A STUDY OF DETECTION OF MYCOBACTERIA BY FLUORESCENCE MICROSCOPY IN IMPRINT SMEAR AND ZIEHL-NEELSEN STAIN IN TISSUE SECTION FROM SKIN BIOPSY SPECIMEN

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ABSTRACT

BACKGROUND
Over the years, a number of methods have been tried to detect the presence of Mycobacteria in tissue samples. A number of previous literatures are available comparing the efficacy of fluorescent microscopy with Ziehl-Neelsen (ZN) stain for the detection of acid-fast bacilli (AFB) in tissue sections, but very few literatures are available on the efficacy of fluorescent method in imprint smears taken from skin biopsy over ZN method in tissue sections. Thus, the present study was undertaken with the following objective- to study the efficacy and advantages of using fluorescent microscopy in imprint smear from skin biopsies over ZN method in tissue sections for detection of mycobacteria in clinically suspected cases of leprosy and cutaneous tuberculosis.

MATERIALS AND METHODS
80 patients with a clinical suspicion of leprosy and cutaneous tuberculosis were biopsied & imprint smears taken. Imprint smears were air-dried and stained with Auramine O, and the skin biopsy samples were processed for routine histopathology and ZN Staining. Results were then analysed.

RESULTS
Out of 80 imprint smears, 65 were studied (remaining 15 were excluded from the study as per exclusion criteria), of which 40% (26/65) were positive for AFB on ZN method, while the smear positivity increased to 49.2% (32/65) on the fluorescent method.

CONCLUSION
Fluorescent microscopy has the advantage of speed and ease of screening and reduces observer fatigue and found to be more advantageous than conventional ZN method, particularly in paucibacillary and low mycobacterial load cases. Hence, fluorescent method can be an adjuvant when used with routine histopathology for the identification of AFB.

KEY WORDS
Imprint Smear, Fluorescent Method, Ziehl-Neelsen Stain.

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BACKGROUND

Over the years, a number of methods have been tried to detect the presence of Mycobacteria in tissue samples. Culture is the reference method for detection of mycobacteria, but it is time consuming and requires specialized procedures in laboratories. Serological techniques have the disadvantage of lack of sensitivity and specificity.[1] ZN Stain plays a major role in diagnosing and monitoring patients who are on treatment for leprosy and cutaneous tuberculosis.

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Its major disadvantage is low sensitivity ranging from 20% to 43%.[1,2] Numerous studies have indicated, the superiority of fluorescence microscopy over ZN stain in detection of mycobacteria in tissue sections.[3,4,5] In these studies, it was evident that fluorochrome staining offered greater ease and speed in detection of Mycobacteria. Newer molecular techniques such as polymerase chain reaction (PCR), although rapid, are costly to be routinely used in developing countries where most leprosy and cutaneous TB cases occur. Hence, a method for the identification of acid-fast bacilli (AFB) which is more sensitive and time saving than the ZN method is required for early detection of Mycobacterium.

The aim of this study is to correlate the fluorescent method in imprint smear with the ZN method in tissue section for the detection of AFB and, also to study the efficacy and advantages of using the Auramine O stain on imprint smear under fluorescent microscopy as a fast, efficacious and alternative method of detecting Mycobacterial infection.

Aim of The Study

To study the efficacy and advantages of using fluorescent microscopy in imprint smear over ZN method in tissue sections from skin biopsy specimens for detection of Mycobacteria in clinically suspected cases of leprosy and cutaneous tuberculosis.

Objectives of The Study

1. To determine the frequency of Mycobacterium positive cases in ZN stained tissue section.
2. To determine the frequency of Mycobacterium positive cases in imprint smear under fluorescence microscopy.
3. To correlate the fluorescent method in imprint smear with the ZN method in tissue sections for the detection of AFB in clinically suspected cases of leprosy and cutaneous tuberculosis.

MATERIALS AND METHODS

80 patients suspected clinically of having leprosy and cutaneous tuberculosis were biopsied from May 2015 to April 2016 were included in the study. Exclusion criteria were patients having chronic skin diseases other than leprosy and cutaneous tuberculosis, inadequate imprint smear and patient's refusal to participate in the study. Relevant details, such as history, duration of symptoms, and clinical findings were reviewed in these patients. All the patients were biopsied with a disposable skin biopsy punch and processed for routine histopathology with HE staining and ZN staining (5%/20% H_2SO_4) by direct microscopy. Imprint smears were taken from the punch biopsy specimen, air-dried and stained with Auramine O. Results were then compared with the findings of the ZN Staining method for detection of Mycobacteria and the bacteriological index noted down.

The following modified fluorescent staining procedure was implemented[6]

1. The heat-fixed smears were stained with the filtered Auramine-O mixture at 37°C for 15 minutes.
2. The slide was rinsed with deionized water for 2 minutes.
3. Decolorization was performed with 0.5% hydrochloric acid in 70% ethanol for 2 minutes.
4. The slide was rinsed with deionized water for 2 minutes.
5. Counterstaining was performed with 0.5% aqueous potassium permanganate for 2 minutes.
6. The slide was rinsed with deionized water for 2 minutes, and air dried and examined under high power (×400) which was confirmed under oil immersion (×1000).

Study Type and Design

Descriptive Study.

Study Place

NBMC&H.

Sample Size

65 patients.

Inclusion Criteria

All clinically diagnosed cases of Tuberculosis and Leprosy attending OPD/medicine dept.

Exclusion Criteria

1. Debilitated patients.
2. Patients not giving consent.
3. Bleeding disorder.
4. Immunocompromised state.

Controls

Positive and negative controls were included with each batch of smears stained by all the above methods. The AFB appear as yellow to orange, slender, rod-shaped bacilli under fluorescent microscopy. Smears stained by the ZN method, directly, were examined for AFB under oil immersion (×1000) using light microscopy. The data was processed statistically.

RESULTS

A total of 80 imprint smears and tissue sections were studied during the study period. Of these, 65 specimens were evaluated and the remaining 15 were eliminated. Out of the 15 cases, 13 were not diagnosed as cutaneous TB or leprosy on histopathology & they did not show mycobacteria in imprint smear also. The remaining 2 cases had inadequate imprint smear.

Age in Years	Number of Cases	Percentage (%)
1-10	03	5
11-20	15	23
21-30	13	20
31-40	11	17
41-50	12	18
51-60	11	17
Total	**65**	**100**

Table 1. Age Wise Distribution of Cases (n = 65)

The age ranged from 8 yrs., to 60 years. Maximum cases (23%) were in the age group of 11-20 yrs., of age with the mean age of 32.7 years.

Gender	Number	Percentage (%)
Male	35	53
Female	30	47

Table 2. Gender Wise Distribution of Cases (n = 65)

Slight male preponderance was noted accounting for 53% of cases with female 47% of cases.

Diagnosis	Total No. of Cases	(%)	AFB Positive on ZN Method	AFB Positive on Fluorescent Method
TT	17	26.1	02 (11.7%)	03 (17.6%)
BT	11	16.9	03 (27.2%)	05 (45.4%)
BL	05	7.7	04 (80%)	05 (100%)
LL	15	23	15 (100%)	15 (100%)
LV	13	20	00	00
Scrofuloderma	03	4.6	02 (66%)	03 (100%)
TVC	01	1.5	00	01 (100%)
Total	**65**	**100**	**26 (40%)**	**32 (49.2%)**

Table 3. Morphological Types of Cases (N = 65) and The Comparison of Results of Fluorescent Method in Imprint Smears and ZN Method in Tissue Sections

TT: Tuberculoid Leprosy, BT: Borderline Tuberculoid Leprosy, BL: Borderline Lepromatous Leprosy, LL: Lepromatous Leprosy, LV: Lupus Vulgaris, TVC: Tuberculosis Verrucosa Cutis.

TT accounted for the most number of cases in 17 (26%) cases, followed by LL in 15 (23%) cases & LV in 13 (20%) cases. There was only a single case of TVC. There were no cases of borderline borderline leprosy (BB).

Fluorescent method of staining showed a better positivity in 3 (17.6%) cases and 5 (45.4%) cases in paucibacillary conditions than ZN method which showed positivity in 2 (11.7%) cases and 3 (27.2 %) cases respectively. The methods showed equivalent positivity for LL. In this study, there was no detection of Mycobacteria in LV cases by any of the methods. Fluorescent method showed an overall positivity of 49.2% as compared to 40% for ZN Stain method.

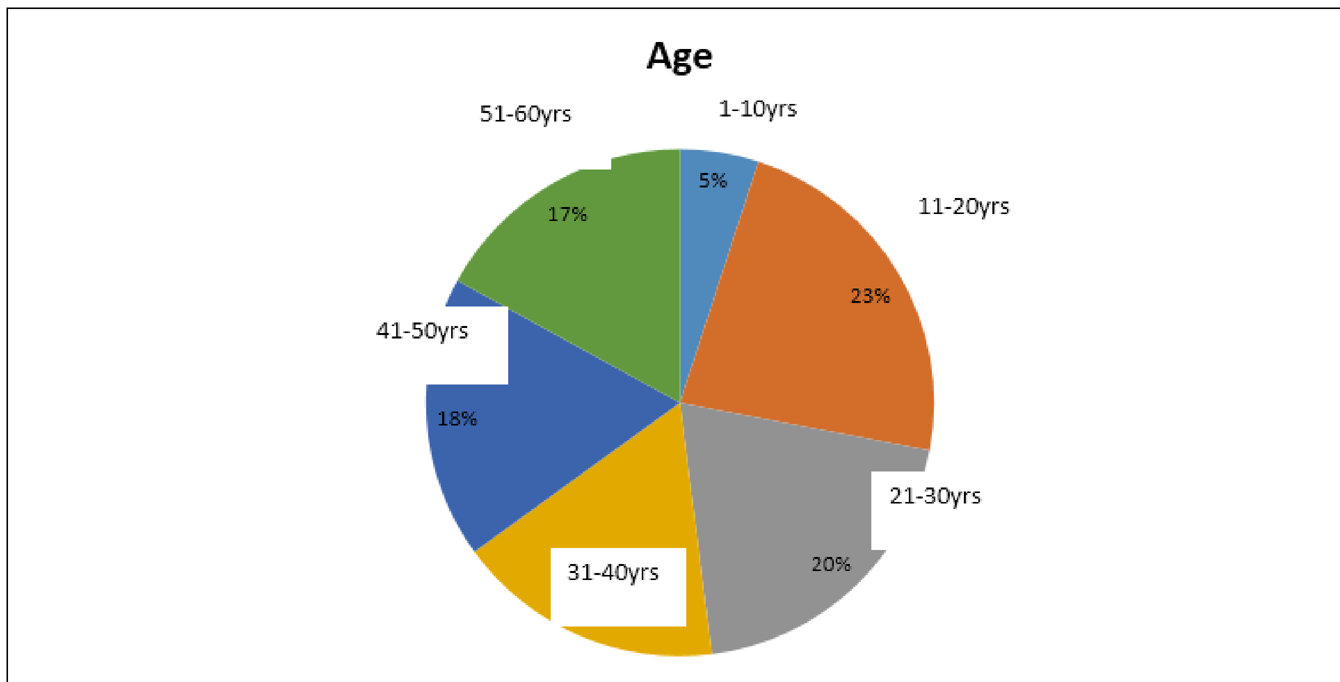

Figure 1. Pie-Chart Showing Distribution of Cases According to Age

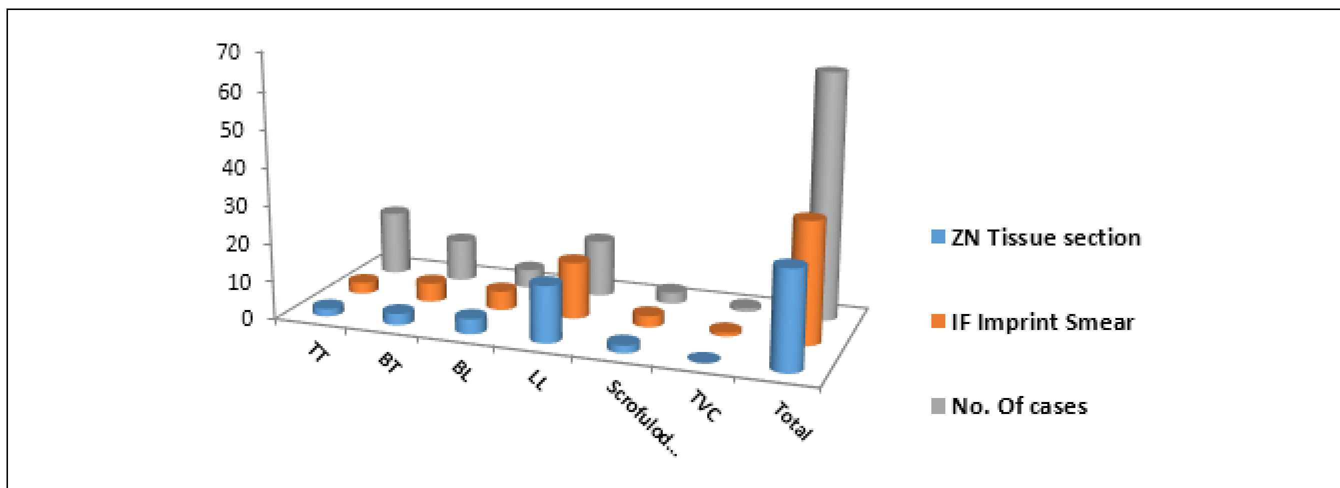

Figure 2. Comparison Between Fluorescent Method in Imprint Smears & ZN Method in Tissue Sections for Mycobacteria

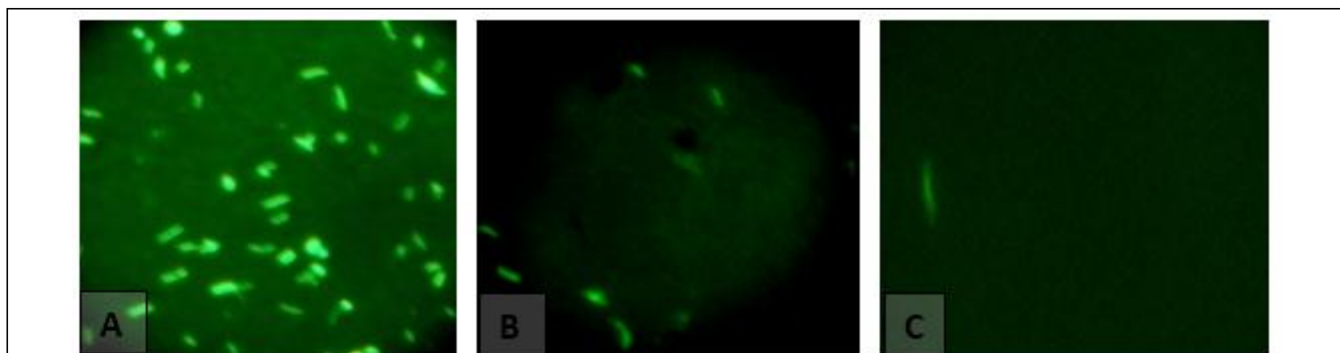

Figure 3. Photomicrograph of Mycobacteria with Frequency in Different Cases
a- Lepromatous, b- Borderline Tuberculoid, and c- Tuberculoid Leprosy

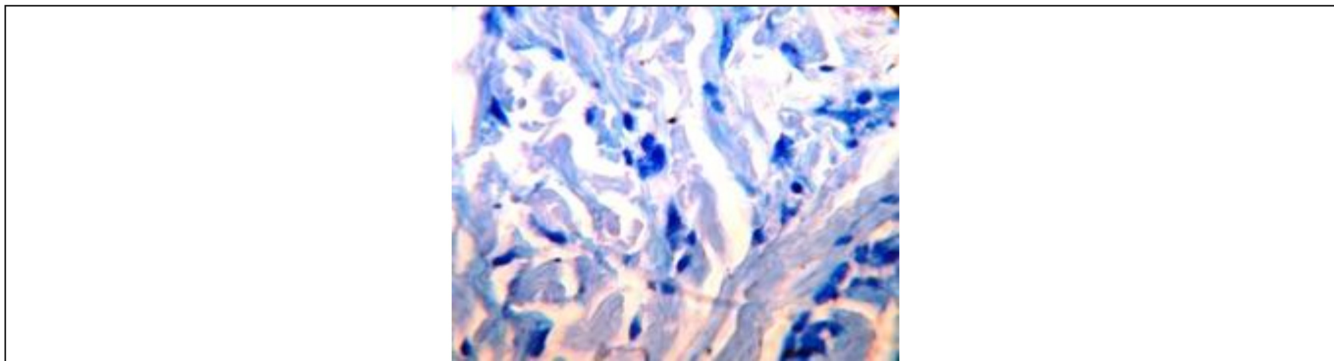

Figure 4. Photomicrograph of Tissue Sections of Lepromatous Leprosy Showing Mycobacteria in ZN Stain

Bacteriological Index by ZN Method in Tissue Section

Diagnosis	Number and Percentage of Cases							Total Nos.
	0+	1+	2+	3+	4+	5+	6+	
TT	15 (88.3%)	02 (11.7%)	0 (0%)	0 (0%)	0(0%)	0(0%)	0 (0%)	17
BT	08 (72.8%)	03 (27.2%)	0 (0%)	0 (0%)	0(0%)	0 (0%)	0 (0%)	11
BL	0 (0%)	0 (0%)	0 (0%)	03 (60%)	02 (40%)	0 (0%)	0 (0%)	05
LL	0 (0%)	0 (0%)	0 (0%)	0 (0%)	05 (33.3%)	06 (40%)	04 (26.6%)	15
LV	13 (100%)	0 (0%)	0 (0%)	0 (0%)	0(0%)	0 (0%)	0 (0%)	13
Scrofuloderma	01 (33.3%)	02 (66.7%)	0 (0%)	0 (0%)	0(0%)	0(0%)	0 (0%)	03
TVC	01 (100%)	0 (0%)	0 (0%)	0 (0%)	0(0%)	0(0%)	0 (0%)	01

Table 4. Bacteriological Index By ZN Method in Tissue Section

Bacteriological Index by Fluorescent Method

Diagnosis	Number and Percentage of Cases							Total Nos.
	0+	1+	2+	3+	4+	5+	6+	
TT	14 (82.4%)	03 (17.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	17
BT	06 (54.5%)	04 (36.3%)	01 (9.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	11
BL	0 (0%)	0 (0%)	0 (0%)	01 (20%)	04 (80%)	0 (0%)	0 (0%)	05
LL	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	05 (33.3%)	10 (66.6%)	15
LV	13 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	13
Scrofuloderma	0 (0%)	02 (66.7%)	01 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	03
TVC	0 (0%)	01 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0(0%)	01

Table 5. Bacteriological Index by Fluorescent Method in Imprint Smear

Bacterial Index	Ziehl-Neelsen Method	Fluorescent Method
0	38	33
1+	07	10
2+	0	02
3+	03	01
4+	07	04
5+	06	05
6+	04	10

Table 6. Comparison of Bacterial Index Between ZN Method in Tissue Sections & The Fluorescent Method in Imprint Smears

	Nayak SV et al		Jariwala HJ et al,		Our Study	
	No.	(%)	No.	(%)	No.	(%)
Total Number of Cases Studied	56	100%	50	100 %	65	100 %
Positivity in Fluorescence Method	39	69.64 %	22	44 %	32	49.23 %
AFB Detected in Tissue Section	25	44.64 %	20	40 %	26	40 %

Table 7. Comparison Between Different Study and Our Study of Sensitivity of Zn Method in Tissue Sections & The Fluorescent Method

There were 5 such cases which did not show bacilli in ZN method had a bacterial index of 1+ or 2+ in Fluorescent method. Fluorescent staining depicted a higher bacteriological index in most cases, signifying a better detection of bacillary load.

DISCUSSION

Prompt detection of Mycobacterial infection is a necessity for diagnosis as well as treatment. Early detection is important to avoid deformities. The diagnosis is confirmed by demonstration of Mycobacterium in tissue samples taken from the lesion.

Hagemann, in 1937, was the first to demonstrate the fluorescence of both Mycobacterium tuberculosis and Mycobacterium leprae when these bacteria were stained with berberine sulphate.[6] In 1938, he demonstrated better fluorescent-staining of these mycobacteria with phenol-auramine.[7]

We found that the positivity of fluorescent stain from imprint smears were 100% when compared to modified ZN Stain in tissue sections while detecting multibacillary M. leprae, scrofuloderma and tuberculosis verucosa cutis. These results were similar to the finding of Nayak,[8] Jariwala,[5] Bhatia[9] and Mansfield et al.[4] who carried out fluorescent staining in

tissue sections. However, Lacordaire [10] observed that fluorescent staining method was inferior.

The positivity for fluorescence staining from smears were 45.4% and 17.6% while detecting BT and TT cases compared to 11.7% and 27.2% respectively for ZN stained tissue sections.

The AFB typically fluoresce as golden, slender, rod-shaped bacilli, but they may appear curved or bent. Also, some individual AFB may display heavily stained areas referred to as beads and/or alternating light and dark areas of stain producing a banded appearance. Although the ability to retain aryl methane dyes, such as Auramine O, after washing with alcohol or weak acids is a primary feature of the genus Mycobacterium, it is not entirely unique to the genus. Other bacteria, which contain mycolic acids, such as Nocardia, can also exhibit this feature. The exact method by which the stain is retained is unclear, but it is thought that the stains become trapped within the cell or may form a complex with the mycolic acids.

A good observation is required to distinguish with certainty AFB from other small, naturally fluorescent particles present in some smears. When first using fluorescent microscopy, it is necessary to examine all small fluorescent objects seen both with the ×10 and ×40 objectives. With practice, it becomes possible to distinguish bacilli with a fair degree of certainty under the ×10 objective only, so that almost all negative smears can be examined with this objective only.

Pitfalls

The study process was not without its share of problems and hitches. The main problem which we faced was that of thin quality imprint smears, artifactual fluorescence & photobleaching of fluorescent stain. If the smear is too this, there is a possibility of the smear getting washed off during the staining procedure and yielding negative results.

Secondly, artefacts from the fluorochrome staining, together with interference from primary or secondary fluorescence of tissue compounds, increased the likelihood of false-positive results.

Moreover, morphological differentiation between Mycobacteria was not possible by fluorescent staining. Subsequent H/P examination remains a necessity.

These problems and obstacles can however be overcome by regular practice and carefully following procedures.

CONCLUSION

The use of the fluorescent method greatly improves the diagnostic value especially in patients with a low density of bacilli that are likely to be missed on ZN-stained smears. The speed of observation and the rapidity of finding the bacilli by fluorescent microscopy reduced observer's fatigue because the bright bacilli stand out in dark background. The fluorescent-stained smears can be examined under low magnification allowing for much larger areas of the smear to be examined in a much short span of time. Prompt detection of Mycobacterium would provide an important clue to the clinician and act as an aid to diagnosis & early treatment. Detection of bacilli by fluorescent method greatly improves its diagnostic value in AFB negative ZN-stained sections. It can be considered as an effective screening tool. Large scale study is needed to corroborate the findings.

Hence, it would be beneficial to use fluorescence method as an adjunctive, fast, efficacious method of detecting mycobacterial infections.

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