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Original Research Article

Evaluation of Fluorescent Staining for the Diagnosis of Leprosy in Tissue Sections and its Histopathological Correlation : Comparision with ZN Staining

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Abstract

Introduction: to study histopathological evaluation and its correlation with fluorescent staining used in the diagnosis of leprosy in tissue section and comparision of fluorescent staining with zn staining. *Materials and Methods:* 40 Skin biopsies cases from leprosy patients were received and processed as for routine histopathology. Sections of 5 microns were taken and routine H & E staining, ZN staining and fluorescent (auramine-rhodamine) staining is done. Various Stained sections were examined under light and fluorescent microscope there after result were ploted in tabulated form and various graphs. *Results:* Age group of 31–40 years were affected most with 13 cases (32.5%) Males were affected the most, with 23 cases (57%.5) and females being 17 cases (42.5%) with sex ratio is 1.35, that 80% of the patients belong to rural areas, Borderline Tuberculoid Leprosy was the most common histopathological diagnosis which constitutes 20 (50%) Positivity rate with fluorescent stain was 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen that shows improvement in Positivity rate by 47.5% and hence sensitivity as evident by present study. *Conclusion:* Hitopathological correlation with fluorescent (auramine-rhodamine) staining is significant. Fluorescent microscopy has higher case detection rates when compared to ZN stain. It can be used as a supplementary tool when ZN staining method fail to detect the bacilli. The procedure is valuable in cases where negativity of sections is to be certified. Positivity rate with fluorescent stain was 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen that shows improvement in Positivity rate by 47.5% and hence sensitivity as evident by present study. Comparision between ZN stain and fluorescent stain was found significant with $p < 0.001$ (calculated statistically by chi square test).

Keywords: Fluorescent stain (Auramine-Rhodamine); ZN stain; tissue section; Histopathological diagnosis.

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Introduction

Leprosy is a chronic infectious disease caused by Mycobacterium leprae (M. leprae) [1]. It is an ancient disease known to mankind for many years. Leprosy is also known as Hansen disease, named after G.A. Hansen, who is credited with the 1873 discovery of M. leprae [2]. The incubation period of M. leprae can range between nine months and twenty years. It has been estimated that 0.1-1% of the human population develops clinical disease suggesting the important role of host immunity. Leprosy is an old age disease in general and affecting mankind with myriad clinicopathological forms [3,4]. Mycobacterium leprae, is an obligate intracellular rod-shaped bacteria. The immune response of the patient and the density of the bacteria in the lesion (ie. Bacterial Index) determine the various clinical manifestation and the infectivity of disease. Histopathological examination of tissue sections of skin in leprosy patients exhibits different morphological patterns depending on the immune status of the host. Ziehl-Neelsen (ZN) staining method is the old and conventional method of detection of the organism [5]. Modified Fite-faraco technique is the routinely used method now a days to demonstrate Mycobacterium leprae in tissue sections in a cases of leprosy [4]. Though modified Fite-faraco is more sensitive than Ziehl Neelsen method in detection of Mycobacterium leprae in tissue sections, but it is tiresome, time consuming and leads to observer fatigue. Hence, fluorescent microscopy has been used for rapid screening to reduce observer fatigue and specially to increase sensitivity. Early detection of leprosy is important to avoid deformities in a developing country like India where the majority of cases are of paucibacillary type and deformities can be avoided by early detection. The diagnosis is confirmed by demonstration of Mycobacterium leprae (M. leprae) in tissue sections taken from the lesion. Ziehl Neelsen (ZN) staining technique is now replaced by modified Fite-Faraco method as lepra bacilli are not as acid fast as tubercle bacilli. As this method is tedious and less sensitive, there is need for simple and sensitive procedure.

Although the incidence of leprosy is declining, it continues to prevail in practically every corner of the world. As far as tropical countries like India are considered, it is still one of the major health problems of population. This problem can be overcome by correct diagnosis and early treatment. Leprosy is a chronic granulomatous inflammatory disease primarily of the peripheral nervous system, skin, and reticuloendothelial system [6]. It presents clinically as an erythematous or hypopigmented anesthetic patch of skin and a thickened and/ or tender cutaneous peropheral nerve. Leprosy is also called Hansen disease. Leprosy is a great mimiker of other skin diseases, and it can present with different morphological lesions, hence an expert eye is needed to diagnose it [7]. Leprosy manifests as a spectrum of disease beginning from lesions having low immunity and high infectivity to those having high immunity and low infectivity. This clinicopathological spectrum determines the treatment regimen for individual cases.

We hereby undertook the study with the aim of comparing the sensitivity of fluorescent microscopy with that of ZN staining in detecting Mycobacterium leprae in tissue sections and correlation of sensitivity of fluorescent microscopy with that of histopathological diagnosis of leprosy [8]. fluorescent staining is clearly shown to be superior to conventional Ziehl-Neelsen staining both in sensitivity and quality of staining. Also in paucibacillary cases it can be used as a supplementary tool when modified Fite-Faraco method fail to detect the bacilli in tissue sections. Hence the present study will be done to evaluate the diagnostic utility of fluorescent microscopy in the diagnosis of leprosy in tissue sections of clinically diagnosed leprosy patients in this institute (MLB medical college Jhansi).

Materials and Methods

Forty (40) Skin biopsies cases from patients clinically diagnosed as leprosy were received and Punch biopsy (4-5 mm) was used for obtaining samples of skin biopsy collected in 10% formalin/ NBF after injection of local anaesthetic. Sections of 5 microns were taken for routine H & E staining, ZN staining and for fluorescent (auramine-rhodamine) staining.

For Histopahological Examinaion: H & E Staining

Procedure: The following procedure has been used -

1. Warmed the slide and treat with xylene to remove wax.
2. treated with alcohol in (decreasing order of concentration) to remove xylene.
3. Rinsed in water.
4. Stained the slide with harris hematoxylene for 10–20 min.
5. Dipped of stain and washed thoroughly with water ¼–1/2 min.

6. Differentiated with 1% alcohol for 10–20 seconds till nuclei only are stained.
7. Washed off the 1% acid alcohol with tap water.
8. section to be carried out in warm tap water for atleast 5 min.
9. Counter stained with 1% aqueous eosin till section is bright red (1–5 min).
10. Washed in running water
11. Dehydrated with 70% & 95% & absolute alcohol1
12. Made the slide Clear ion xylene & Mounted with DPX

Results: Nuclei – Blue. Cytoplasm – shades of pink

Ziehl- Neelsen Stain

A. Procedure.

1. Paraffin sections placed in xylene for 30 min two changes each.
2. Hydrated by passing through 90%, 70% and 50% alcohol.
3. Stained with preheated carbol fuchsin for 10 minutes.
4. Decolorized in 1% alcohol.
5. Washed in running water.
6. Counterstained with methylene blue.
7. Dried, cleared in xylene and mounted.

Results: Acid fast bacilli- Red, Background – Light blue.

All the H & E stained as well as ZN stained sections were examined under microscope. Pathological findings were noted at the level of epidermis, dermis and sub-cutis and were segregated into different histological patterns. Sections showing organisms with typical morphology of Mycobacterium leprae by the 40X objective were confirmed using 100X objective. The typical rod shaped organisms which stained red were taken positive. Bacteriological index was calculated under the oil immersion field.

Fluorescent Staining

For fluorescent staining, ribbons containing four to five serial sections has been taken on clean scratch free slides. No adhesives like egg albumin has been used. After deparaffinisation in xylene, the auramine–rhodamine staining has been done according to the procedure of *Kuper and May* (1954). For each batch of sections that had stained, sections from a skin biopsy of a typical lepromatous leprosy patient and a skin biopsy from a normal individual has been used as controls.

Procedure

Routinely processed unstained paraffin sections mounted on glass slides has been used. They are deparaffinized in three changes of xylene leaving the slide in each change for one and a half minutes and they are then hydrated by dipping four or five times in (I) absolute alcohol, (2) 95% alcohol, (3) 70% alcohol, (4) water, in succession. This is then followed immediately by staining with an fluorescent stain.

The stain should be thoroughly mixed before use and preheated for 20 minutes to a temperature of 60°C. The slides are placed in the heated stain and kept in a 60°C. incubator for 10 minutes. Preheating provides a convenient and time-saving means of applying the stain at the proper temperature. Upon removal of the slides from the stain they has been:

1. Washed for two minutes.
2. Decolorized in 0.5% HCL for two to three minutes.
3. Rinsed in a slow running tap water bath for two minutes.
4. Counterstained with 0.5% potassium permanganate for one minute (this gives the sections a pale brown appearance grossly).
5. Washed for two minutes, Blotted dry & Dehydrated in absolute alcohol for 15 seconds.
6. Cleared in xylol & mounted with DPX.

Tissue sections were observed immediately under Carl Zeiss fluorescent microscope. All sections were screened with 10X and 40X objectives. Sections showing organisms with typical morphology of Mycobacterium leprae bacilli by the 40X objective were confirmed using 100X objective. Only solidly fluorescing organisms were considered for a definitive diagnosis. Bacillary fragments were not taken into consideration.

The typical morphology of the bacilli showing bright yellow fluorescence emitted by the bacilli when interspersed with the artifact was considered the diagnostic criteria for labeling the biopsy positive for Mycobacterium leprae. Mycobacterium leprae appeared as rod shaped organisms that emitted bright yellow fluorescence.

Statistical Analysis

For ZN stain:

Staining Method	No. of Positive cases	No. of Negative Case
ZN	7	33
Non-ZN	26	14

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\text{Sensitivity} = \frac{7}{7+26} \times 100 = \frac{7}{33} \times 100 = 21\%$$

$$\text{Specifity} = \frac{TN}{TN+FP} \times 100$$

$$\text{Specifity} = \frac{14}{33+14} \times 100 = \frac{14}{47} \times 100 = 29\%$$

$$\text{Positive predictive value} = \frac{TP}{TP+FP} \times 100$$

$$\text{Positive predictive value} = \frac{7}{7+33} \times 100 = \frac{7}{40} \times 100 = 17.5\%$$

For fluorescent stain

Staining Method	No. of Positive cases	No. of Negative Case
Fluorescent	26	14
Non-Fluorescent	7	33

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\text{Sensitivity} = \frac{26}{26+7} \times 100 = \frac{26}{33} \times 100 = 78.8\%$$

$$\text{Specifity} = \frac{TN}{TN+FP} \times 100$$

$$\text{Specifity} = \frac{14}{33+14} \times 100 = \frac{14}{47} \times 100 = 29.7\%$$

$$\text{Positive predictive value} = \frac{TP}{TP+FP} \times 100$$

$$\text{Positive predictive value} = \frac{26}{26+14} \times 100 = \frac{26}{40} \times 100 = 65.0\%$$

Results and Discussion

Table 1: Age wise distribution of cases (N-40).

Age	No. of cases	Percentage (%)
≤ 20	5	12.5%
21–30	9	22.5%
31–40	13	32.5%
41–50	4	10%
> 50	9	22.5%
Total	40	100%

In the present study, patients in the age group of 31-40 years were affected most with 13 cases (32.5%) out of 40 cases. The least affected age groups are those < 20 years and 41–50, comprising 5 cases (12.5%) and 4 cases (10%).

According to one study in Uttar Pradesh, published in journals/ISRN/2013, concluded that patient with more than 59 years are most commonly affected by leprosy followed by 50–59 years and 40–49 years of age.

Table 2: Gender distribution of patients (N-40).

Sex	No. of cases	Percentage
Male	23	57.5%
Female	17	42.5%
Total	40	100%

In the present study males were affected the most, with 23 cases (57%.5) and females being 17 cases (42.5%) with sex ratio is 1.35 which shows that leprosy is more common in male patients

Table 3: Different histological patterns in present study (N-40).

Histopathological Diagnosis	No. of Cases	Percentage (%)
Intermediate leprosy	4	10%
Tuberculoid Leprosy	0	0%
Borderline Tuberculoid Leprosy	20	50%
Borderline Borderline Leprosy	0	0%
Borderline Lepromatous Leprosy	12	30%
Lepromatous Leprosy	4	10%
Total	40	100%

In our study Borderline Tuberculoid Leprosy was the most common histopathological diagnosis which constitutes 20 (50%) out of 40 cases, followed by Borderline Lepromatous Leprosy 12(30%), lepromatous leprosy 4 (10%) and Intermediate leprosy 4 (10%). There was no borderline borderline & no tuberculoid leprosy cases in our study.

In a study conducted by Shenoi SD *et al.* [9] in 1988, most common histological pattern was borderline tuberculoid leprosy (50%) followed by tuberculoid leprosy (22%), indeterminate leprosy (11%), borderline borderline (6%), lepromatous leprosy (6%) and borderline lepromatous(5%).

Kar P.K *et al.* [10]., in their study, in 1994, found that the most common histological pattern of leprosy was borderline tuberculoid leprosy (31.66%) followed by indeterminate leprosy (29.16%), tuberculoid leprosy (18.33%), borderline lepromatous leprosy (8.33%), borderline borderline leprosy (6.66%) and lepromatous leprosy (5.83%).

Table 4: Percentage of histological diagnosis positive for ZN Stain

Histo-Pathological Diagnosis	ZN Staining		
	No. of Positive cases	No. of Total cases	%
Intermediate leprosy	0	4	0%
Tuberculoid Leprosy	0	0	0
Borderline Tuberculoid Leprosy	1	20	5%
Borderline Borderline Leprosy	0	0	0
Borderline Lepromatous Leprosy	2	12	16.7%
Lepromatous Leprosy	4	4	100%
Total	7	40	17.5%

In the present study various histological patterns showed varied positivity rates for ZN stain. 0 (0%) out of 4 patients of indeterminate leprosy, 1 (5%) out of 20 cases of borderline tuberculoid leprosy, 2 (16.7%) out of 12 cases of borderline lepromatous leprosy and 4 (100%) out of 4 cases of lepromatous leprosy were positive by ZN stain.

Table 5: Percentage of histological diagnosis positive for fluorescent Stain.

Histo-Pathological Diagnosis	Fluorescent Staining		
	No. of Positive cases	No. of Total cases	%
Intermediate leprosy	1	4	25%
Tuberculoid Leprosy	0	0	0
Borderline Tuberculoid Leprosy	11	20	55%
Borderline Borderline Leprosy	0	0	0
Borderline Lepromatous Leprosy	10	12	83.33%
Lepromatous Leprosy	4	4	100%
Total	26	40	65%

In the present study various histological patterns showed varied positivity for fluorescent stain. 1 (25%) out of 4 patients of indeterminate leprosy, 11 (55%) out of 20 cases of borderline tuberculoid leprosy, 10 (83.3%) out of 12 cases of borderline lepromatous leprosy and 4 (100%) out of 4 cases of lepromatous leprosy were positive by fluorescent stain.

Table 6: Comparison of ZN stain and fluorescent stain in tissue section (N=40).

Staining Method	No. of cases	Result	Percentage
ZN	40	7	17.5%
Fluorescent	40	26	65%

The results obtained after staining the biopsy slides with H & E stain, ZN stain, and Fluorescent stain were analysed. In the present study, patients in the age group of 31–40 years were affected most with 13 cases (32.5%) out of 40 cases and the least affected age groups are those < 20 years and 41-50, comprising 5 cases (12.5%) and 4 cases (10%) respectively. Males were affected the most, with 23 cases (57%.5) and females being 17 cases (42.5%) with sex ratio is 1.35 which shows that leprosy is more common in male patients. This clear reflected in my study that 80% of the patients belong to rural areas. Borderline Tuberculoid Leprosy was the most common histopathological diagnosis which constitutes 20 (50%) out of 40 cases, followed by Borderline Lepromatous Leprosy 12 (30%), lepromatous leprosy 4 (10%) and Intermediate leprosy 4 (10%). There was no borderline borderline & no tuberculoid leprosy cases in our study.

Positivity rate with fluorescent stain in our study is 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen. Hence apart from its higher probability of detecting a case, fluorescent microscopy has an additional value in more accurate grading of Hansen's disease, which affects therapy and outcome.Thus our study shows that fluorescent stain is better than ZN stain in detecting lepra bacilli in tissue sections.

Hitopathological correlation with fluorescent (auramine-rhodamine) staining is significant suggested by the presence of high load of bacteria towards lepromatous leprosy. Fluorescent microscopy has higher case detection rates when compared to Ziehl–Neelsen as evident by its higher sensitivity. Fluorescent microscopy can be used as a supplementary tool when tissue sections stained by Ziehl–Neelsen staining method fail to detect the bacilli in tissue sections. The procedure is valuable in cases where negativity of sections is to be certified.

Lepromatous leprosy shows higher positivity rate, more intense positivity as compared with other histological types and clumps of bacteria which conclude that lepromatous leprosy has more bacterial load and clumps showing macrophages full of bacteria. Positivity rate with fluorescent stain was 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen that shows improvement in Positivity rate by 47.5% and hence sensitivity as evident by present study. Comparision between ZN

stain and fluorescent stain was found significant with $p < 0.001$ (calculated statistically by chi square test).

Lepra bacilli first infect the neural tissue close to site of inoculation of the bacteria. Primary target are the schwann cells. Subsequently fate and type of lesion depend on immune states of the host. Bacilli multiply within the schwann cell as well as perineural cells, later the bacilli destroy them to librate which again enter the neighboring schwann cells and spread the infection intraneurally. When the intraneural infection is recognized, lymphocytes and macrophages infiltrate the nerve, and later macrophages engulf the these bacilli. The bacilli multiply within the macrophages and then are carried to other parts of the nerve and other nerves of various part of body. Later they spread to other parts of the body through blood, lymph as well as tissue fluids. The major factor which determines the outcome is the immune status of the host. The macrophages become foamy due to utilization of oxygen and nutrition from the cytoplasm, by the lepra bacilli.

Later the macrophage ruptures, librating the bacilli into the skin and other structures. These bacilli are again picked up by fresh macrophages. The body responds by a number of lymphocytes and phagocytic macrophages to the site of infection.

In majority of the cases the bacilli are killed by the phagolysosome of the macrophage and the infection fails to establish uaually. But In about 5% of cases the bacilli multiply in the macrophage probably by preventing the formation of phagolysosome.

There is cell mediated immunity and delayed hypersensitivity in the pathogenesis of leprosy disease. This complete immune response involves mainly T-lymphocytes, macrophages, to some extent B Lymphocytes and the cytokines mediators. Whether an individual has tuberculoid or lepromatous leprosy determined by the T-helper lymphocyte response to M. leprae.

Patients with tuberculoid leprosy have a defective TH1 response or a dominant TH2 response along with production of IL-4, IL-5 and IL-10, which will suppress macrophage activation. In tuberculoid leprosy there are fair number of CD4+T lymphocytes and in lepromatous leprosy there are CD4+T lymphocytes are decreased.

In case of Tuberculoid leprosy - CD4+ T cells increase and CD8+ T cells decrease. In case of Lepromatous leprosy- CD4+ T cells decrease and CD8+ T cells increase. In lepromatous patients, CD4+ T helper 2 cells when stimulated by the antigen presenting cell secrete IL-4 and IL-5 which activate B-lymphocytes to secreting plasma cells leading to formation of antigen – antibody complexes. This causes type II reaction (Erythema Nodosum leprosum).

Table 7: Comparison of positivity rates of Ziehl–Neelsen staining, modified Fite-faraco and fluorescent stain with that of other studies.

Various studies	Ziehl–Neelsen stain	Fite-Faraco stain	Fluorescence stain
	No. of Positive Cases	No. of Positive cases	No. of Positive Cases
Present study	7 (17.5%)	-	26 (65%)
Mukkamil AS *et al.* [11]	-	25 (44.64%)	39 (69.64%)
Jariwala *et al.* [12]	-	20 (40.0%)	22 (44.0%)
Bhatia *et al.* [13]	57 (67.8%)	-	75 (89.2%)
Lacordaire Lopes de Faria [14]	-	26 (86.6%)	10 (33.3%)

The present study shows a higher positivity rate in detecting the bacilli with fluorescent staining as compared to that of Ziehl–Neelsen staining which is comparable to the studies done by Bhatia et al. Positivity rate with fluorescent stain in our study is 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen.

Hence, apart from its higher probability of detecting a case, fluorescent microscopy has an additional value in more accurate grading of Hansen's disease, which affects therapy and outcome.

Hitopathological correlation with fluorescent (auramine-rhodamine) staining is significant as suggested by the presence of high load of bacteria towards lepromatous leprosy denoted by staining pattern. Fluorescent microscopy has higher case detection rates when compared to Ziehl–Neelsen as evident by its higher sensitivity. Fluorescent microscopy can be used as a supplementary tool when tissue sections stained by Ziehl–Neelsen staining method fail to detect the bacilli in tissue sections. The procedure is valuable in cases where negativity of sections is to be certified.

Lepromatous leprosy shows higher positivity rate, more intense positivity as compared with other histological types and clumps of bacteria which conclude that lepromatous leprosy has more

bacterial load and clumps showing macrophages full of bacteria. Positivity rate with fluorescent stain was 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen that shows improvement in Positivity rate by 47.5% and hence sensitivity as evident by present study.

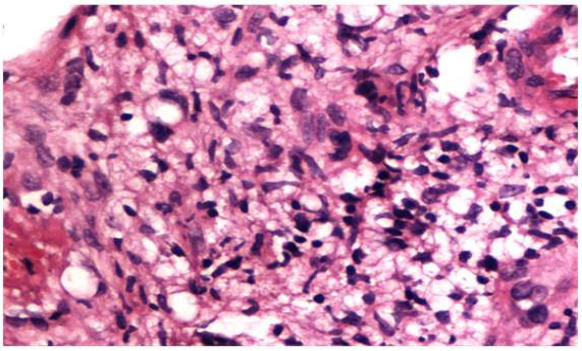

Fig. 1: H&E Stained: HP (40X) view showing granuloma

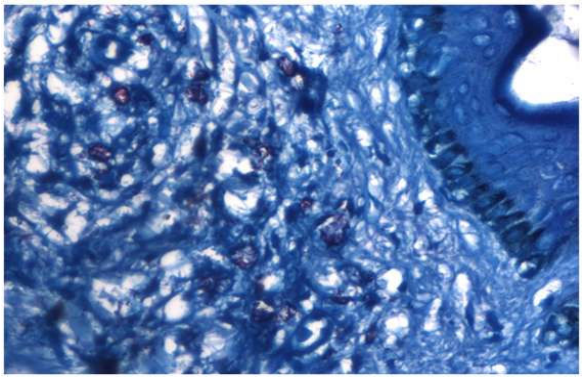

Fig. 2: ZN Stained: HP(40X) view showing pink acid fast bacilli

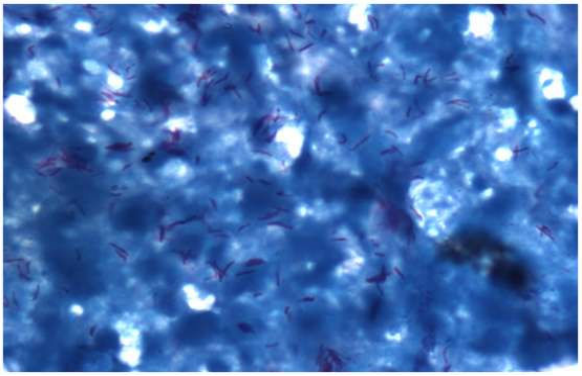

Fig. 3: ZN Stained: oil(100X) view showing pink acid fast bacili

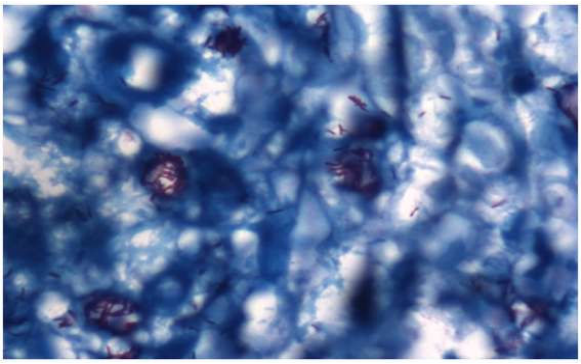

Fig. 4: ZN Stained: oil (100X) view showing pink acid fast bacilli in groups

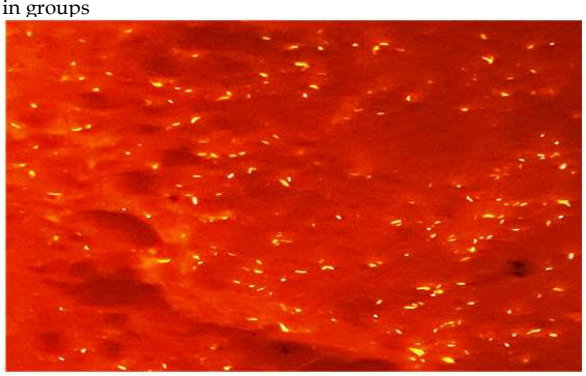

Fig. 5: Fluorescent Stained: LP(10X) view showing bacilli

Fig. 6: Fluorescent Stained: HP(40X) view showing bacill

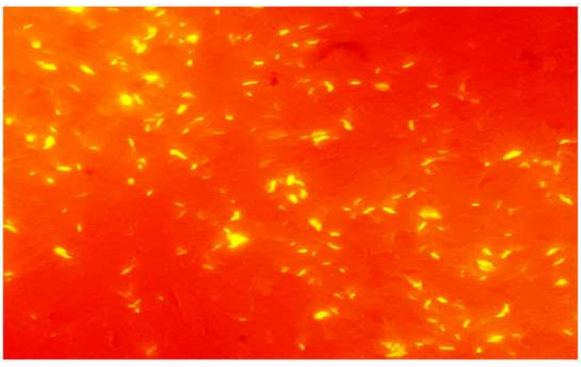

Fig. 7: Fluorescent Stained: HP(40X) view showing bacilli

Conclusion

Maximum number of patients were in 4th decade, least affected being those in 5th decade. Males were affected more compared to females. Borderline Tuberculoid Leprosy was the most common histological type, and borderline borderline least common. Early detection is prudent and crucial in case of leprosy because by doing this one can establish treatment soon thereby consequent deformities can be avoided.

Positivity rate with fluorescent stain was 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen.

Hence apart from its higher probability of detecting a case, fluorescent microscopy has an additional value in more accurate grading of Hansen's disease, which affects therapy and outcome.

- Hitopathological correlation with fluorescent (auramine-rhodamine) staining is significant.
- Fluorescent microscopy has higher case detection rates when compared to Ziehl – Neelsen as evident by its higher sensitivity.
- Fluorescent microscopy can be used as a supplementary tool when tissue sections stained by Ziehl–Neelsen staining method fail to detect the bacilli in tissue sections
- The procedure is valuable in cases where negativity of sections is to be certified.
- Lepromatous leprosy shows higher positivity rate, more intense positivity as compared with other histological types and clumps of bacteria which conclude that lepromatous leprosy has more bacterial load and clumps showing macrophages full of bacteria.
- Positivity rate with fluorescent stain was 65%, whereas with Ziehl–Neelsen was 17.5%. Staining by fluorescent method detected an additional case which was missed by Ziehl–Neelsen that shows improvement in Positivity rate by 47.5% and hence sensitivity as evident by present study.

Although fluorescent staining is not cost effective but as it is more sensitive therefore it is better for the purpose to diagnose, to classify as well as for the treatment purpose and hence can be used as routine staining.

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