"COMPARISON OF FLUORESCENT STAINING AND CONVENTIONAL STAINING OF SKIN BIOPSY IN DIAGNOSIS OF LEPROSY- A HOSPITAL BASED STUDY."

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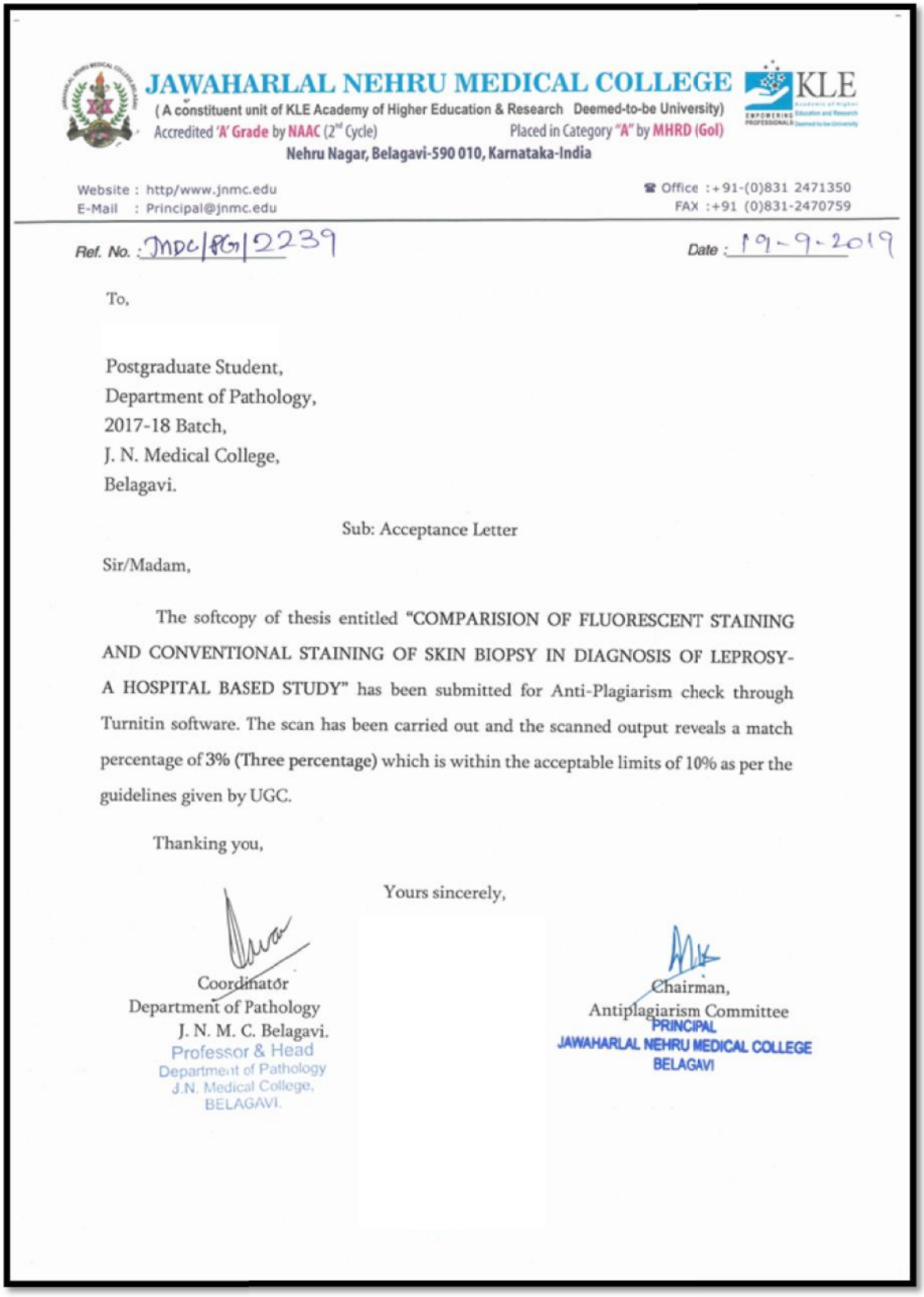

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ABSTRACT

"COMPARISON OF FLUORESCENT STAINING AND CONVENTIONAL STAINING OF SKIN BIOPSY IN DIAGNOSIS OF LEPROSY- A HOSPITAL BASED STUDY."

BACKGROUND: Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. The correct diagnosis of leprosy is done by histopathological examination of skin lesions. In this study, we evaluate the use of fluorescent microscopy for rapid and accurate diagnosis and compare fluorescent staining with the conventional staining in the diagnosis of leprosy.

OBJECTIVES: 1.To compare fluorescent staining and conventional staining of skin biopsy in diagnosis of leprosy.

2. To study the histological spectrum of skin lesions in leprosy.

METHODOLOGY: Skin biopsies of forty-nine cases of leprosy patients were received in the Department of Pathology, Jawaharlal Nehru Medical College, Belagavi, Karnataka were stained with Hematoxylin and Eosin(H and E), Wade-Fite (WF)stain and Fluorescent stain(FL). Each case was evaluated for presence of bacilli on 10X, 40X and 100X.

RESULTS: The sensitivity of Fluorescent stain over Wade-Fite stain was found out to be 82.50 and specificity was found out to be 100 %. The positive predictive value of the fluorescent stain was found out to be 100 % and negative predictive value of 56.25%.

CONCLUSION: Fluorescent staining is sensitive than the conventional Wade Fite stain in detecting lepra bacilli in tissue sections particularly in cases with lower Bacteriological Index.

KEY WORDS: Leprosy; Fluorescent microscopy; Wade-Fite stain; Auramine Rhodamine.

LIST OF ABBREVIATIONS USED

AFB	Acid fast bacilli
AR	Auramine-rhodamine
BB	Mid borderline leposy
BI	Bacteriological index.
BL	Borderline lepromaous leprosy.
BT	Borderline tuberculoid leprosy.
DNA	Deoxyribonucleic acid.
ENL	Erythema nodosum leprosum.
FL	Fluorescent stain
IL	Indeterminate leprosy.
LL	Lepromatous leprosy.
MB	Multibacillary
PB	Paucibacillary
PCR	Polymerase chain reaction.
TT	Tuberculoid leprosy.
WF	Wade-Fite stain.
WHO	World Health Organisation
ZN	Ziehl Neelsen

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INTRODUCTION

"Leprosy" is an ominous age-old disease associated with a heavy stigma around it. It is a chronic infectious disease which is caused by *Mycobacterium leprae*. "Leprosy" was known as "Kushtaroga" since ancient times.

The stigma that revolves around this disease is because it is extremely contagious. Leprosy affects many parts of the body but it has more inclination towards the cooler areas such as the peripheral nerves and skin. And hence a proper skin and neurological examination can only establish the diagnosis.[1]

This disease affects all age groups. As physical disabilities are the most common sequelae of this disease, early diagnosis is very essential which will in turn, leads to proper treatment and hence having an impact on an individual's life. This will reduce the stigma and prejudice revolving around this disease.[1]

Leprosy is a grievous public health crisis in most of the developing countries. So control of communicable disease is based on identifying and destroying or attacking the causative organism.

The first case of leprosy has been documented as old as biblical times around 3000 years ago. Gerhard Armauer Hansen, a Norwegian physician identified the causative agent of leprosy as Mycobacterium leprae in the year 1873.[2]

This disease is known to be endemic in the tropical regions, mainly in the underdeveloped or developing countries.

Since the introduction of Multi Drug Treatment (MDT) at the beginning of 1980s, the prevalence of leprosy has markedly decreased. Till date, many countries are still

endemic, specifically the countries located in America, South-East Asia, Eastern Pacific, Western Mediterranean and Africa.

The goal of elimination of leprosy as a public health issue has yet not been achieved by India (defined by the prevalence lower than 1 case per 10,000 inhabitants), ranking first in terms of the total number of cases, with Brazil ranking the second.

This problem can be managed by accurate diagnosis and timely treatment. The clinical diagnosis of leprosy has to be established by a diagnostic procedure like skin biopsy and slit skin smear.

Histopathological examination of the skin biopsies in leprosy exhibits a different morphological pattern which depends on the host's immune status. The conventional method of detection of the organism in clinical specimens is Ziehl-Neelsen (ZN) staining method.[3]

The Ziehl Neelsen stain was modified by Fite and Faraco. Modified Fite-Faraco technique, also known as Wade-Fite stain is now routinely used as a standard method to illustrate *Mycobacterium leprae* in skin biopsy sections.[3]

Even though modified Wade Fite stain is sensitive than Ziehl Neelsen stain in the detection of *Mycobacterium leprae* in skin biopsy sections, it is very tiresome, time taking and often also leads to observer fatigue especially in the paucibacillary case.

Therefore fluorescent staining has been employed by several researchers for rapid screening to decrease observer fatigue and to increase sensitivity.

In this study, a comparison was done between the sensitivity of fluorescent microscopy with that of Wade-Fite stain in detecting *Mycobacterium leprae* in skin biopsies of leprosy.

OBJECTIVES

1. Compare fluorescent staining with conventional staining in diagnosis of leprosy.
2. To study the histological spectrum of skin lesions in leprosy.

REVIEW OF LITERATURE

Historical Aspects:

Leprosy is one of the oldest diseases known to humans.[4] Leprosy was discovered by G.H. Armauer in 1873. He found that leprosy was caused by the collection of rod-shaped bacilli in the lesion. It was initially observed by him in skin, nerves and visceral lesions in unstained tissue specimens and in due course he found that they could be better visualized if treated with dilute osmic acid. [5]

The research work on the classification and diagnosis of leprosy was going hand in hand with research on the treatment since the time this disabling disease was discovered. Greater emphasis was made on the proper diagnostics, as it determined the basis of the treatment and thereby causing a reduction in the associated disability and death. Hence, a lot of research was done which suggested and demonstrated different ways to identify lepra bacilli in slit skin smears and tissue sections.

The main challenge posed by most researchers was the difficult staining properties of *Mycobacterium leprae* due to its acid fastness. Moreover, the obscurity of staining *Mycobacterium leprae* in paraffin sections of skin biopsy is mainly due to the capacity of these bacilli to adsorb paraffin wax, and also due to the destruction of the acid-fastness of the bacteria as a result of removal with fat solvents. In both these respects, this bacilli differs from *M.tuberculus*.

One of the oldest methods of detecting the organisms in tissue sections is conventional ZN method of staining[3]. Later a modification was adapted to this method commonly known as the Wade-Fite method which requires a combination of peanut oil and xylene to reduce exposure of the bacteria's cell wall to organic solvents

and protect acid-fastness of these organisms. Now, this has become one of the most commonly used methods for demonstrating lepra bacilli in tissue sections [6]. One of the other methods with which bacilli can be demonstrated is the fluorescence method using Auramine and Rhodamine stains and also with PCR technique.[7]

Another modality of recent diagnostic importance is the use of Polymerase Chain Reaction. The use of DNA amplification based on PCR provides an exquisitely sensitive method for detecting *M. leprae*. This technique could be used to assess the treatment of paucibacillary patients and to detect the presence or persistence of bacteria in detecting sub-clinical infection.[8,9]

Studies using this technique have detected *M. leprae* DNA on swabs taken from nasal mucosa of normal individuals staying in an area where leprosy is endemic. The only major setback it has is the higher cost and lack of expertise.

EPIDEMIOLOGY

There has been a drastic decline in the global prevalence of this disease over the past twenty years due to the initiation of multi-drug therapy. Since 1985, as many as 20 million patients around the world have been treated by MDT. [10, 11]

According to the WHO, the global registered prevalence of leprosy is 1,76,176 cases at the end of 2015 collected from 138 countries of 6 WHO regions. During the same year, 2,11,973 new cases were reported.[12]

"A total of 88,166 leprosy cases are on record in India in April 2017, giving cumulative Prevalence Rate (PR) of 0.66 per 10,000 populations, as against 86028 cases April 2016. A total of 554 districts (81.23%), out of 682 districts had reported PR<1/10,000 population. 128 district reported with PR>1/10000 population. Total

number of cases released as cured during 2015-16, is 1,25,302 (94.33%) which calculates as the total number of persons affected by this disease, cured of the disease, with multi-drug treatment in India, till date to 130.59 lakh (13.05 million)."[13]

"Leprosy continues to be a key public health problem in India with a yearly new case detection rate of 10.17 per 100,000 population, as against 1,27,334 cases in 2015-16. According to the national leprosy eradication programme report of 2017-2018, the total new case detection is highest in the state of Bihar followed by Uttar Pradesh, Maharashtra, Chhattisgarh and Odisha. The prevalence rate per 10,000 was highest in the state of Chhattisgarh and Lakshadweep closely followed by Odisha and Bihar."[13]

Leprosy has always remained as a chief public health problem in many extremely endemic countries around the world, namely Madagascar, Congo, India, Brazil, Mozambique, Nepal, Tanzania and Angola African Republic. Elimination of leprosy as public health problem was achieved globally in the year 2000.[12]

Leprosy occurs at all ages right from early infancy to extreme old age. Therefore the age of onset remains very variable with respect to time, place and country.[14]

Even though leprosy affects both the sexes, the male to female ratio is mostly 2:1. The main reason behind this could be that leprosy workers are mostly men, and moreover, the examination of women is usually less complete and unsatisfactory particularly in most cultural situations resulting in under detection of leprosy cases among the females.[15]

With regards to the global prevalence, major steps have been made towards the objective of leprosy control. The global yearly incidence proves much more complicated to decipher. Theoretically, incidence can be estimated by new case

detection rates, which holds factual only if a stable proportion of all existing cases are duly notified. However, this postulation is not met with regards to leprosy.[16]

The diverse environmental coverage, the varied intensity of case-finding, re-registration of defaulted patients, and the leprosy information systems varies for every country and has changed a lot over time. Thus, it is very difficult to use new case detection rates as a substitute for incidence.[16]

BACTERIAL PROPERTIES

Leprosy is caused by *Mycobacterium leprae*, which was discovered by Gerhard Henrick Armauer Hansen in 1873.[17]

The exact taxonomy of mycobacterium was described in the Bergey's manual and is classified as[18]

Class	Schizomycetes
Order	Actinomycetales
Family	Mycobacteriacae
Genus	*Mycobacterium*
Species	*Leprae*

GENERAL CHARACTERISTICS

M.leprae is usually slender, straight or slightly curved rod-shaped organism which ranges from 1µm to 8 µm in length and measures about 0.3 mm in breadth. These organisms have parallel sides and rounded ends. These organisms are acid-fast and alcohol fast. They are also gram-positive, non-capsulated, non-motile, non-cultivable, non-sporing and obligate intracellular bacterium. They are predominantly seen in

macrophages, either singly or as large aggregates termed as globi. These organisms divide by binary fission.[18,19]

SPECIAL CHARACTERISTICS

1. Due to the presence of mycolic acid in the cell wall, these bacteria have a unique property of acid fastness.[18]

2. Pyridine extraction: When *M.leprae* is treated with pyridine, it removes the acid fastness of the bacteria but not gram positivity. [18]

3. Ultra-structure: *M.leprae* has the following characteristics:

a. The cell wall of this bacteria has a thickness of about 15-20 nm, consisting of an inner electron-dense and an outer electron transparent layer.

b. The cytoplasm of *M.leprae* is electron-dense and also contains structures common to gram-positive organisms, such as DNA, mesosomes, storage granules and continuous plasma membrane below the cell wall.[21]

BIOLOGICAL PROPERTIES

The generation time of *M.leprae* is 11-13 days on the footpads of mice.[20]The optimum temperature required for the growth of *M.leprae* is 27-30° C.[21] The minimal infective dose of *M.leprae* is 40-43 live solid staining bacteria [22]. It has viability of 7 to 10 days at 4° C. [18]

BIOCHEMICAL PROPERTIES.

1) Capsule: The most characteristic feature of the capsule of *M.leprae* is the presence of an abundant amount of lipid component which is responsible for the foamy material seen in macrophage of lepromatous patients.[18]

Two types of capsular lipids are described.

1. Wax ester phthiocerol dimycocerosate (PDIM).[23]
2. Phenolic glycolipid-I (PGL-I)

PGL-I reacts with free radical compounds. Draper and Rees in 1970 also suggested that the lipid capsule protects the bacteria from the toxic effects of all the lysosomal enzymes and reactive oxygen metabolites which are produced by host macrophages during infection by this bacteria.[18]

2) Cell wall: The cell-wall has cross-linked peptidoglycan. In *M.leprae* peptidoglycan, glycine is present at place of L-alanine at the amino terminus of the tetrapeptide side chain. This unique pattern of cell wall associated with mycolic acids distinguishes *M.leprae* from other mycobacteria.[24]

A second most important component of the cell wall is lipoarabinomannan (LAM) and has similar features with that of other mycobacteria, although a few monoclonal antibodies to LAM have been reported to differentiate among *M.leprae* and *M.tuberculosis*.[25]

ANTIGENIC STRUCTURE:

As many as 20 different antigens can be detected by immunodiffusion. Out of these antigens, two are specific to *M.leprae*. These antigens have been identified as well as purified. The first antigen is a phenolic glycolipid-1(PGL-1) which is serologically active and has also been biochemically synthesized. Many serological tests such as ELISA are based on the use of this antigen. The other antigen is lipoarabinomannan which is a dominant antigen of *M.leprae* and has immunological cross-reactivity with similar products from tuberculosis.[26]

CULTIVATION:

The two main animals used for in-vivo cultivation are mouse and nine-banded armadillo (Dasypus novemcinctus).[27]

Mice are more convenient as well as more sensitive in detecting a small number of *M.leprae*. Armadillos are more inconvenient to work with and even more costly but the bacterial population produced is up to 10^{12} leprae or 300 mg. A few T cell deprived models like thymectomized irradiated mice, neonatally thymectomized Lewis rats and nude rats have been used. Many other experimental models have been tried such as the Pangolian, Syrian and Chinese hamsters, the Koren chipmunk and a few species of mastomys.[28]

TRANSMISSION

The route of transmission of *M.leprae* is still a grey area of research. The most plausible mechanism is believed to be the transmission via the naso-respiratory route. There is very few evidence to support transmission via fomites or skin abrasions.[29]

The virulence of this organism is based on features of its cell wall.[30] These bacteria have specific targets:

1. Peripheral neural tissue (Schwann cells)
2. Small vessels (pericytes and endothelial cells)
3. Monocyte-macrophage system

The most common histopathological landmark of this disease is the formation of granulomas.[31]

Another area of discussion is the likelihood of a subclinical carrier condition in which a subset of patients can carry the disease and infect others, but not develop leprosy by themselves.[32]

As thought earlier, leprosy does not appear to be a highly infectious disease. Also as a known fact, the majority of people exposed to *M. leprae* never develop this disease. Strong data suggests that extended and close contact with a leprosy patient is obligatory to pass on the disease.[33,34]

INCUBATION PERIOD

The incubation time of *M. leprae* is very unsure, generally thought to be between 2 to 5 years.[35,36] However, some researchers also say that the incubation time could be as less as a few months and as extensive as many years.[37]

BACTERIAL INDEX

This index tells about the number of bacilli which can be observed in a microscopic field. Minimum of at least 25 microscopic fields should be examined. In this method, both live and dead bacilli are counted. It is assessed in 5µm thick tissue sections in the same way as it is done in smears. The dermis is not assessed. Oil immersion objective is used.

1+	1 to 10 bacilli per 100 OIF – Examine 100 OIF
2+	1 to 10 bacilli per 10 OIF – Examine 100 OIF
3+	1 to 10 bacilli per OIF – Examine 25 OIF
4+	10 to 100 bacilli per OIF – Examine 25 OIF
5+	100 to 1000 bacilli Per OIF – Examine 25 OIF
6+	>1000 per bacilli per OIF – Examine 25 OIF

Morphology of individual bacilli varies in tissue sections and smears which is stained by ZN method or Fite stain.[38, 39]

Morphological Index:

"In this 200 living bacilli are counted and expressed as a percentage. Living (viable), leprosy bacilli appear as solid staining i.e., bright pink rods having rounded tips and are uniformly stained throughout their complete length. Non-living (dead) leprosy bacilli appear as fragments (showing small gaps in stain) or granular (showing unstained zones across the width of the bacilli)" [38,39]

Auramine-Rhodamine staining technique and fluorescent microscopy.[40]

Fluorescent microscopy has often been used by many researchers for quick screening of mycobacterium leprae and also to reduce observer fatigue. There are quite a few studies done on skin biopsy section to detect M. leprae by fluorescent microscopy. There are various conflicting views about the sensitivity of fluorescent staining in detecting M. leprae in skin biopsy sections.

In almost all paucibacillary cases this method has an added advantage over the conventional modified Wade-Fite method and it can also be used as an additional tool when skin biopsy sections stained by Wade-Fite method fails to identify the bacilli.

Fluorescent microscopy for detecting *Mycobacteria* was first used by Hagemann in1937.[41]

After this in 1952, the advantages of fluorescent microscopy for detecting *Mycobacterium leprae* in smears was described by Gohar.[42]

Later in 1956, Khanolkar and Nerurkar used fluorescent microscopy in their study of diagnosis of leprosy in smears.[43]

In 1960, Kuper and May introduced the use of fluorescent microscopy for the recognition of acid-fast bacilli in skin biopsy sections. They also added Rhodamine to the older method of staining which greatly improved the appreciation of bacilli and contrast by fluorescent microscope.[44]

In a study conducted by, Silver et al in 1966, various modifications in the procedure of fluorescent staining was suggested which included time variation in the duration of exposure to stain and counter-stain and also mountant to be used.[45]

In the further course of development in 1970, Mansfield observed that with the use of fluorescent microscopy helped in easy identification of the bacilli. He also concluded from his study that xylene peanut oil mixture for deparaffinization produces much brighter staining of the bacilli in cases of both fluorochrome and carbol-fuchsin stained tissue sections.[41]

However, in 1972 Lacordaire conducted a study in which he established the Wade-Fite stain to be better compared with the fluorescent staining in detecting *Mycobacterium leprae* in skin biopsy sections.[46]

In 1979 Jariwala et al observed that fluorescence stain was better than the Wade-Fite stain in detecting *Mycobacterium leprae* in skin biopsy sections mainly in paucibacillary cases. They also observed that the field covered was 16 times larger, so that an average section could, therefore, be scanned in two to three minutes.[47]

Later in the year 1981, Hardas and Lele, concluded after their study on 117 smears and 69 biopsies that granular and dusty forms of the organisms were altogether

missed by the fluorescent method. He also remarked that the higher frequency of artefacts makes the staining less advantageous.[48]

A little further in the timeline, Bhatia et al carried out a study in 1987 in which a large number of histopathological sections from various cases of indeterminate, tuberculoid and borderline tuberculoid cases by fluorescent method. They found in their study that in comparison to ZN staining, fluorescent staining was superior and that it can be used to supplement ZN staining when there is a conflict in decision regarding negativity of smear.[49]

A year later in 1988, Bhatia et al again conducted a similar study on 84 skin smears and it was found that 75 smears showed AFB by Auramine staining as compared to only 57 smears by ZN staining. And also with regards to bacterial index (BI), it was higher by Auramine staining as compared to BI by ZN staining. The study also revealed a minimal inter-observer variation by Auramine method.[50]

715 clinical specimens were studied in 2002 by Jain et al, which included sputum, fine needle aspirate, pus and body fluids and examined them by ZN and Auramine Rhodamine staining techniques, simultaneously. They found the sensitivity of Auramine Rhodamine staining to be as high as 98% when compared to 78.8% by ZN staining. Also, fluorescent stain was more advantageous in paucibacillary cases.[51]

In 2003, Nayak and Shivarudrappa promoted fluorescent microscopy for leprosy diagnosis after a study that was conducted in a hospital in South India. It was found that fluorescent staining had a higher positive rate as compared to Wade-Fite. The rapidity of finding the bacilli and the speed of observation also reduced observer fatigue.[40]

In a recent study done by Deepa et al in 2016 concluded on similar lines that fluorescent method is more sensitive in detecting lepra bacilli in skin biopsy sections with main emphasis in case having low BI than three.[52]

CLASSIFICATION

The clinical manifestations of leprosy are based on the status of immune system of an individual, thus expressing in two extreme forms. Between these two forms, various other types are present. The overlapping of the clinical as well as histological features is the main reason for marked difficulties in its classification.

Box 1 Classifications of leprosy

1. Pre-Manila classifications
    a) Danielssen & Boeck (1847)
    b) Danielssen & Boeck (1848)
    c) Hansen & Looft (1895)
    d) Neisser (1903)
2. The Manila classification (1931)
3. The Cairo classification (1938)
4. The Strassbourg classification (1923)
5. The Pan American classification (1946)
6. The Havana classification (1948)
7. WHO expert committee (1952)
8. The Madrid classification (1953)
9. The Indian classification (1955)
10. Ridley-Jopling classification (1966)
11. The New IAL classification (1981)
12. Job & Chacko classification
13. WHO classification (1982)
14. WHO classification (1988)
15. WHO classification based on number of lesions (1998)
16. Classification under NLEP, India (2009)

The Manila classification

In the year 1931, a round table conference was held in Manila by Leonard Wood Memorial, Philippine. This was the first time when a first-ever international system of classification was attempted dividing leprosy into three types. [53]

Box 2. The Manila classification (1931) [53]

1.	Cutaneous
2.	Neural
3.	Mixed

WHO Expert Committee classification (1952)

The expert committee of WHO in 1952 at its first meeting recommended adding a new group as borderline group in the already accepted three groups at Havana.[54]

Box 3 WHO expert committee (1952) [54]

1. Lepromatous
2. Tuberculoid
3. Indeterminate
4. Borderline

The Madrid classification

Immediately in the next year in 1953, one of the major improvements was made at Madrid, at International leprosy congress. In this classification, the four classes proposed by WHO was further divided into various clinicohistological type.[55]

Box 4. The Madrid classification (1953) [55]

"Lepromatous type (L)

- Macular
- Diffuse
- Infiltrated
- Nodular
- Neuritic, pure

Tuberculoid type

- Macular
- Minor tuberculoid (micropapuloid)
- Major tuberculoid (plaques, annular lesion)
- Neuritic, Pure

Indeterminate group

- Macular
- Neuritic type

Borderline (Dimorphous) group

Infiltrated "

The Indian classification

As the disease burden in India was at a rise, a need for a practical classification arose. Therefore a classification was proposed by Indian authors in 1955 where maculoanesthetic and pure neuritic were kept separate for the ease of field workers.[56]

Box 5.The Indian classification (1955) [56]

- Lepromatous (L)
- Tuberculoid (T)
- Maculoanesthetic (MA)
- Polyneuritic (P)
- Borderline (B)
- Indeterminate (I)

Ridley-Jopling classification

About a decade later, in 1966 a simpler classification to the one given in Madrid was sought. Ridley and Jopling proposed a classification which divided leprosy into two stable polar forms with unstable borderline in between the two.[57, 58]

The main advantage of this classification was that it was based on bacteriological, histopathological, immunological and clinical features of leprosy.

Box 6. Ridley-Jopling classification (1966) [57, 58]

- "Tuberculoid leprosy (TT)
- Borderline tuberculoid leprosy (BT)
- Mid-borderline leprosy (BB)
- Borderline lepromatous leprosy (BL)
- Lepromatous leprosy (LL)"

The New IAL classification

In 1981, Indian Association of Leprologists (IAL) general meet in Agra classified leprosy into five groups .[59]. There was a modification done to the previous Indian classification in which the maculoanesthetic leprosy was combined with tuberculoid leprosy.

Box 7.The New IAL classification (1981) [59]

Lepromatous (L)
Tuberculoid (T)
Polyneuritic (P)
Borderline (B)
Indeterminate (I)

WHO classification (1982)

Next in line was classification by WHO in 1982 outlined a simple classification system devised on the probable number of *M. leprae* being carried by an individual and classified it into multibacillary leprosy paucibacillary.[60]

Box 8.WHO classification (1982) [60]

- Paucibacillary leprosy (BI<2+)
- Multibacillary leprosy (BI≥2+)

BI=Bacterial index

Bacteriological index < 2+ on Ridley scale was termed paucibacillary and that with bacteriological index > 2+ was termed multibacillary.

■ It was changed in 1988, all cases with BI-O were considered paucibacillary (all smear-negative cases) and all cases with BI-1+ or above were considered as multibacillary (all smear-positive cases)

Box 9.WHO classification (1988) [61]

1. *Paucibacillary leprosy*: includes smear-negative cases which belong to

• Indeterminate (I), tuberculoid (TI), and borderline tuberculoid (BT) cases as classified under Ridley-Jopling classification and

• Indeterminate (I), and tuberculoid (T) cases under Madrid classification

2. *Multibacillary leprosy*: included all

• Mid-borderline(BB), borderline lepromatous (BL), and lepromatous (LL) under Ridley-Jopling classification

• Borderline (B) and lepromatous (L) cases under Madrid classification

• Any other smear-positive case

■ Leprosy patients with 1 to 5 skin lesions are considered as the paucibacillary (PB) group for treatment purpose without taking into account the extent and size of lesions or the number of nerves involved.

Box 10. WHO classification based on the number of lesions (1998)[62]

Paucibacillary single lesion leprosy (SLPB);

Paucibacillary leprosy (2-5 skin lesions);

Multibacillary leprosy- 6 or more than 6 skin lesion and also, all smear-positive cases.

Classification under NLEP, India (2009)

As per criteria laid down under the "National Leprosy Eradication Programme (NLEP) of Government of India", the number of nerves involved is also taken into consideration for classification of leprosy .[63]

Box 11. Classification under National Leprosy Eradication Programme, India (2009) [63]

Paucibacillary (PB)

Skin lesions - 1-5 lesions

Peripheral nerve involvement - No nerve or only1 nerve with or without 1 to 5 lesions

Skin smears - Negative at all sites

Multibacillary (MB)

Skin lesions – 6 and above

Peripheral nerve involvement - More than 1 nerve irrespective of the number of skin lesions

Skin smears - Positive at any site

Clinical Aspects of Leprosy Lesions

In some individuals, leprosy involves only one peripheral nerve (mononeuritic) or causes a single skin blemish which persists indefinitely or disappears on its own, while in many others it produces multiple lesions and nodules, together with polyneuritis and damage to vital organs, such as bones, eyes, larynx, and testes.[64]

WHO has set guidelines based on at least two of the following[66]

1. Characteristic skin lesions

2. Thickened nerves/AFB positive skin smear.

3. Sensory loss.

VARIOUS CLINICAL TYPES

1. Indeterminate Leprosy

Indeterminate leprosy is the earliest form of leprosy which manifest in the form of small one or few hypopigmented macules, about 1 cm or less than 5 cm in diameter, rarely erythematous. Thickening of the nerve is usually absent. Skin smears are usually negative. The histopathological examination helps in confirming the diagnosis. Indeterminate leprosy usually heals spontaneously or remains indeterminate for a long period of time. The lesions may sooner or later progress to determinate forms of leprosy lesions.[65, 66, 67]

2. Tuberculoid Leprosy

Lesion of tuberculoid leprosy is commonly seen on the face, dorsum of extremities and lowers back, and affects both skin and peripheral nerves. These lesions are usually single with well-defined borders. Many of the lesions appeared mostly as macules or rarely plaques. They may be erythematous or hypopigmented with hair losses on their surface. Affected nerves are thickened with absence of tenderness. Sensations of touch are usually preserved. Skin smears are mostly negative for AFB.[65, 66, 67]

3. Borderline Tuberculoid Leprosy

The most common type of leprosy is borderline tuberculoid leprosy. Lesions of borderline tuberculoid leprosy may be single or multiple with varying size and shape and are mostly well defined, symmetrical and often with raised margins and hair loss. Dryness and hypopigmentation are often less severe than the tuberculoid type. Few satellite lesions are seen near the edges of larger lesions.

Asymmetrical nerve thickening is present with loss of sensation over the lesions. One of the most striking features of borderline tuberculoid leprosy is its tendency to present with type I reaction. Skin smears sometimes are positive for AFB.[66, 67, 68]

4. Mid- Borderline Leprosy

This is one of the most unstable and also one the rare form of leprosy. It comes in the spectrum between lepromatous and tuberculoid poles. It constitutes multiple lesions of skin with varying size, shape and distribution. They may be macules, papules or plaques with ill-defined margins, having moderate hair loss. It also causes nerve thickening which is asymmetrical, with mild to moderate decrease of sensation. Skin smears are positive due to the presence of many bacilli. [66, 67]

5. Borderline-Lepromatous Leprosy

Classically the lesions begin as macules, localized at first and later it is wide-spread as seen in lepromatous type. These macules are wide-spread over the lower back and extremities. Lesions are mostly symmetrical and often vary in size. Peripheral nerve thickening is present which also causes impairment of sensation. Skin smears are positive with many bacilli often in clumps and globi. [66, 67, 68]

6. Lepromatous Leprosy

Lesions are seen all over the body as macules, papules or nodules. Lesions are present over face, both upper and lower extremities and ears. They are symmetrical and are slightly hypopigmented and sometimes erythematous with ill-defined margins. Sensations are slightly impaired with hair loss which is known as leprous alopecia. Often the nodular lesions over the face coalesce together, with loss of eyelashes which is called as madarosis and depression of nasal bridge more commonly known as leonine facies. Trophic ulcer formations may also be seen on the extremities. [66, 67]

Muscle weakness along with wasting may be seen. There may be involvement of eyes, lymphadenopathy, testes or other systemic organs. Hands and feet may be swollen and edematous. Involvement of the mucosa of upper respiratory tract is seen in 80% of new lepromatous cases. Skin smears show plenty of AFB with multiple globi.[68,69.]

7. Pure Neuritic Leprosy

Pure Neuritic Leprosy is also known as pure neural, primary neural, primary neurotic, primary polyneuritic. This type of leprosy is common in India. It usually presents with neurological deficit without any skin lesions. It may present as anaesthesia in an extremity which progresses to a gradual foot drop. Mono-neuritic is the most common form but multiple nerve involvement may be present.[66]

Histological Features of Leprosy Lesions

Histologically all types of leprosy are examined under following criteria.[66]

1. Cell Type:

In all lesions of leprosy, lymphocytes are present in varying numbers. In indeterminate leprosy the predominant cell type is lymphocytes.

In tuberculoid types (BT and TT), epithelioid cells and granulomas are found and in lepromatous types (LL & BL), foamy macrophages are predominantly seen.

2. Bacterial Load:

The bacterial load is diverse from almost absence of *Mycobacterium leprae* in tuberculoid types to macrophages packed with bacilli in lepromatous types.

3. Nerves:

One of the main diagnostic features is the association of nerve and also the existence of bacilli within the nerve.

I.Indeterminate Leprosy:

Clinical diagnosis of indeterminate leprosy is mostly varied in the majority of the times. The histopathological study is always necessary to make a definitive diagnosis.

This subtype of leprosy shows features which are usually non-specific with epidermis showing no noteworthy changes. But the dermis usually shows lymphocytic and macrophage collection around the dermal vessels, neurovascular bundles, erector pilli muscle and along the sweat glands. Sometimes confined lymphocytic presence in the lower part of epidermis and even in the nerves of the dermis may be seen. In a few

cases, hyperplasia of Schwann cell is also seen but this feature is highly subjective. One of the key points in diagnosis relies on finding acid-fast bacilli in the various sites of predilection under the epidermis, in erector pilli muscle, in nerve or a macrophage in the proximity of a vessel. If bacteria are not present, the diagnosis can only be presumptive.

II.Tuberculoid Leprosy (TT):

The epidermis is thinned out showing atrophy, but occasionally few areas also show hypertrophy. The dermis is usually filled with many granulomas containing few aggregates of epithelioid cells along with Langhans type of giant cells. The granulomas more or less replace the nerves, sweat glands, hair follicles, erector pilorum muscles and also sebaceous glands. Dense lymphocytic infiltrate is seen surrounding them. Usually, there is no clear zone present (Grenz zone) and the granulomas are seen almost reaching up to the epidermis. Acid-fast bacilli are few and quite often difficult to demonstrate.

III.BorderlineTuberculoid Leprosy (BT):

In this, the epidermis shows minimal atrophy depending on the size and extensiveness of the granulomas. Along with that, the dermis shows granulomas along with lymphocytes that often follows the neurovascular bundles and also infiltrate sweat glands and even erector pili muscle. Few Langhans giant cells are found which are smaller in size.

Frequent granulomas lie next to the superficial vascular plexus but they do not infiltrate up to the epidermis. Nerve erosion and obliteration are very typical. Acid-fast bacilli are sparse and are very commonly seen in the Schwann cells of nerves.

IV. Mid Borderline (BB):

It is one of the rare types of leprosy and is often unstable. The epidermis is atrophic and thinned out. The dermis has a grenz zone which is a clear zone which separates the epidermis from the granulomas. Many granulomas are ill-defined and composed of a good number of epithelioid cells, many scattered lymphocytes and few macrophages. In this type of leprosy, all the macrophages are evenly activated to help in the formation of epithelioid cells but are not localized into discrete granulomas. There are no Langerhans giant cells.

Involvement of nerves is seen with lesser destruction of those nerves but often reactive proliferation and edema of the perineurium is seen. AFB can be found in Schwann cells and in scattered macrophages.

V.Borderline Lepromatous Leprosy (BL) :

The epidermis is highly atrophic. The dermis at many times shows a mixture of macrophages with large clusters of lymphocytes, which are divided from the epidermis by a thin clear region (Grenz zone). All the macrophages are foamy with abundant granular pink cytoplasm.

These mixed inflammatory cells are also seen around hair follicles, sweat glands, sebaceous glands and erector-pilorum muscles in aggregates which damage them.

There is marked infiltration around nerves, which show proliferation of perineural cells with formation of a distinct onion skin perineurium appearance (concentric layers around the nerves) on cross-section.

A large number of acid-fast bacilli can be often established, which are distributed as singles, clumps or occasionally in globi.

VI. Lepromatous Leprosy

This subtype of leprosy has atrophy of epidermis with flattening of rete ridges. The dermis shows band of mixed cellular infiltration, consisting predominantly of macrophages with few lymphocytes. This layer is separated by a clear grenz zone from the epidermis. Macrophages show vacuolated and foamy, pale cytoplasm. Often few plasma cells are also be seen. The macrophages infiltrate around the hair follicles, sebaceous glands and also sweat glands which appear atrophic. Macrophages are also seen to be surrounding the nerves, but there is very minimal proliferation of perineurium.

Large numbers of acid-fast bacilli arranged in clumps (globi) are also present in macrophages, perineurium, Schwann cells, sweat glands, sebaceous glands hair follicles, erector pilorum muscle and even in some endothelial cells.

VII. Histoid Leprosy

Histoid leprosy is a very unusual form of lepromatous- leprosy. In this form, the epidermis is usually thinned out by an expanding pseudoencapsulated dermal mass which often consists of interlacing bands and whorls of spindle shaped histiocytes. In most early lesions, predominant cells are polygonal or irregular histiocytes. The adjacent sub-epidermal zone does not contain any cellular infiltrate. The histoid masses contain excessively large numbers of acid fast bacilli which are packed tight bundles and groups without disturbing the rest of the cellular detail.

The lesion often resembles a dermatofibroma and must be differentiated from other fibrohostiocytic and histiocytic skin tumours. It can be easily differentiated by demonstrating intracellular acid-fast organisms.

PATHOGENESIS AND IMMUNOLOGY OF LEPROSY

Leprosy is known to be caused by mycobacterium leprae which is known by well recognized definitive pathological changes. These pathological characteristics are very remarkably different from most of the other infectious diseases because of the unique features of *M. leprae*.[70]

Peripheral nerves and skin are most commonly affected tissues. Other tissues like respiratory mucosa; lymph nodes etc. can be quite often affected.

Amongst all the leprosy patients, the ones having lepromatous patients prove to be as the main basis of infection in a given population. The *Mycobacterium leprae* bacilli are liberated into the surrounding environment through many different routes. Most common route of spread is through the oro-nasal sinuses and skin ulcers of these patients. Many times it is not certain how this *M. leprae* enters and infects the human host often.

The first tissue infected by Lepra bacilli is the neural tissue. The main target of these bacilli is the Schwann cells. Rest of the development of the type of lesion and its fate depends mainly on the immune status of the host. As the bacilli multiply and proliferate within the Schwann cell and perineural cells, thereby destroying them in this process. These infected schwann cells liberate the bacilli, which enters the neighbouring cells and causes the spread of the infection intra-neurally. As the intra-neural infection is recognized, lymphocytes and macrophages infiltrate the nerve; later on, these macrophages engulf the bacilli to limit the infection. The bacilli within these macrophages multiply and then spread to other parts of the nerve as well as other nerves. Later as the disease progresses they spread to other body parts through blood, lymph and tissue fluids.[71,72]

Many experimental studies have concluded that there are two portals of entry.

a) Abraded skin at the cooler parts of the body.

b) Nasal mucosa.

The major factor which determines the outcome of this disease is the host's immune status and many other factors which influence the outcome of this infection are age, skin, race, nutrition and inter-current disease.[72]

The macrophages utilize oxygen and nutrition from the cytoplasm and the bacilli and become foamy. As the disease progress, the macrophage ruptures, thereby releasing its bacilli into the skin and other surrounding structures. Then these bacilli are picked up by other nearby macrophages. The body's immune system responds by sending phagocytic macrophages and lymphocytes to the site of infection.

In many of the cases, these bacilli are phagocytosed by the phagolysosome of the macrophage and the infection thus fails to establish. In the remaining few cases the bacilli continue to multiply in the macrophages mainly by preventing the formation of phagolysosome.

Role of Immunology in Pathogenesis:

The pathogenesis of leprosy involves cell-mediated immunity and delayed hypersensitivity. These not only lead to the development of leprosy but also determine the type of leprosy. The immune system response involves mainly T-lymphocytes, macrophages, to some extent B Lymphocytes and the mediators.[73]

The response of the immune system with lymphocyte (T-helper cells) to *M.leprae* decides if an individual has lepromatous or tuberculoid leprosy.

Patients with tuberculoid leprosy have a defect in the TH1 response or a dominant TH2 response with the formation of interleukin-5, interleukin-10 and interleukin-4, which leads to suppression of macrophage activation.

In tuberculoid leprosy, there are good numbers of CD4+T lymphocytes and in lepromatous leprosy; there are decreased CD4+T lymphocytes.

Tuberculoid leprosy - CD4+ T cells increase, CD8+ T cells decrease. Lepromatous leprosy- CD4+ T cells decrease with an increase in CD8+ T.

In lepromatous patients, CD4+ T helper 2 cells (TH2 cells) when stimulated by the antigen presenting cell secrete IL-5 and IL-4 which in turn activate B-lymphocytes to secret plasma cells leading to formation of antigen–antibody complexes. This causes type II reaction (Erythema Nodosum leprosum).[73]

MATERIALS AND METHODS

Source of data: Skin biopsies of patients diagnosed with leprosy were received in the Department of Pathology, Jawaharlal Nehru Medical College, Belagavi from September 2017 to August 2019. Skin biopsies were obtained after taking informed consent.

Inclusion criteria: All cases clinically diagnosed as leprosy were taken in the study.

Exclusion criteria

1) Inadequate biopsy.
2) Bacillary fragments seen in case of fluorescent microscopy.

Sample size: Universal sampling was done and 49 cases were included in the study.

Method of collection of data: After taking relevant clinical history, consent of the patient was taken and biopsy was performed under aseptic precautions after giving local anesthesia. The skin biopsy was submitted in toto for routine processing.

Processing of specimen: After the process of fixation, the tissue was dehydrated by passing through various ascending grades of alcohol. Thereafter, clearing was done by passing it through two changes of xylene. The tissue was then embedded in paraffin wax and blocks were made and then serial sections each of 5 microns were taken.

Deparaffinization: Normal method of deparaffinization was done for Haematoxylin and Eosin stain. Deparaffinization for Wade Fite and fluorescent staining was done using a mixture of one part peanut oil and three part of xylene; two changes of 5 minutes each.

Staining: One section was taken for routine H and E stain and one for Wade-Fite stain and Fluorescent stain. For staining with fluorescent stain, clean scratch free slides were used. Sections were directly placed on the slides without the use of egg albumin or any other adhesive. For staining, K061-1KT (HIMEDIA) was used. The standard Kuper and May staining protocol was used with few minor changes .The whole process of staining as well as reporting of slides was done in a dark room. The time of staining was increased to 20 minutes instead of 10 minutes. Distilled water was used to wash the slides in place of normal tap water.

The tissue sections that were stained with fluorescent stain were observed immediately under Olympus BX 41 fluorescent research microscope with 100 watts mercury halogen lamp. Controls of skin biopsy from typical lepromatous leprosy patient was used for each batch of Auramine-Rhodamine stain. (Refer annexure IV)

The sections were first screened at 10X and then 40X objectives. Sections showing organisms at 40X objective were confirmed using the 100X objective. Solidly fluorescing organisms were considered for diagnosis. Broken fragments of bacteria were not taken into consideration.

The morphology of the bacilli having bright green yellow fluorescence was considered as diagnostic criteria for tagging the biopsy positive for *Mycobacterium leprae*. *Mycobacterium leprae* appeared as linear, rod shaped organisms that emitted bright green yellow fluorescence.

Method of Statistical Analysis: Statistical analysis of this study was done using Microsoft Excel and SPSS (SPSS Inc, Chicago, and Version 20.0).The result for each

parameter was calculated as mean and standard deviation represented as categorical data in the form of tables and figures.

Statistical analysis:

- Data was represented by diagrammatic presentations and tabulations.
- Sensitivity, specificity, positive predictive value and negative predictive values were calculated.
- Data collected was also analysed using by Spearman's correlation coefficient. ('p' value of < 0.05 is considered as statistically significant) and kappa statistic.

RESULTS AND OBSERVATIONS

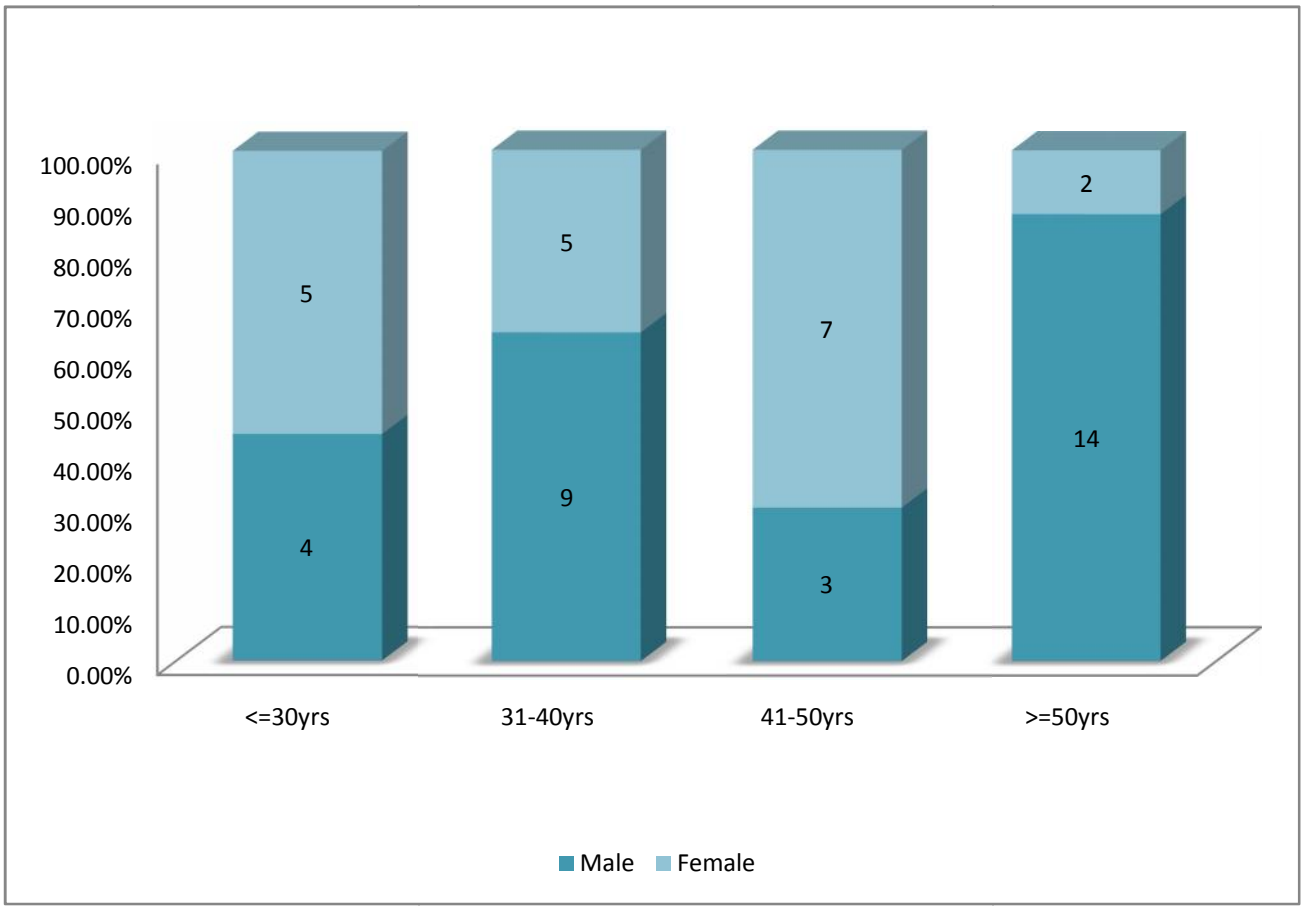
The present study was done on a total of 49 clinically diagnosed leprosy patients attending the Department of Dermatology, Venereology and Leprosy Jawaharlal Nehru Medical College, Belagavi from September, 2017 to August, 2019. The results obtained after staining the biopsy slides with Wade-Fite and Fluorescent stain were compared.

In this study, patients in the age group of >50 years were affected most with 16 cases (32.65%). The least affected age group was that of < 30 years, comprising 9 cases (18.37%). The mean age was found out to be 45.37.

Table 1: Distribution of cases according to age and gender

Age groups	Male	%	Female	%	Total
<=30yrs	4	44.44	5	55.56	9
31-40yrs	9	64.29	5	35.71	14
41-50yrs	3	30.00	7	70.00	10
>=50yrs	14	87.50	2	12.50	16
Total	30	61.22	19	38.78	49
Mean	47.47		42.05		

Graph 1: Distribution of cases according to age and gender



Patients in the age group of >50 years were affected most with 16 cases (32.65%). The least affected age group was that of < 30 years, comprising 9 cases (18.37%). The mean age was found out to be 45.37.

Amongst the present population, males were most affected in the 6th decade comprising of 87.50% whereas in the females, the most commonly affected group was the 5th decade of life constituting about 70%. The mean age affected in males were 47.47 and in females were 42.05.

Table 2 : Distribution of cases according to clinical diagnosis

Clinical Diagnosis	Number	Percent
BL	17	34.69
BT	16	32.65
IL	6	12.24
LL	7	14.29
TL	3	6.12
Total	49	100.00

Borderline lepromatous leprosy was the most common clinical diagnosis constituting 17 (34.69%) cases, followed by borderline tuberculoid leprosy 16(32.65%), indeterminate leprosy 6 (12.24%), lepromatous leprosy 7 (14.29%) and tuberculoid leprosy 3(6.12%). There was no borderline borderline case in our study.

GRAPH 2 : DISTRIBUTION OF CASES ACCORDING TO CLINICAL DIAGNOSIS

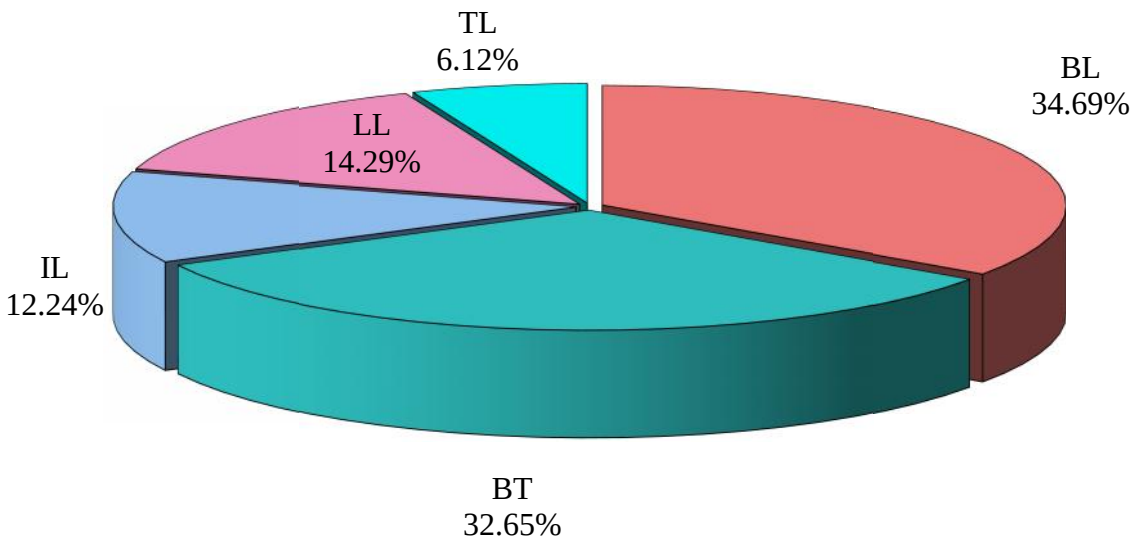


Table 3: Distribution of cases according to histopathological diagnosis

Histopathological Diagnosis	Number	Percent
BL	19	38.78
BT	12	24.49
IL	8	16.33
LL	8	16.33
TL	2	4.08
Total	49	100.00

Borderline lepromatous leprosy was the most common histopathological diagnosis constituting 19 (38.78%) cases, followed by borderline tuberculoid leprosy 12(24.49%), indeterminate leprosy 8 (16.33%), lepromatous leprosy 8(16.33%) and tuberculoid leprosy 2(4.08%). There was no borderline borderline case in our study.

GRAPH 3:DISTRIBUTION OF CASES ACCORDING TO HISTOPATHOLOGICAL DIAGNOSIS

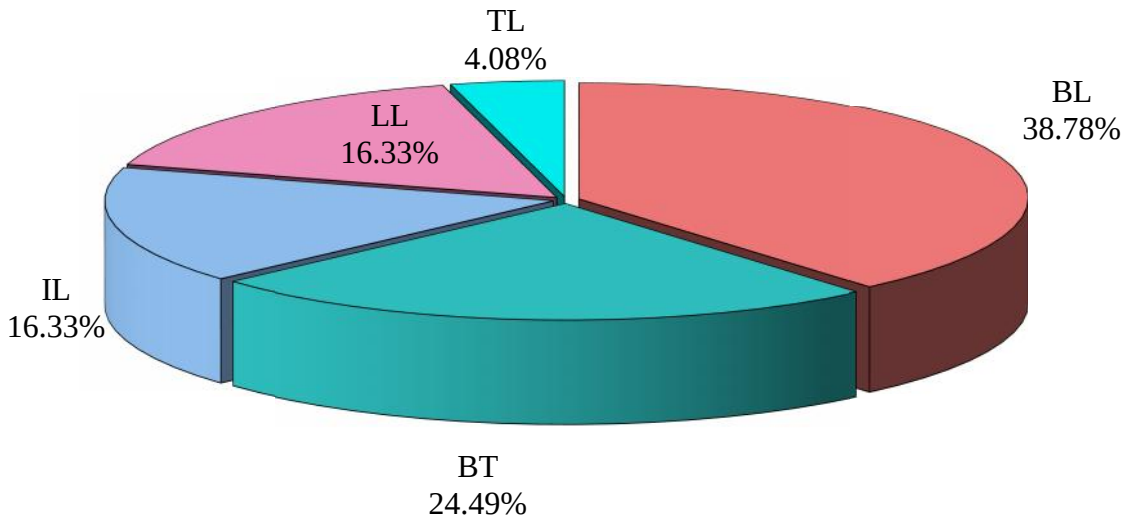


Table 4: Correlation between Clinical Diagnosis and Histopathological Diagnosis by Spearman's correlation coefficient

Diagnosis	Correlation between Clinical Diagnosis with			
	N	Spearman R	t-value	p-level
Histopathological Diagnosis	49	0.8174	9.7278	0.0001*

*p<0.05

The correlation between clinical diagnosis and histopathological diagnosis by Spearman's correlation coefficient was found out to be 0.8174 with a p value of 0.0001.

Table 5 : Agreement between Clinical Diagnosis and Histopathological Diagnosis

Clinical Diagnosis	Histopathological Diagnosis						
	BL	BT	IL	LL	TL	Total	% of Agreement
BL	15	1	0	1	0	17	88.2
BT	4	11	0	0	1	16	68.8
IL	0	0	6	0	0	6	100
LL	0	0	0	7	0	7	100
TL	0	0	2	0	1	3	33.3
Total	19	12	8	8	2	49	

This study showed, out of the 17 BL lesions diagnosed clinically, one was diagnosed as BT and one was diagnosed as LL on histopathology. And of the 16 cases diagnosed as BT, 4 were diagnosed as BL and one case was diagnosed as TL on histopathology. Out of the 3 lesions clinically diagnosed as TL, 2 were histopathologically diagnosed as IL. IL and LL showed complete concordance between the clinical and histopathological diagnosis.

In this study, highest agreement was seen in lepromatous leprosy and indeteminant leprosy of 100 % and lowest agreement was seen in tuberculoid leprosy about 33.3%.

Table 6 : Distribution of number of lesions

Number of lesions	Number	Percent
Single	7	14.29
Multiple	42	85.71
Total	49	100.00

Most of the lesions were multiple constituting about 42 cases (85.71 %) compared to single lesions which were 7 cases (14.29 %).

Table 7: Distribution of cases based on Signs and Symptoms

Signs and Symptoms	Number	%
Hypopigmented lesions	30	61.22
Erythematous lesion	13	26.53
Hypopigmented and erythematous	06	12.22

In the present study, the higher percentages of lesions were hypopigmented, constituting 61.22% .

Table 8: Distribution of lesions in accordance with sensation

Sensation	Number	Percent
Absent	23	46.94
Diminished	10	20.41
Not affected	16	32.65
Total	49	100.00

Maximum number of cases showed absence of any sensation in the lesion (46.94 %), followed by diminution of sensation (20.41 %). In rest of the lesion, the sensation was not affected (32.65 %).

Table 9: Distribution of types of lesions

Types of lesions	Present	%
Symmetrical	8	16.33
Asymmetrical	41	83.67
Well defined	22	44.90
Ill defined	27	55.10

A vast majority of the cases were asymmetrical constituting 83.67% and most of the cases were ill defined constituting about 55.10%.

Table 10: Distribution of lesions according sensation

Sensation	Number	Percent
Absent	23	46.94
Diminished	10	20.41
Not affected	16	32.65
Total	49	100.00

The lesions which presented with absence of sensation constitute about 46.94%.

Table 11: Distribution of lesions based on different types

Skin lesions	Present	%	Absent	%
Nodule	2	4.08	47	95.92
Papule	12	24.49	37	75.51
Macule	7	14.29	42	85.71
Plaques	29	59.18	20	40.82

Most leprosy cases presented as plaque summing up to 59.18% followed by papule constituting about 24.49 % followed by macule as 14.29% and nodule as 4.08%.

Table 12 : Distribution of histopathological features of skin biopsy

Histopathology of skin biopsy		Present	%	Absent	%
Epidermis	Unremarkable	5	10.20	44	89.80
	Atrophied	44	89.80	5	10.20
	Ulcerated	0	0.00	49	100.00
Dermis	Grenz zone	12	24.49	37	75.51
	Lymphohistiocytic aggregate neurovascular bundle	49	100.00	0	0.00
	Errector pillaris	40	81.63	9	18.37
	Granuloma	33	67.35	16	32.65
	Giant cells	14	28.57	35	71.43
	Foamy macrophage	14	28.57	35	71.43

Table 13: Distribution of lesions according to Bacillary index using Wade Fite stain

	Grades	Number	Percent
Paucibacillary	0	10	20.41
	1+	12	24.49
	2+	1	2.04
	3+	5	10.20
Multibacillary	4+	8	16.33
	5+	10	20.41
	6+	3	6.12
	Total	49	100.00

Since BI is a variable which is continuous, we divided the cases into two groups ie, those with BI <3 and those with BI >3, for definitive comparison between groups. The cases which were having bacillary index less than three were grouped as Paucibacillary cases and more than three were grouped as Multibacillary.

Table 14 : Comparison of Wade -Fite stain and Fluorescent stain with respect to leprosy types

	Wade-Fite				Fluorescent stain			
	Positive		Negative		Positive		Negative	
IL	1	12%	7	88%	2	25%	6	75%
LL	8	100%	0	0%	8	100%	0	0%
BL	18	95%	1	52%	18	95%	1	52%
BT	6	50%	6	50%	12	100%	0	0%
TL	0	0%	2	100%	0	0%	2	100%
Total	33	67.3%	16	32.6%	40	81.6%	9	18.3%

Amongst the 33(67.3%) positive cases of Wade Fite, highest positivity was seen in lepromatous leprosy where all the 8 cases of lepromatous leprosy showed positivity (100%) and least was seen in tuberculoid leprosy where out of 2 cases both were negative (0%) followed by indeterminate leprosy in which out of the 8 cases only 1 showed positive Wade-Fite staining (12%).

In the comparision with fluorescent stain, maximum positive cases was seen in lepromatous leprosy and borderline tuberculoid leprosy in which all 8 cases and 12 cases respectively showed positive fluorescent staining (100%) and least positivity was seen in tuberculoid leprosy where out of the 2 cases, none were positive (0%) followed by indeterminent leprosy in which out of the 8 cases 2 were positive (25%).

Table 15: Comparison of agreement between Wade-Fite stain and Fluorescent stain

Wade-Fite statin	Fluorescent stain			
	Present	Absent	Total	%
Present	33	0	33	67.35
Absent	7	9	16	32.65
Total	40	9	49	100.00
%	81.63	18.37	100.00	

A comparison was made between Wade Fite stain and Fluorescent stain. Highest overall positivity rates were seen with Fluorescent stain 81.63% compared to 67.35% of Wade Fite method.

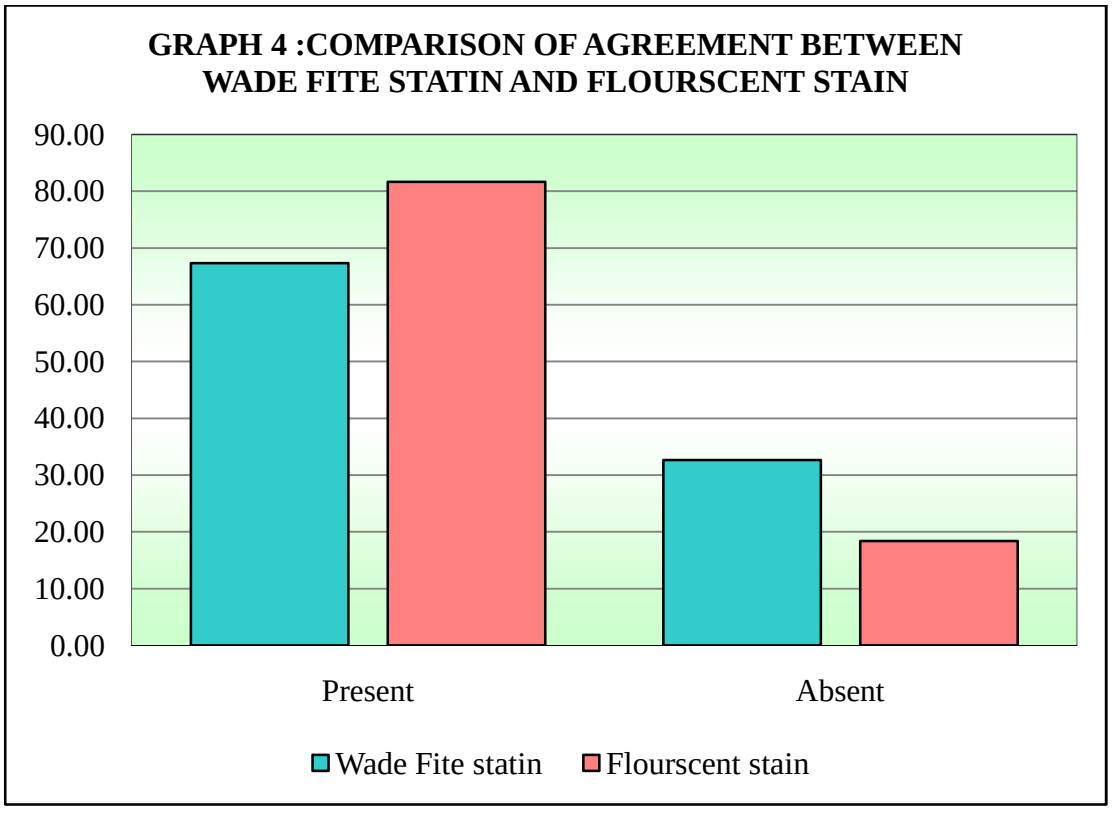


Table 16 : Agreement between Wade Fite satin and Fluorescent stain by Kappa statistic

Agreement between Wade Fite stain and Fluorescent stain					
Agreement	Expected Agreement	Kappa	Std. Err.	Z-value	p-value
85.71%	60.97%	0.6339	0.1329	4.7700	0.0001*

*p<0.05

According to kappa statistics, this study achieved an agreement of 85.71% to an expected agreement of 60.97 %, with a p value of 0.0001.

Table 17: Sensitivity and specificity of Fluorescent stain over Wade Fite statin

Sensitivity	82.50
Specificity	100.00
Positive predictive value	100.00
Negative predictive value	56.25

The sensitivity of fluorescent stain over Wade Fite stain was found out to be 82.50 and specificity was found out to be 100 %. The positive predictive value of the fluorescent stain was found out to be 100 % and negative predictive value of 56.25%.

ANNEXURE –V - PHOTOGRAPHS

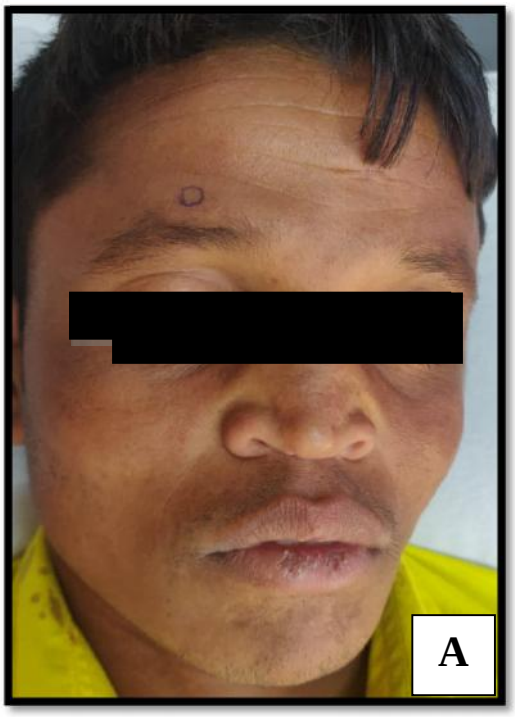


Photo 1(A): Lepromatous leprosy case with saddle shape appearance of the nose.

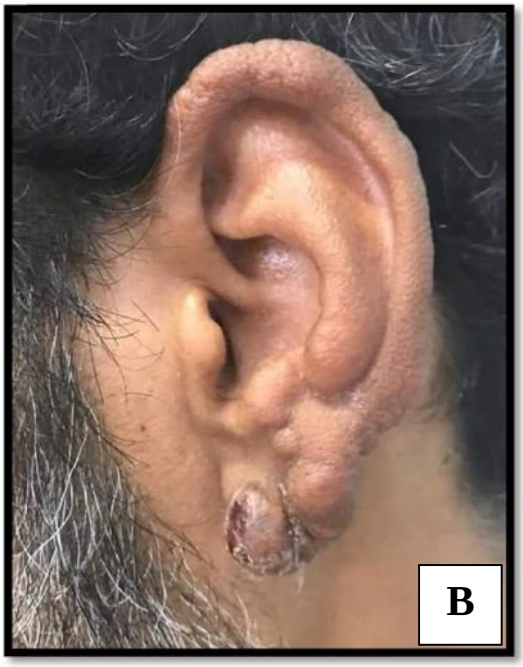


Photo 1 (B)Lepromatous leprosy case with multiple nodules over ear lobe, few of which are ulcerated

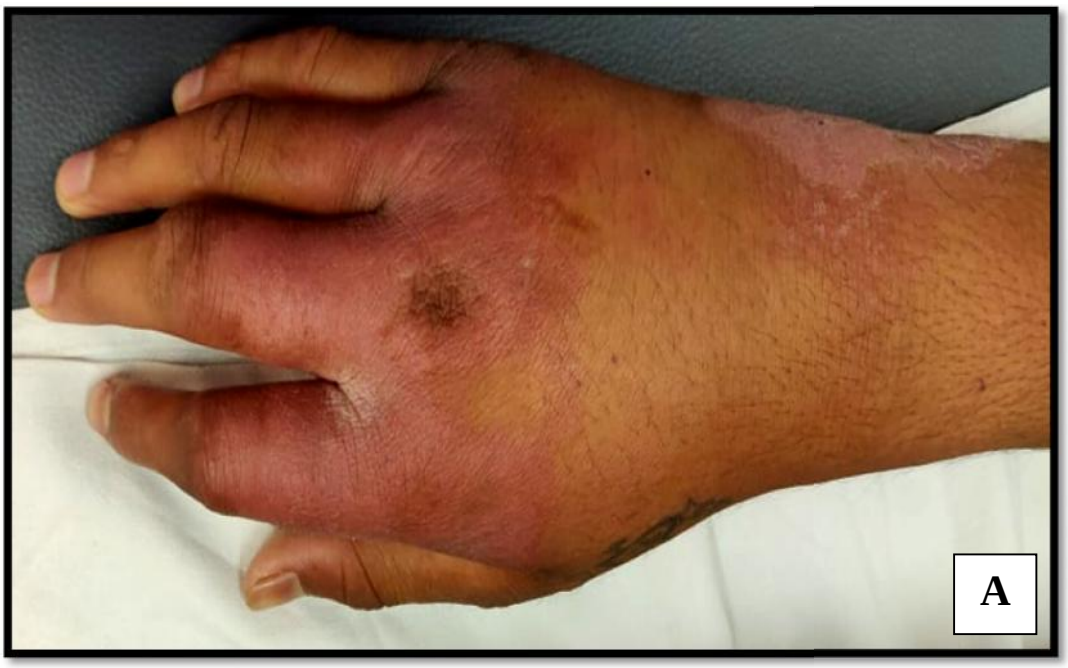


Photo 2 (A) Borderline tuberculoid leprosy with erythamatous plaque present on fingers and hypopigmented patch present on lateral aspect of wrist

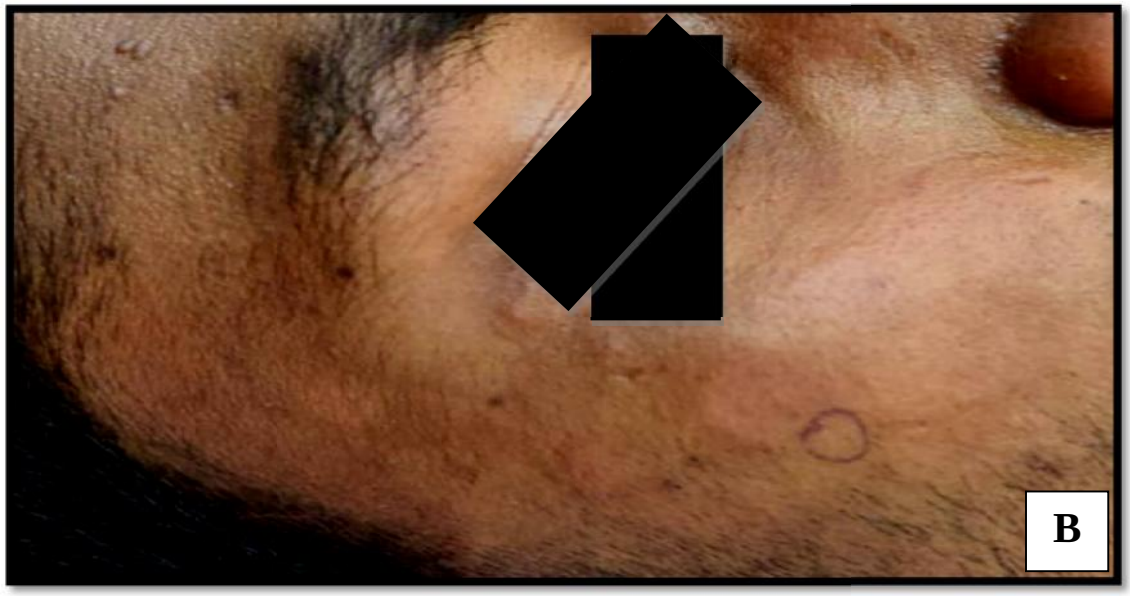


Photo 2 (B) Boderline tuberculoid leprosy with ill-defined multiple hypopigmented patches on face

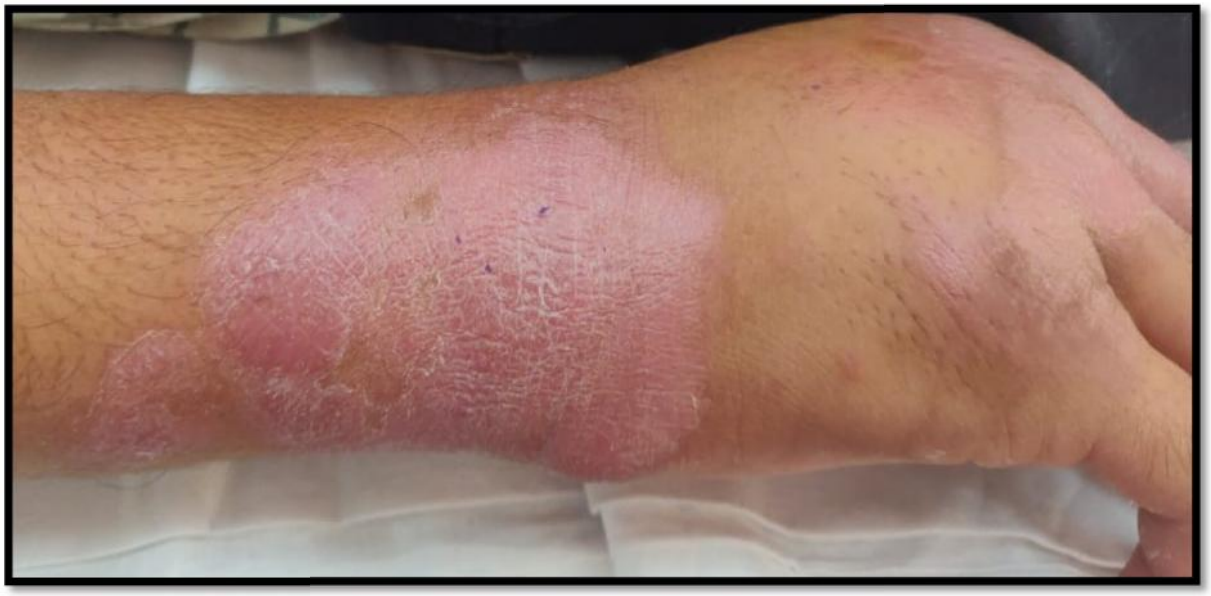


Photo 3: Boderline lepromatous leprosy.Well to ill defined hypopigmented patch present on lateral aspect of wrist and knuckles

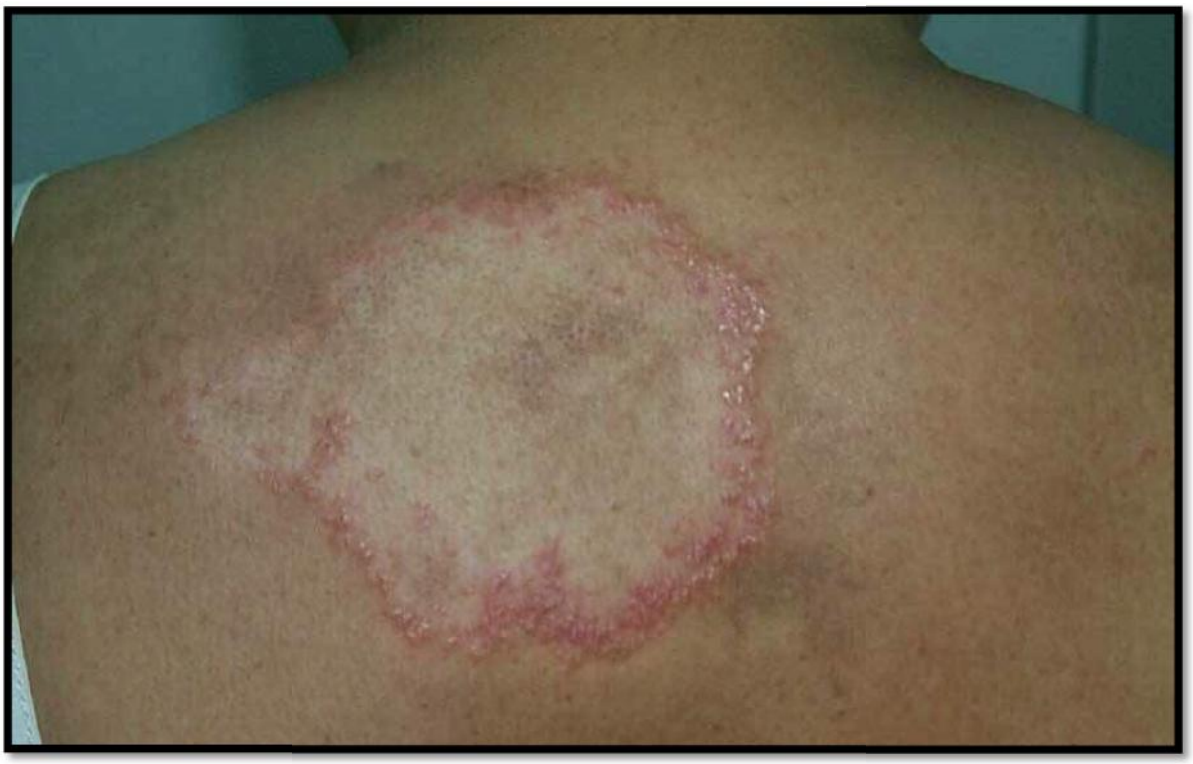
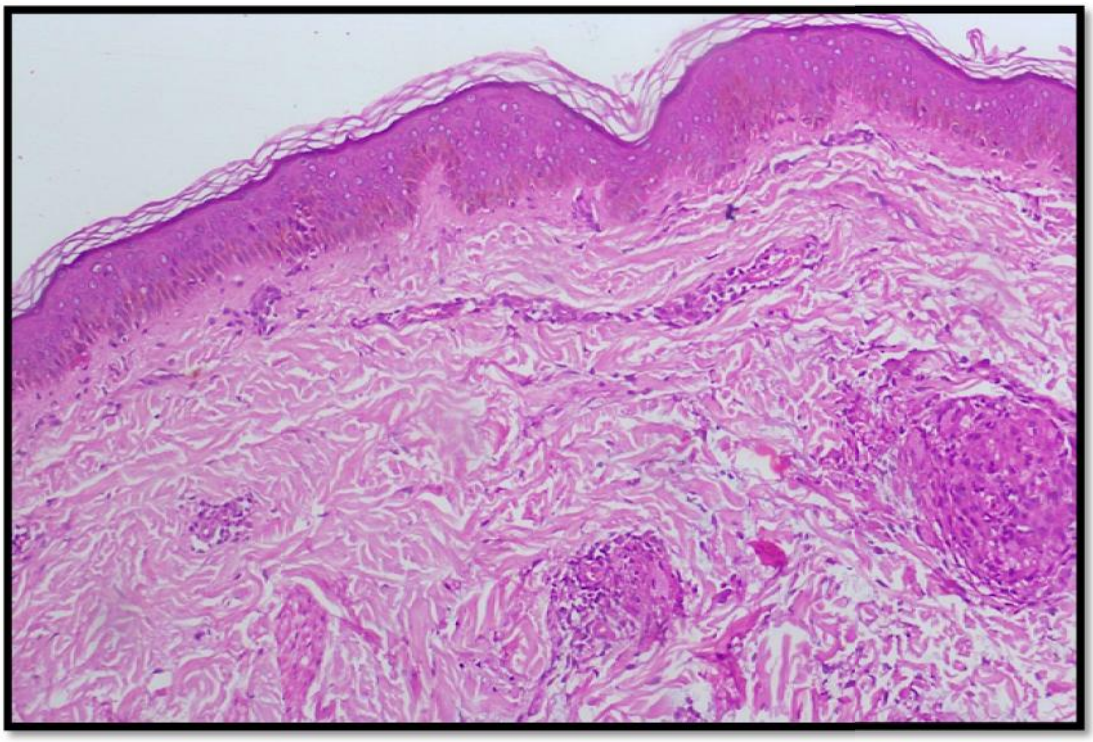
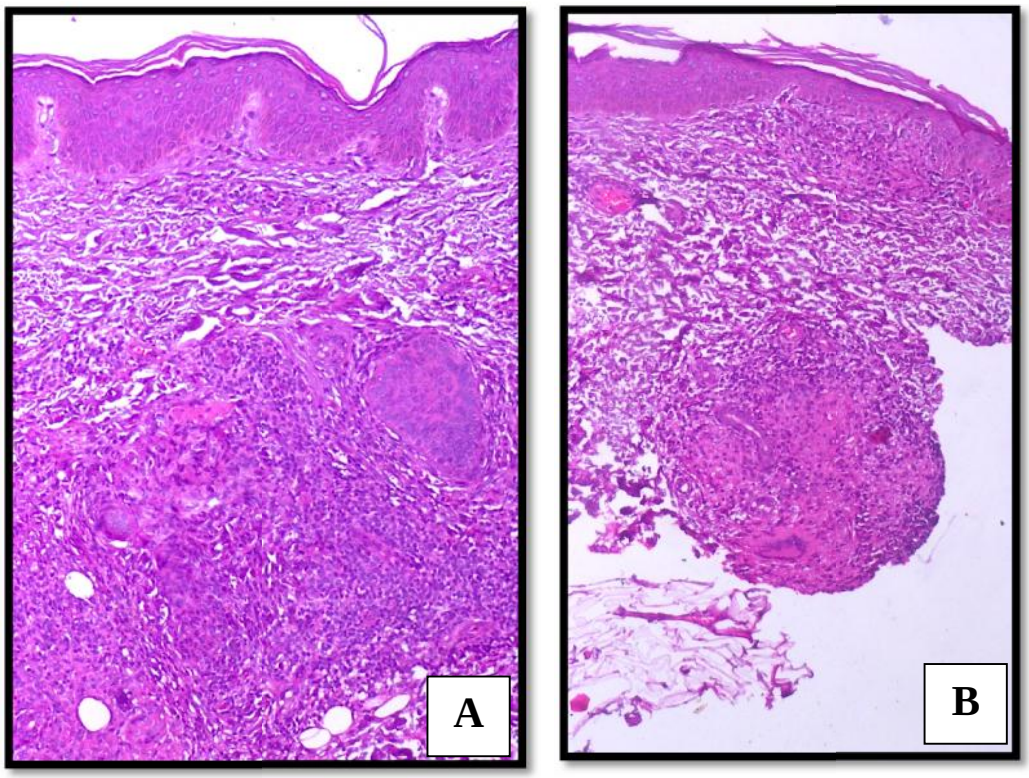


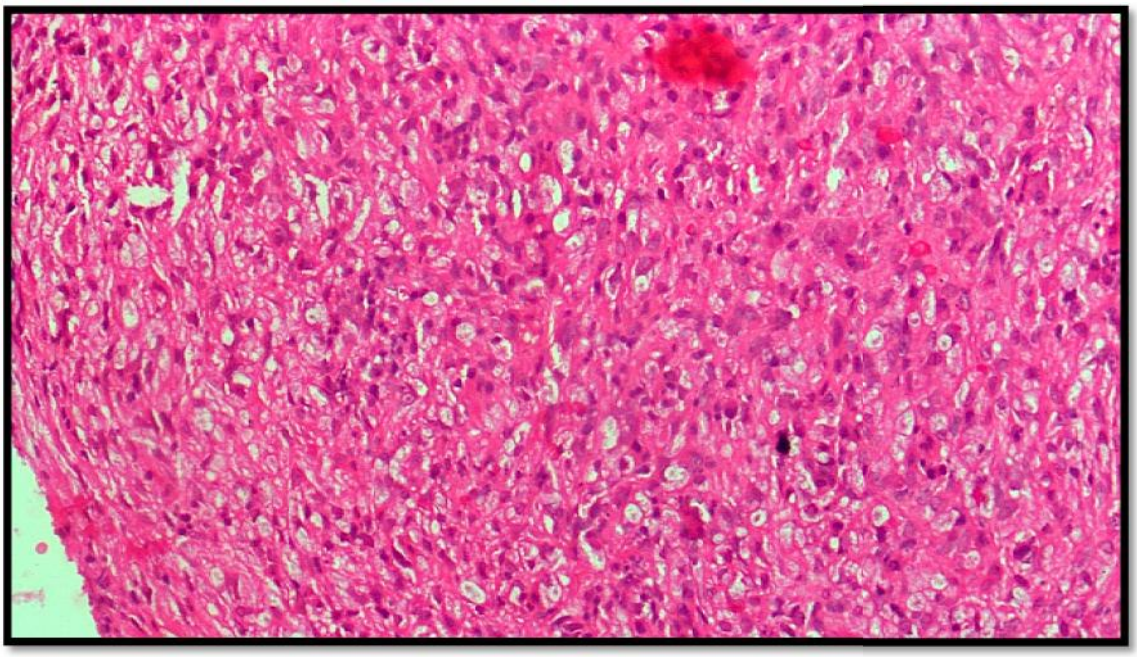
Photo 4: Tuberculoid leprosy, well defined , hypopigment lesion with hyperemic raised borders.



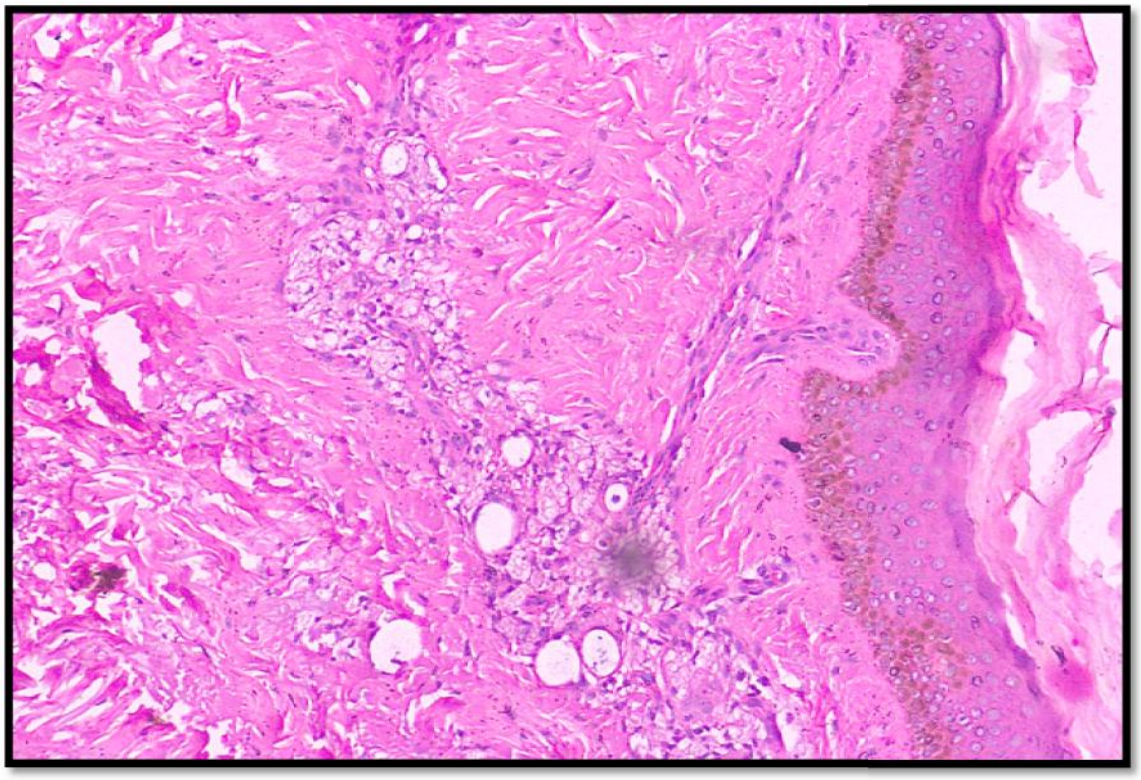
Photomicrograph 1: Indeterminate leprosy showing scant superficial and deep lymphohistiocytic infiltrate. (H and E stain ,20X)



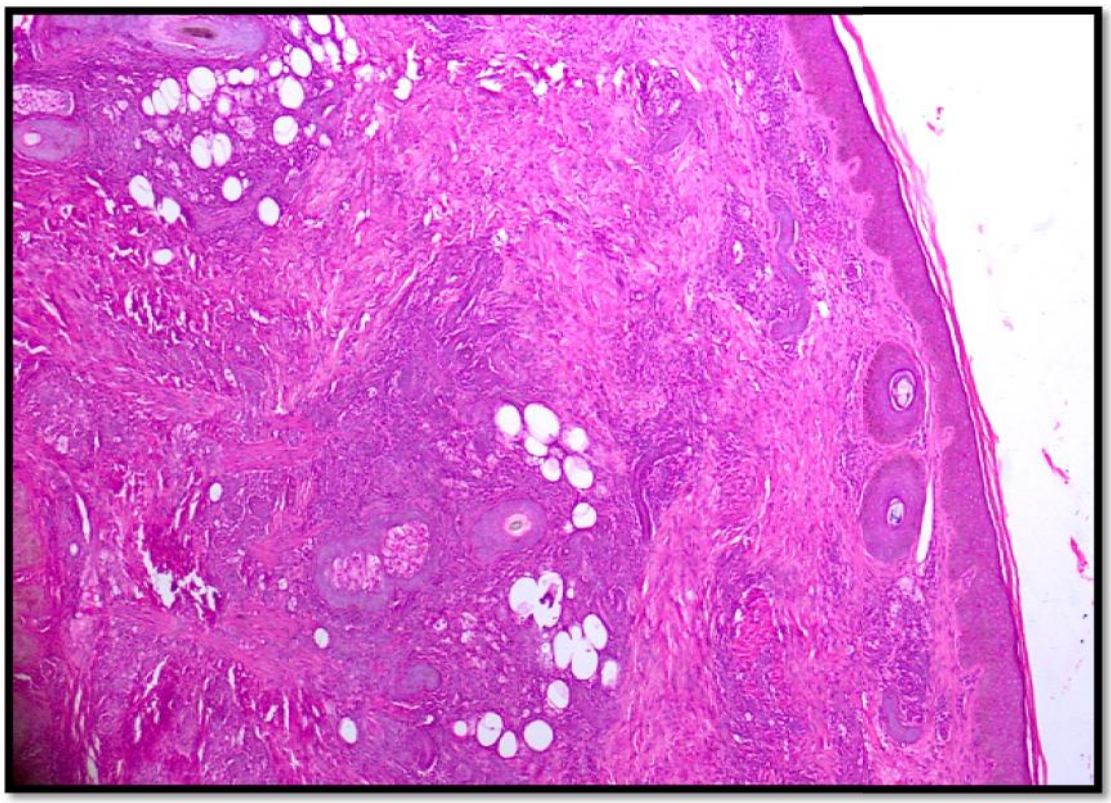
Photomicrograph 2 (A and B): Photomicrograph of Tuberculoid leprosy showing a granuloma with dense lymphocytic infiltrate reaching upto the epidermis along with numerous langhans giant cells (H and E stain 20X)



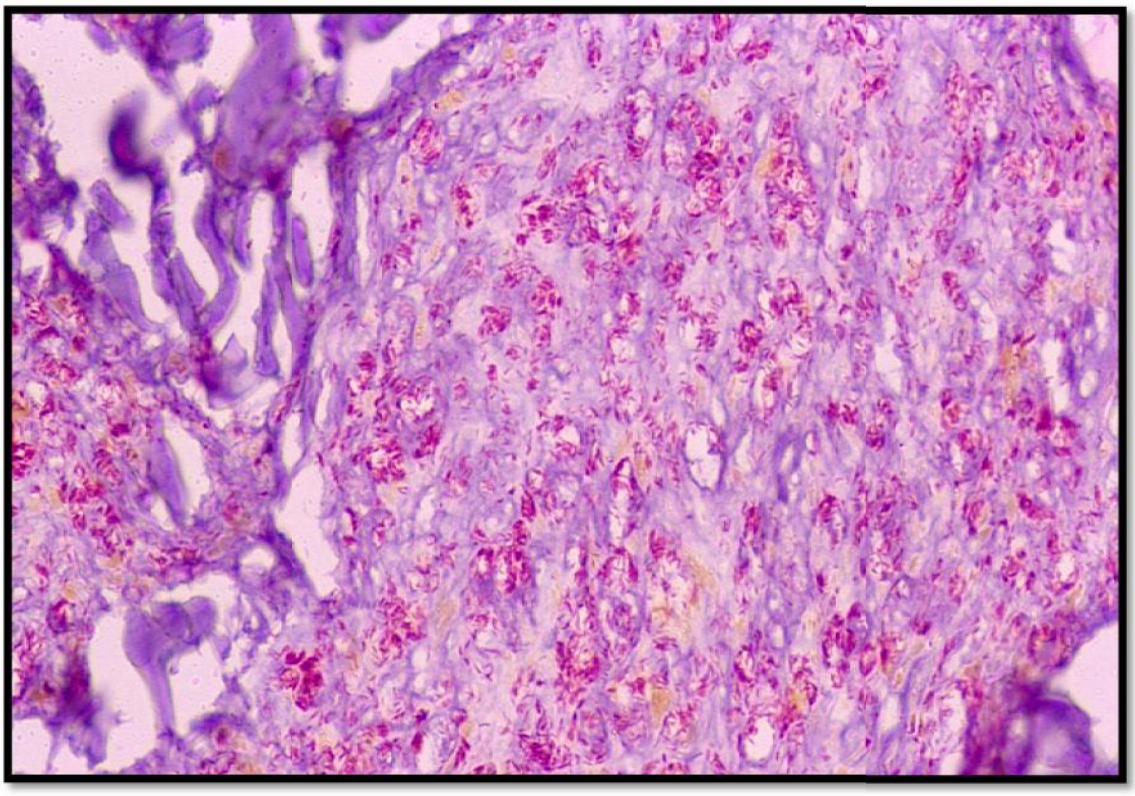
Photomicrograph 3. Lepromatous leprosy showing numerous foamy macrophages and chronic inflammatory infiltrate. (H and E stain ,40X)



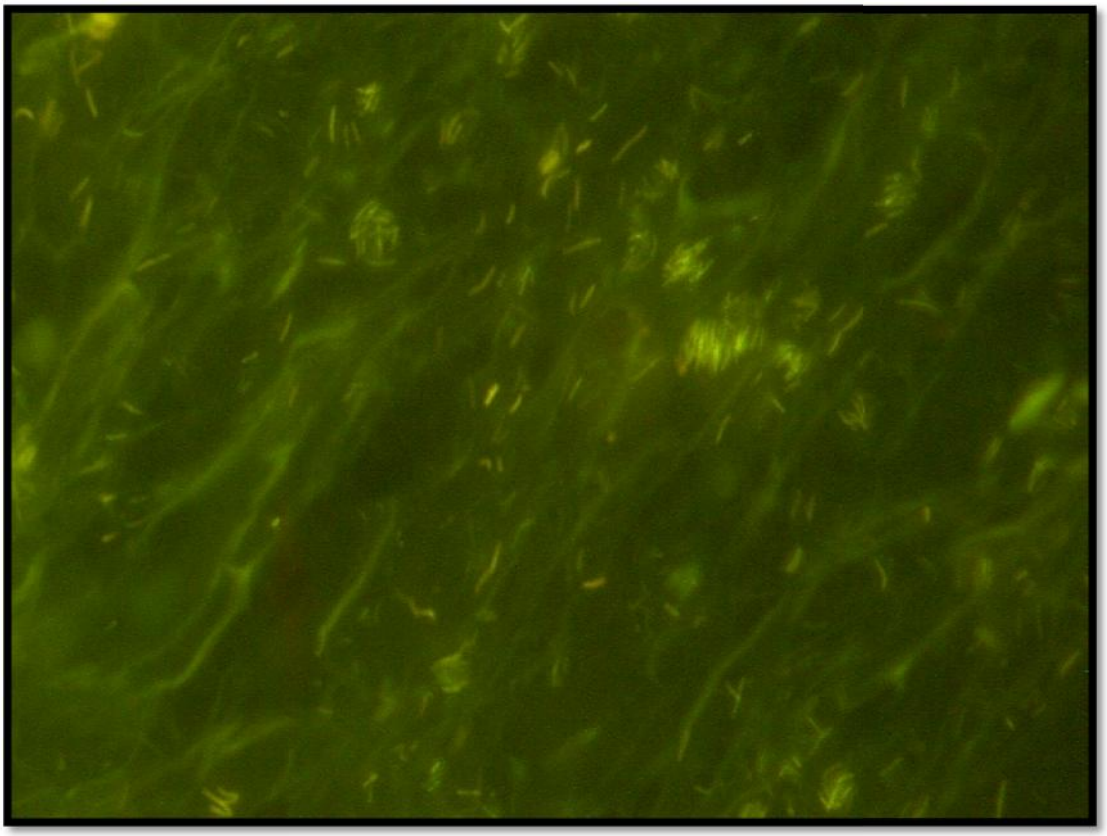
Photomicrograph 4: Boderline lepromatous leprosy showing a grenz zonein the dermisand chronic inflamatory infiltrate along with foamy macrophages in the deeper dermis (H and E stain, 40X)



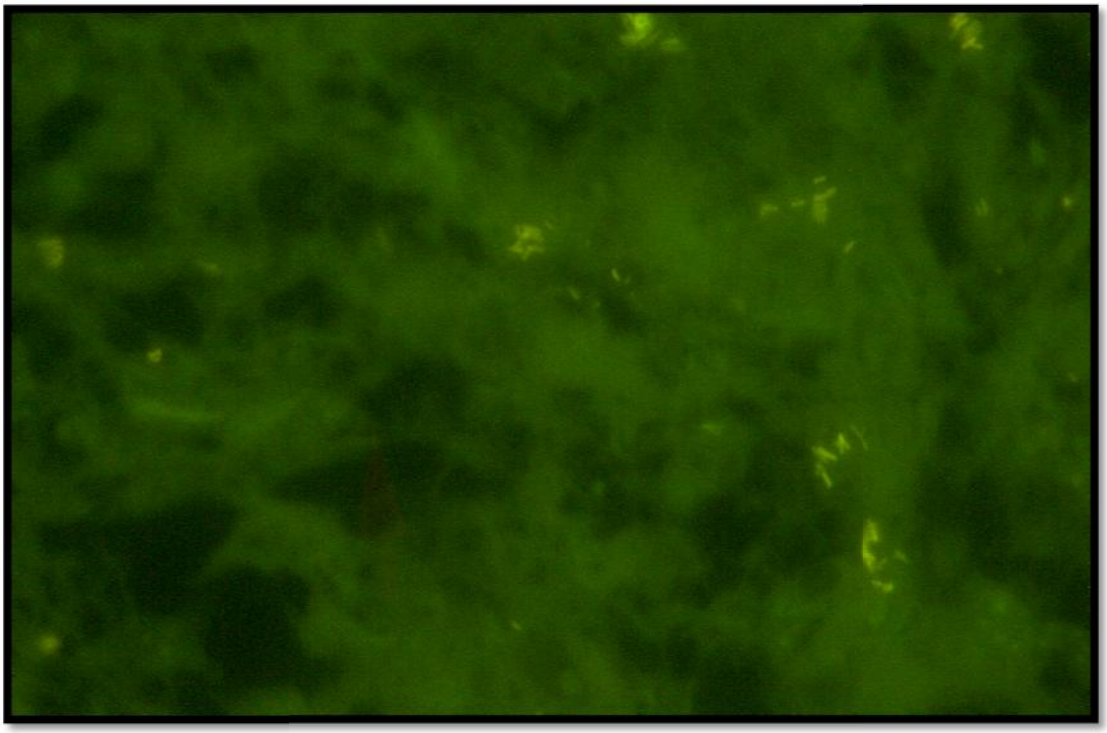
Photomicrograph 5 : Boderline tuberculoid leprosy showing numerous granulomas around the adenexal tissue along with lymphocytes. (H and E stain 20X)



Photomicrograph 6 : Lepromatous leprosy showing clusters of bacilli forming globi .BI +6 (WF stain ,20X)



Photomicrograph 7: Boderline lepromatous leprosy showing numerous green yellow rod shaped bacilli in clusters as well as singly. (Fluorescent stain 40X)



Photomicrograph 8: Boderline tuberculoid leprosy showing very few green yellow rod shaped bacilli lying singly. (Fluorescent stain 40X)

DISCUSSION

The dreadful disease of leprosy has seen changing trends in its incidence and prevalence in recent times. Even after decades of sincere efforts in eradication of leprosy, it still poses a grave threat as a main community health crisis in India where a yearly case detection rate is of 0.84 per 10,000 populations. Leprosy affects most parts of the body such as peripheral nerves, skin and few organs which lead to progressive and often serious permanent deformities in the patient. This disease presents with a wide-ranging and diverse clinical as well as histopathological features.

The main diagnosis is histopathological examination and categorization of leprosy into a spectrum of immunological type which determines the treatment. Wade-Fite staining is the most commonly used method to observe *Mycobacterium leprae* in skin biopsy sections which is often a tiresome and time taking process leading to observer fatigue. In a quest for rapid screening and diagnostic modality, fluorescent microscopy is used in many studies to decrease observer fatigue and also increase sensitivity.

This study was done in the Department of Pathology, Jawaharlal Nehru Medical College, Belagavi, over a period of 24 months from September 2017 to August 2019. The aim of the study was to compare fluorescent staining with conventional staining in the diagnosis of leprosy and to study the histological spectrum of skin lesions in leprosy.

In this study, we compared the performance of Fluorescent microscopy with Wade-Fite in detecting *Mycobacterium leprae* in skin biopsies of leprosy.

During the study period of two years, a total of 49 skin biopsies of leprosy were received.

Disease occurrence in leprosy is related to age at detection rather than age at onset of disease. Leprosy can occur at all age groups ranging from early infancy to very old age.[74] In the present study, patients in the age group of >50 years were affected most with 16 cases (32.65%) which was coherent with the study done by Mathur et al. Often the younger age is more susceptible to leprosy but due to its relatively long incubation period symptoms manifest in older age group.[75]

Leprosy is more commonly prevalent amongst males. Even in the present study, the prevalence amongst males was found to be higher 61.22% which is in concordance with Thakkar and Pate[76], Giridhar M et al[77], Manandhar et al.[78]

Male predominance can often be due to reasons like urbanization, industrialization and greater opportunities for contact with leprosy cases in males, which accounts for the fewer number of female patients reporting for treatment in hospitals.[75]

There has been an increased awareness of leprosy amongst the general population due to various National Programme and sincere eradication efforts by many government and non government run organizations. All these reasons have led them to report at an initial stage to hospitals, contributing directly to an increase in reporting of borderline groups of leprosy.

On histopathological study , borderline lepromatous leprosy was the most common histopathological diagnosis constituting 38.78% of the cases, followed by borderline tuberculoid leprosy constituting about 24.49% of the cases , indeterminate leprosy about 16.33% of the cases, lepromatous leprosy with 16.33% of the cases and

tuberculoid leprosy being the least with 4.08% of the cases. There was no borderline borderline case in our study.

Often there is discordance between the clinical and histological diagnosis as the clinical diagnosis was made based on of Ridley Jopling classification even when histopathological diagnosis was not done.

In our study, the correlation between clinical diagnosis and histopathological diagnosis by Spearman's correlation coefficient was found out to be 0.8174

(p= 0.0001)

A Positive clinico-histopathological agreement was better demonstrated in indeterminant leprosy and lepromatous leprosy and least agreement was seen in borderline tuberculoid leprosy which was coherent with the findings made in a study by Moorthy BN et al[79] and Nadkarni NS et al[80].

Table 18: Comparison of positivity rates of Wade Fite and fluorescent stain with that of other studies.

Various studies	Wade fite	Fluorescence method
	Positive cases	Positive Cases
Present study	33 (67.35 %)	40(81.63 %)
Nayak AS et al [40]	25 (44.64 %)	39 (69.64 %)
Jariwala et al [47]	20 (40.0 %)	22 (44.0 %)
Lacordaire Lopes de Faria [46]	26 (86.6 %)	10 (33.3 %)
Deepa et al [52]	19 (31.7 %)	26 (43.3 %)

This study shows higher positivity rates in finding the bacilli with fluorescent staining as compared to that of Wade Fite which is comparable to the studies done by Nayak AS et al[40] and Jariwala et al[47] and Deepa et al[52.]

In the study conducted by Lacordaire Lopes et al[46] observed that Wade Fite staining was better than fluorescent staining. In their study, egg albumin was used as adhesive and phenol was used which often leads to staining artefacts. These confounding artefacts were not found in the present study as well as a study done by Nayak AS et al[40].

In the present study, such problems did not occur as neither egg albumin nor any other adhesive was used. The bacilli have to be differentiated from artefacts because the

artefacts gave pale yellow fluorescence, and the bacilli have bright solid green-yellow fluorescence.

Some of the modifications done during the process of staining to obtain maximum fluorescence were that the whole process of staining and observation was carried out in a dark room. Moreover, the exposure time of each slide to fluorescent stain was increased by 10 minutes than the standard protocol as stated by Kuper and May[44]. Instead of washing the slides with tap water, distilled water was used as the chlorine present in tap water can interfere with the fluorescence properties of the mycobacterium.

The exposure time of each slide with potassium permanganate was 3 minutes. Increased exposure time to potassium permanganate proved useful in reducing the fluorescent staining of the skin tissue, hence reducing artefacts in staining. Thus our study shows that fluorescent stain is better than Wade-Fite in detecting lepra bacilli in tissue sections.

Taking into account the Wade-Fite method of staining as the standard stain, we compared it with the performance of fluorescent staining. Fluorescent staining showed a sensitivity of 82.50% and specificity of 100 % was observed which was in concordance with a study done by Deepa at al.[52]

The bacterial genus *Mycobacterium* has a primary feature of retaining the aryl methane dyes like Auramine O, but it is not highly specific. Other bacteria, like Nocardia, also often exhibit this feature. Even dead bacteria and bacteria rendered inactive by treatment often take up the stain as they still have a certain amount of mycolic acid left in the cell.[81]

The speed of screening the fluorescent slide and the ease of going through many slides needs to be mentioned. This is because the bacilli which are fluorescent easily stand out against the dark background. This rapidity also helps to reduce observer fatigue.[40]

One major disadvantage is the staining of other artefacts which have to be extremely carefully differentiated from the fluorescent mycobacterium. The slide should be first examined at 10X and then confirmation at 40X and 100X needs to be done. Also, the fluorescent stained slides on exposure to intense ultraviolet light tend to fade out often with areas of focal fading, a phenomenon known as "quenching" effect which could be only slightly managed by carrying out the whole process of staining as well as observation in a dark room.[41]

Another setback of this method is that the fluorescence of the organisms is eventually lost over a short period of time and hence the slides can neither be stored nor reviewed.[41]

The other drawback of fluorescent microscopy is the availability of fluorescent microscope which proves to be a costly asset in a developing country like India.

In this study as well as few other studies it is evident that the positivity rate with the fluorescent method was more as compared to Wade-Fite[40]. In addition to that, the greater positivity rates with fluorescent stain were seen in cases with lower bacillary index while the disparity evened out with LL and BL cases. This emphasizes the advantage of fluorescent stain particularly in cases with lower bacillary index. In paucibacillary cases, the fluorescent stain was found to be more beneficial than the Wade-Fite stain.

LIMITATIONS OF THE STUDY

As far as the effect of staining method in bacteriological indexing of leprosy cases are concerned very few studies are available. More such studies with larger sample size are required. Also use of combined molecular diagnostic techniques along with staining using fluorescent microscope with clinical correlation with lab results involving the clinicians and pathologists will help in early diagnosis and management of leprosy patients. More number of female patients could not be evaluated as compared to males due to the heavy stigma associated with the disease.

CONCLUSION

- Fluorescent microscopy has higher case pick-up rates when compared to Wade-Fite stains as evident by its higher sensitivity.
- Fluorescent microscopy is more reliable for demonstrating bacilli in earlier case of leprosy and also in case categorized low bacillary index (BI<3) which often proves very important for precise categorization of leprosy and also treatment.
- In cases where Wade-Fite stain fails to detect the bacilli, Fluorescent staining can be used as a supplementary tool.

SUMMARY

Skin biopsies from 49 leprosy patients were received in the Departmentof Pathology, Jawaharlal Nehru Medical College, Belagavi from September 2017 to August 2019.

Each case was evaluated for the presence of acid fast bacilli, after staining with H&E, Wade-Fite and Auramine- Rhodamine stains.

Maximum number of patients were in > 50 years of age, least affected being those <30 years.

Males were affected more as compared to females. Boderline lepromatous leprosy was the most common histological type, and tuberculoid leprosy was the least common.

Positivity rate with fluorescent stain was 81.6 % whereas with Wade-Fite was 67.3%.

The sensitivity of fluorescent stain over Wade-Fite stain was found out to be 82.50 and specificity was found out to be 100 %. The positive predictive value of the fluorescent stain was found out to be 100 % and negative predictive value of 56.25%.

Hence fluorescent microscopy proves to have higher probability of detecting a case, with better sensitivity and specificity.

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ANNEXURE –I - INFORMED CONSENT

COMPARISON OF FLUORESCENT STAINING AND CONVENTIONAL STAINING OF SKIN BIOPSY IN DIAGNOSIS OF LEPROSY, A HOSPITAL BASED STUDY.

Purpose of the study: You are being asked to enroll in this study as you are eligible for participation in this study. If you are diagnosed with leprosy you will be included in this study.

The purposes of this study are comparison of fluorescent staining and conventional staining in diagnosis of leprosy and determine the histomorphological study of skin biopsies in leprosy.

Procedure: During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge. The principal investigator of the study is Dr _____ under the guidance of Dr. _____ (guide).

If you agree to enroll yourself in this study, you will be interviewed regarding your sociodemographic details, present, past and family history.

Risks and benefits: There are no risks involved in taking part in this study and benefit is we will be able to know the histomorphological co-relation of leprosy.

Alternatives: Taking part in this study is voluntary. You may choose not to take part in this study, or if you decide to take part now, you can later change your mind and withdraw from the study. The study doctor or sponsor may terminate your participation in this study anytime.

Privacy and confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.
2. If required by law.

Financial incentives for participation: You will not be paid / offered any gift /incentives for participating in this study.

Authorisation to publish results: The results of this study would be forwarded to the KAHER, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

Questions: In case you have any questions related to the study in future you can contact:

1. If you have any queries about your rights as a study subject, you may contact Dr Roopa M. Bellad Professor, Department of Paediatrics, Chairman of J.N.Medical College Institutional Ethical Committee of Human Subjects Research, at J.N. Medical College, Belagavi.

CONSENT STATMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read, or it has been read to me, this entire consent form, and have had all my questions answered.

In case of the queries during the study or in future you may contact following person.

Principal Investigator :Dr _____

Guide :Dr _____

Name of the participant:

(signature/thumbprint)

Name of the witness:

(signature)

Name of the investigator:

(signature)

Date:

Address:

Phone no:

ANNEXURE –II – ETHICAL CLEARANCE CERTIFICATE

K.L.E.UNIVERSITY'S
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)
(Accredited 'A' Grade by NAAC)

Website: http//www.jnmc.edu
E-Mail : dome@jnmc.edu

Phone: (+ 91-(0)831 Office : 2471350
Principal: 2471701
Fax No. +91 (0)831 – 2470759

Ref: MDC/DOME/ 04 **Date: 22/11/2017**

To,

PG student in Pathology,
J.N.Medical College,
BELAGAVI.

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"COMPARISON OF FLUORESCENT STAINING AND CONVENTIONAL STAINING OF SKIN BIOPSY IN DIAGNOSIS OF LEPROSY – A HOSPITAL BASED STUDY",** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Arathi Darshan)
Member Secretary
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

(Dr. Roopa M Bellad)
Chairman,
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

ANNEXURE –III - PROFORMA

1.Name:

2.Age:

3.Sex:

4.IP/OP no.

5.Address:

6.Biopsy number:

7.Presenting symptoms:

a) Skin lesion (visible)	1- 5	>5
b) Loss of sensation		
c) Numbness/ tingling		
d) Muscular weakness/wasting		
e) Other features		

8.Past history / family history:

H/o previous leprosy	
Type	

9.Physical examination :

Skin lesion		Macules / Papules / Plaques / Nodules					
Site							
Number							
Symmetrical		Asymmetrical					
Well defined		Ill defined					
Hypopigmented		Erythematous					
Sensation		Altered		Impaired			
Complete loss of		Touch		Pain		Temperature	

10.CLINICAL DIAGNOSIS:

11. Histopathology**:** Skin Biopsy

Epidermis	Unremarkable/Atrophied /Ulcerated	
Dermis	Grenz zone ±	
Lymphohistiocytic aggregate around	Neurovascular bundle	
	Erector Pilormuscle	
	Appendage	
Granuloma: ±		
Giant Cells:±	Foamy Macrophage:±	

12. Reactions:

Type I	Disorganized granuloma/ Oedema / Lymphocytes / PMN
Type II	PMN infiltrate/Microabscess damage to collagen±/ Elastic fibres Ulceration /Vasculitis
Lucio Phenomena	Necrotizing Epidermis / Necrotizing Vasculitis

13.Fite stain: AFB – status ±

14.Bacterial Index of granuloma

1+	1-10 bacilli/100 OIF
2+	1-10/bacilli 10 OIF
3+	1-10 bacilli/OIF
4+	10-100 bacilli/OIF
5+	100-1000/OIF
6+ >	1000/OIF

14. Flourscent stain: Positive Negative

15.Histopathological diagnosis:

ANNEXURE –IV - STAINING PROTOCOL

STAINING PROTOCOLS

HAEMATOXYLIN AND EOSIN STAIN:

a) Haematoxylin

b) Xylene I and II

c) Absolute alcohol I and II

d) 90% alcohol

e) 1% eosin

Procedure:

1) Paraffin sections placed in xylene for 2 minutes.
2) Transferred to absolute alcohol for 1 minute.
3) Section drained and placed in 90% alcohol for 1 minute
4) Section transferred to haematoxylin for 10-40 minutes
5) Slides transferred to slide washing tray for blueing for 10 minutes
6) Section dipped in acid alcohol, agitated for few seconds for differentiation.
7) Section dipped in 1% eosin for 3 minutes and washed in water.
8) After draining, section transferred to 90% alcohol agitated for 10-15 seconds.
9) Slides transferred to absolute alcohol agitated for 10-15 seconds.
10) Slides transferred to absolute alcohol I and then to absolute alcohol II for 30 seconds.
11) Sections transferred to Xylene I and Xylene II until completely clear.
12) Sections mounted with DPX.

Results:

Nuclei – Blue. Cytoplasm – Shades of pink

All the 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.

WADE FITE STAIN

a) Carbol fuchsin
b) 1% acid alcohol.
c) Methylene blue.
d) 1 part Peanut oil & 3 part Xylene mixture.
e) Xylene

Procedure

1) Paraffin sections placed in Xylene & Peanut oil mixture 30 min two changes each.
2) Drain off excess oil.
3) Blot the section lightly on filter paper 3 times.
4) Sections were stained with Carbol fuchsin for 20 minutes.
5) Sections were washed in running water for 5 minutes.
6) Sections were decolorized with 1 % acid alcohol.
7) Sections were washed in running water.
8) Sections were counterstained with methylene blue.
9) Sections were washed in running water for 5 minutes.
10) Sections were blotted and dried..
11) Sections were cleared in xylene and mounted.

Results

Acid fast bacilli – Red, Background – Light blue.

All stained sections were observed under 40X objective. 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 STAIN

For fluorescent staining, scratch free glass slides were taken without egg albumin or any other adhesive. These tissue sections were stained with fluorescent dye (Auramine-rhodamine, HIMEDIA, Mumbai K061-1KT) and examined under fluorescent microscope.

Procedure

Auramine Rhodamine fluorescent stain as recommended by Kuper and May was used. Following procedure was used.

1) Deparaffinization was performed with a combination of 1 part peanut oil and 3 parts xylene mixture; in which two changes of 5 minutes each was done and then blotted carefully.
2) The slide was stained with filtered Auramine Rhodamine mixture for 20 minutes.
3) The slide was washed in distilled water for 1 minute.
4) Decolorization was performed in 0.5 % hydrochloric acid in 70 % ethanol for 1 minute.
5) The slide was washed in distilled water for 1 minute.

6) Counterstaining was performed with 0.5 % aqueous potassium permanganate for 3 minutes.
7) The slide was washed in distilled water for 2 minutes.
8) Dehydration was performed in absolute alcohol by dipping the slide just once and blot dried immediately.
9) The slide was mounted with DPX using a scratch free cover slip.

The tissue sections that were stained with fluorescent stain were observed immediately under Olympus BX 41 fluorescent research microscope with 100 watts mercury halogen lamp. Controls of skin biopsy from typical lepromatous leprosy patient was used for each batch of Auramine-Rhodamine stain.

The sections were first screened at 10X and then 40X objectives. Sections showing organisms at 40X objective were confirmed using the 100X objective. Solidly fluorescing organisms were considered for diagnosis. Broken fragments of bacteria were not taken into consideration.

ANNEXURE –V- MASTER CHART KEY

BB	Mid borderline leposy
BI	Bacteriological index.
BL	Borderline lepromaous leprosy.
BT	Borderline tuberculoid leprosy.
FL	Fluorescent stain
IL	Indeterminate leprosy.
LL	Lepromatous leprosy.
MB	Multibacillary
PB	Paucibacillary
TT	Tuberculoid leprosy.
WF	Wade-Fite stain.
N	Negative
P	Positive
M	Male
F	Female

							Types of lesions				Signs and Symptoms		Sensation				Skin lesion				Epidermis			Dermis	Lymphohistiocytic aggregate								
No	Clinical Diagnosis	Histopathological Diagnosis	Age	Sex	Site of lesion	Number	Symmetrical	Asymmetrical	Well defined	Ill defined	Hypopigmented lesions	Erythematous lesion	Absent	Diminished	Impaired	Not affected	Nodule	Papule	Macule	Plaques	Unremarkable	Atrophied	Ulcerated	Grenz zone	NVB	EP	Granuloma	Giant cells	Foamy macrophage	Wade Fite statin	Grades	Grades	Flourscent stain
1	TL	TL	65	M	Face , Back	Multiple	N	P	P	N	P	N	P	N	N	N	P	N	N	N	P	N	N	N	P	P	P	P	N	N	0	1	N
2	LL	LL	30	F	Upper Limbs, Lower Limbs, Back ,Face	Multiple	P	N	N	P	N	P	N	P	N	N	N	P	N	P	N	P	N	N	P	P	N	N	P	P	6	2	P
3	TL	IL	50	F	Face	Single	N	P	P	N	P	N	P	N	N	N	P	N	N	N	N	P	N	N	P	P	N	N	N	P	1	1	P
4	BT	BT	40	F	Cheek	Single	N	P	P	N	N	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	P	P	2	1	P
5	IL	IL	19	F	Back , Extremities	Multiple	N	P	P	N	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	N	N	N	N	0	1	N
6	TL	IL	50	F	Face	Single	N	P	P	N	N	P	P	N	N	N	N	N	P	N	N	P	N	N	P	P	N	N	N	N	0	1	P
7	BT	BT	15	M	Lower Limbs	Multiple	N	P	P	N	P	N	N	P	N	N	N	N	P	P	P	N	N	N	P	P	P	P	N	P	1	1	P
8	BL	BL	70	M	Back	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	3	1	P
9	BL	BT	64	M	Face	Single	N	P	P	N	P	N	P	N	N	N	N	N	P	P	P	N	N	P	P	P	P	P	P	N	1	1	P
10	BT	TL	55	M	Trunk, B/L Lower Limbs	Multiple	N	P	P	N	P	N	P	N	N	N	N	N	P	N	P	N	N	N	P	P	P	P	N	N	0	1	N
11	BT	BT	55	M	Trunk, B/L Lower Limbs	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	P	P	N	N	P	P	P	P	P	P	P	P	1	1	P
12	BL	BL	41	F	Upper Limbs,	Single	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	3	1	P
13	BL	BL	81	F	neck, Upper Limbs,	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	3	1	P
14	BL	BL	34	M	Lower limbs	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	4	2	P
15	LL	LL	22	M	Face, Upper Limbs,	Multiple	P	N	N	P	P	N	N	N	N	P	N	N	P	N	N	P	N	N	P	P	N	N	N	P	5	2	P
16	BT	BT	27	M	Back, Buttock	Multiple	N	P	P	N	P	N	N	P	N	N	N	N	P	N	P	N	P	P	P	P	P	N	N	1	1	P
17	BT	BT	80	F	Face, Upper Limbs, ,Lower Limbs	Multiple	N	P	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	P	1	1	P
18	LL	LL	40	F	B/L Upper Limbs,	Multiple	P	N	N	P	P	N	N	N	N	P	N	P	N	N	N	P	N	N	P	P	N	N	P	P	5	2	P
19	LL	LL	40	F	B/L Upper Limbs	Multiple	P	N	N	P	P	N	N	N	N	P	N	N	P	N	N	P	N	N	P	P	N	N	N	P	5	2	P
20	BT	BL	50	M	Back	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	4	2	P
21	BT	BT	18	M	Back	Multiple	N	P	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	P	1	1	P
22	BL	BL	35	M	Trunk, b/l leg	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	3	1	P
23	BL	BL	35	M	Trunk, b/l leg	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	3	1	P
24	BL	LL	61	M	Face	Single	P	N	N	P	P	N	N	N	N	P	N	N	P	N	N	P	N	N	P	P	N	N	N	P	5	2	P
25	BL	BL	65	M	B/L legs , thighs, Back	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	4	2	P
26	BL	BL	35	M	Trunk, b/l leg	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	5	2	P
27	LL	LL	38	M	Back, Face, B/l limbs	Multiple	P	N	N	P	P	N	N	N	N	P	N	N	P	N	N	P	N	N	P	P	N	N	N	P	6	2	P
28	BT	BT	56	M	upper limb	Multiple	N	P	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	P	1	1	P
29	BL	BL	43	M	B/L Lower limbs , thighs, Back	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	4	2	P
30	BL	BL	70	M	Face, Trunk	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	4	2	P
31	BT	BL	40	F	Right leg and hand	Multiple	N	P	P	N	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	N	0	1	N
32	IL	IL	49	F	Neck	Single	N	P	P	N	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	N	N	N	N	0	1	N
33	BL	BL	60	M	Upper Limbs	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	5	2	P
34	BT	BL	48	F	Face , Back	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	N	P	P	P	N	N	P	4	2	P
35	LL	LL	70	M	Back , Extremities	Multiple	P	N	N	P	P	N	N	N	N	P	N	N	P	N	N	P	N	N	P	P	N	N	N	P	5	2	P
36	BT	BT	50	M	B/L Lower Limbs , thighs, Back	Multiple	N	P	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	N	1	1	P
37	BT	BT	40	M	Nape of neck	Multiple	N	P	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	N	1	1	P
38	IL	IL	23	F	upper limb	Multiple	N	P	P	N	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	N	N	N	N	0	1	N

							Types of lesions				Signs and Symptoms		Sensation				Skin lesion				Epidermis			Dermis	Lymphohistiocytic aggregate								
No	Clinical Diagnosis	Histopathological Diagnosis	Age	Sex	Site of lesion	Number	Symmetrical	Asymmetrical	Well defined	Ill defined	Hypopigmented lesions	Erythematous lesion	Absent	Diminished	Impaired	Not affected	Nodule	Papule	Macule	Plaques	Unremarkable	Atrophied	Ulcerated	Grenz zone	NVB	EP	Granuloma	Giant cells	Foamy macrophage	Wade Fite statin	Grades	Grades	Flourscent stain
39	BT	BL	19	F	Back , Extremities	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	5	2	P
40	BL	BL	35	M	B/L legs , thighs, Back	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	6	2	P
41	BT	BT	27	F	Face , Back , Trunk	Multiple	N	P	P	N	P	P	N	P	N	N	N	N	N	P	N	P	N	N	P	P	P	P	N	N	1	1	P
42	BL	BL	34	F	Face , Back	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	5	2	P
43	BL	BL	35	M	Back , Extremities	Multiple	N	P	N	P	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	P	N	N	P	4	2	P
44	IL	IL	41	F	Back	Multiple	N	P	P	N	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	N	N	N	N	0	1	N
45	BL	BL	64	M	Back	Multiple	N	P	N	P	N	P	N	N	N	P	N	P	N	N	N	P	N	P	P	N	P	N	P	P	4	2	P
46	IL	IL	54	M	Face, Trunk	Multiple	N	P	P	N	P	N	P	N	N	N	N	N	N	P	N	P	N	N	P	P	N	N	N	N	0	1	N
47	BT	BT	65	M	Back , Extremities	Multiple	N	P	P	N	N	P	P	N	N	N	N	N	N	P	P	N	N	N	P	P	P	N	N	N	1	1	P
48	LL	LL	47	F	Trunk, B/L Lower Limbs	Multiple	P	N	N	P	P	N	N	N	N	P	N	N	P	N	N	P	N	N	P	P	N	N	N	P	5	2	P
49	IL	IL	38	M	Back	Multiple	N	P	P	N	P	N	P	N	N	N	N	P	N	N	N	P	N	N	P	P	N	N	N	N	0	1	N