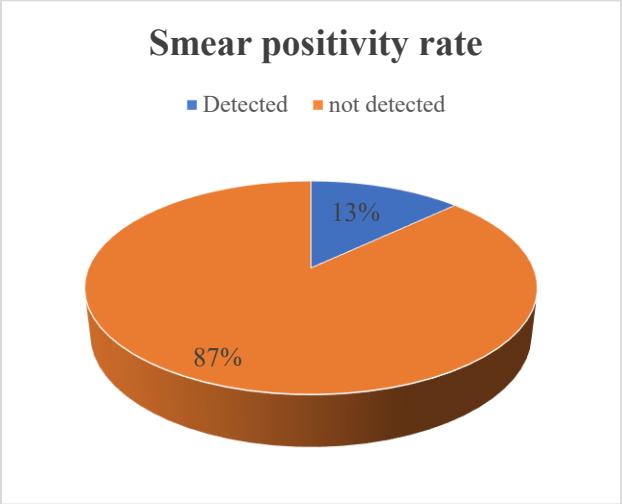

Figure 3: Smear positivity rate for MTB among patients visiting MTH.

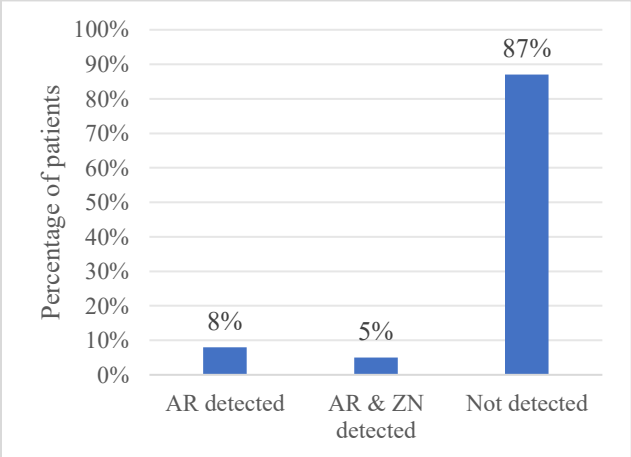

Figure 4: Comparison of AR and ZN staining.

Out of the 100 specimens processed 51 were from males and 49 from females. As shown in fig 3, overall smear positivity was found to be 13%

(13/100). Among these13, 8(8%) were positive by AR only, while 5 i.e. 5% were positive by both AR and ZN (fig 4). However, there were no cases that were negative with AR, but positive by ZN.

Table 1: Smear positivity for MTB in sputum samples of various age groups of patients. (n=100)

Age in years	MTB detected	MTB not detected	Total
20-39	2(9%)	20(91%)	22
40-59	4(22%)	14(78%)	18
60-79	5(11%)	39(89%)	44
80-99	2(12%)	14(88%)	16
(χ^2 =1.705; p = 0.636)			

Table 1 shows the overall smear positivity (MTB detected) and smear negativity (MTB not detected) in different groups of patients i.e. in the age groups of 20-39 years, 40-59 years, 60-79 years, 80-99 years. High rate of positivity was found in the adults belonging to the age group of 40-59 yrs (22% i.e. 4 out of 18) followed by elderly patients aged 80-99 yrs (12% i.e. 2 of 16).

Table 2: Detection of MTB in sputum samples of males vs females. (n=100)

Gender	MTB detected	MTB not detected	Total
Male	7(14%)	44(86%)	51
Female	6(12%)	43(88%)	49
Total	13	87	100
(χ^2 =0.048; p = 0.826)			

Similarly, when smear positivity was compared among males and females (Table 2), it was noted that MTB was detected among 14% of males and 12% of female patients.

Thus, the difference in the MTB positivity rates among various age groups and among males and females were not found to be statistically significant (Table 1 and 2; p = 0.636 and 0.826 respectively).

Table 3: Detection of MTB in sputum samples from smokers vs nonsmokers. (n=100)

Smoking habit	MTB detected	MTB not detected	Total
Smokers	7(14%)	43(86%)	50
Non smokers	6(12%)	44(88%)	50
Total	13	87	100
(χ^2=0.008; p = 0.766)			

Table 4: Detection of MTB in sputum samples from individuals with diabetes and without diabetes. (n=100)

Diabetic status	MTB detected	MTB not detected	Total
Diabetic	1(5%)	17(95%)	18
Non diabetic	12(15%)	70(85%)	82
Total	13	87	100
(χ^2=1.076; p=0.300)			

Exactly similar observation, i.e. statistically non significant correlations were detected when we compared MTB positivity among smokers and nonsmokers. (Table 3; p= 0.766) and diabetic individuals vs non diabetic individuals (Table 4; p = 0.300).

Table 5: Detection of MTB in sputum samples of farmers Vs non farmers. (n=100)

Occupation	MTB detected	MTB not detected	Total
Farmers	9(26%)	25(74%)	34
Non farmers	4(6%)	62(94%)	66
Total	13	87	100
(χ^2=7.696; p=0.006)			

However, comparative analyses of smear results among farmers and non farmers (table 5) and among those with family history of TB vs those without such family history (table 6) showed interesting results.

Table 6: Detection of MTB in sputum samples from individuals with positive family history vs those without positive family history. (n=100)

History of TB	MTB detected	MTB not detected	Total
Family history of TB	8(36%)	14(64%)	22
No family history of TB	5(6%)	73(94%)	78
Total	13	87	100
(χ^2=13.613; p<0.001)			

As shown in table 5, 26% of farmers were MTB positive as compared to only 6% of nonfarmers who were MTB positive and this difference was found to be statistically significant (p=0.006). Similarly, 36% of patients with family history suggestive of TB were MTB positive. Contrary to this only 6% of the patients without family history were MTB positive (p <0.001, table 6).

DISCUSSION

Tuberculosis is the major public health problem worldwide. It comes under top ten causes of mortality in Nepal. [12] Presence of AFB in the sputum smear microscopy is considered as indicative of infective stage of this disease. Role of the laboratory plays crucial role in the detection of the TB cases. Examination of the sample by light microscope is one of the rapid, economic and reliable techniques. Lately fluorescent microscopy was added by RNTCP guidelines due to higher sensitivity to detect

tubercle bacilli even if there was less load of bacilli in the sample. [13]

The aim of this study was to compare the ZN staining and AR staining for the detection of MTB in sputum samples from suspected TB patients. Additionally, the study sought to examine the relationship between MTB detection and various socio-demographic and health-related factors.

In our study, out of 100 samples, overall smear positivity was found to be 13% (13/100). Of these 8%were positive by AR only while 5%showed positivity by both AR and ZN. However, there were no cases that were negative with AR but positive with ZN. In a similar study done by Gurung et.al 5%(8/100) sample showed positivity by AR only. All those smear positive samples by AR only were confirmed by GeneXpert. [14] Contrary to ours, in a study done by Faridi et. al. [15] , it was found that 265 (51.56%) out of 514 samples were ZN smear positive while 326 (63.43%) were positive for AR. The authors were of the view that higher positivity rates observed by AR method could be due to the superiority of fluorescent microscopy over ordinary light microscopy.

As was evident from our study, maximum cases were seen among elderly. In a similar study done by Sha M. K. et. al it was observed that, the prevalence of the notified cases was highest among the patient having >60 years of age i.e. 32.54%(1344 /4131). [16] This may be due to the fact that older age group individuals were immune compromised compared to other age group individuals. In contrast, a study conducted by Raju et.al showed that highest number of the patients i.e. 52(20.71%) out of a total of 251 cases studied belonged to the age group 41-50, followed by 47(18.72%) in the 21-30 age group. [17]

In the present study, 18 patients were diabetic among whom 1(6%) was positive and 17(94%) were negative for MTB. However, 82 patients were non-diabetic amongst whom, 12(15%) showed positivity and 70(85%) showed negativity for MTB. In a similar study by Hu X.et. al it was shown that 11.3% had diabetes and had no significant relation with detection of MTB.

[18] Likewise, Thapa B et. al studied 407 person of whom 37(9.1%) were found to be diabetic. [19] Whereas in another study conducted by Sharma et. al the prevalence of diabetes among 320 TB patients was 11.9% (38/320). According to the authors, their data suggested that diabetes was a comorbid condition with TB. [20]

Our study demonstrated that, the 7(14%) males showed positivity while 6(12%) females were positive for MTB. In contrary to our findings, a report by Seifert M et al. [21] , put forth that MTB positivity was 47% in males compared with females (39%). The higher prevalence of TB among males as evidenced by studies referred to above could be multifactorial involving a complex interplay of biological vulnerability, behavioral and life style factors, environmental exposure and health care access inequalities.

In our study, out of 100 patients, 22 patients had family history of tuberculosis. Among the patients who had positive family history, 36% (8/22) were positive for MTB. Aditama and colleagues, in yet another study showed that, out of 260 cases enrolled, 135(51%) were positive for TB and 125(49%) were negative for TB. Among TB positive cases however 95(75%)had family history of TB, while 40 (25%)were without such family history. [22] This high incidence of TB among those with positive family history could be due to higher chances of re-exposure out of close contact.

Our findings showed that, 50% (50/100) subjects were smokers, and the rest were nonsmokers. MTB positivity as per our study was found in 7 smokers (14%) and 6 nonsmokers. A similar study conducted elsewhere showed the presence of MTB among 12.1% of current smokers, 9.9% of former smokers and 5.9% of nonsmokers. However, no significant association was observed between smoking status and MTB detection. [23] Although smoking was established as one of the predisposing factors in triggering pulmonary tuberculosis, by affecting alveolar macrophage function and compromising lung defense mechanism, in the present study we could not establish any relationship with smoking. This could be due to the fact that 50% of our subjects were not smokers. Additionally, the sample size was too small to draw any conclusion.

In the present study, the total number of farmers were 34 amongst whom 9(26%) were positive and 25(74%) were negative for MTB. Among 66 individuals from other occupations, 4(6%) were positive and 62 (94%) were negative for MTB. Such association between occupational exposure and MTB detection was found to be statistically significant. Another study conducted by Jabeen C et al. in this context documented a significant association of MTB with farming occupation. These findings highlighted tuberculosis as a major health concern among high risk occupational groups, particularly farmers and animal handlers. [24]

Due to limited resources, we could not perform culture and sensitivity testing, as well as molecular diagnostic techniques.

CONCLUSIONS

Overall, our study revealed that AR was superior to ZN in detecting MTB. There was no statistical correlation between demographic and other predisposing factors such as age, sex, smoking and diabetes with the detection of MTB. However, a significant association was found between family history and farming occupation with the occurrence of TB.

RECOMMENDATION

Based upon the findings several recommendations may be put forth which can be helpful for future researchers.

1. Auramine-Rhodamine should be preferred over ZN stain.
2. Household contact of TB cases must be investigated on priority basis without delay, by sputum smear examination using AR fluorescent technique.
3. High occupational risk group especially farmers should also be investigated for MTB by sputum smear examination with the help of AR fluorescent technique.

CONFLICT OF INTEREST

None

SOURCES OF FUNDING

None

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