“A COMPARATIVE STUDY OF ZIEHL-NEELSEN & MODIFIED FITE-FARACO WITH AURAMINE RHODAMINE STAINING IN DETECTION OF MYCOBACTERIUM LEPRAE IN TISSUE SECTIONS”

by

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Dissertation submitted to the

RAJIV GANDHI UNIVERSITY OF HEALTH SCIENCES,

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In partial fulfilment

of the requirements for the degree of

M. D.

in

PATHOLOGY

Under the guidance of

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I hereby declare that this dissertation entitled **"A COMPARATIVE STUDY OF ZIEHL-NEELSEN & MODIFIED FITE-FARACO WITH AURAMINE RHODAMINE STAINING IN DETECTION OF MYCOBACTERIUM LEPRAE IN TISSUE SECTIONS"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. SUREKHA B. HIPPARGI MD,** Professor, Department of Pathology at B. L. D. E. A's Shri. B. M. Patil Medical College Hospital and Research Centre, Bijapur.

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Place: Bijapur

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. . . . to my parents

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LIST OF ABBREVIATIONS USED

(in alphabetical order)

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.
FF	Fite faraco stain.
FL	Fluorescent stain
IL	Indeterminate leprosy.
LL	Lepromatous leprosy.
MB	Multibacillary
PB	Paucibacillary
PCR	Polymerase chain reaction.
TT	Tuberculoid leprosy.
WHO	World Health Organisation
ZN	Ziehl Neelsen

ABSTRACT

BACKGROUND: Leprosy is a chronic infectious disease caused by mycobacterium leprae. In leprosy, histopathological examination of skin offers the correct diagnosis. We evaluate fluorescent microscopy which is increasingly used for rapid screening.

OBJECTIVES:

1. To compare the efficacy of auramine rhodamine stain (FS) with Ziehl-Neelsen(ZN) and modified Fite-faraco(FF) staining in the diagnosis of leprosy in tissue sections.
2. To assess the role of auramine rhodamine staining method in grading of Hansen's disease.

METHODOLOGY: Skin biopsies of sixty clinically diagnosed leprosy patients received in the department of pathology were subjected to ZN stain, FF stain and FS. Each case was evaluated for presence of bacilli as well as bacillary index (BI).

RESULTS: Sensitivity of FS for indeterminate, borderline tuberculoid leprosies were 100% each. Positivity rates and mean BI with FS was higher as compared to that of ZN and Fite-faraco when the bacillary load was less (BI <3). There was significant correlation between the three staining types at lower bacillary load. There was a higher mean BI with fluorescent stain as well as detection of an additional multibacillary case.

CONCLUSION: Fluorescent method is more sensitive than modified fite-faraco method in detecting lepra bacilli in tissue sections especially in cases with BI<3. It has an additional role in grading of Hansen's disease based on BI. Hence it can prove valuable in tissue sections with lower bacillary load where other methods fail.

KEY WORDS : Leprosy; Fluorescent microscopy; Modified Fite – Faraco stain; ZN stain.

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INTRODUCTION

Leprosy is a chronic infectious disease caused by Mycobacterium leprae which expresses itself in different clinicopathological forms, depending on the immune status of the host.[1]

Mycobacterium leprae was discovered by Gerhard Henrik Armauer Hansen in 1873.[2] Leprosy causes permanent and progressive physical deformities in the patient. It affects mainly the peripheral nerves. It also affects the skin, muscles, eyes, bones, testes and internal organs. Leprosy is present in practically every corner of the globe, but in tropical countries like India, it is still one of the problems of public health importance.

This problem can be tackled by correct diagnosis and timely treatment. The clinical diagnosis of leprosy has to be confirmed by diagnostic procedures like slit skin smear and skin biopsy.

Histopathological examination of skin in leprosy 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 in clinical specimens[3]. Modified Fite-faraco technique is the routinely used method to demonstrate Mycobacterium leprae in tissue sections.

Though modified Fite-faraco is more sensitive than Ziehl Neelsen method in detection of Mycobacterium leprae in tissue sections, it is tedious, time consuming and leads to observer fatigue.

Hence fluorescent microscopy has been used by some for rapid screening to reduce observer fatigue and to increase sensitivity.

This study was done to compare the sensitivity of fluorescent microscopy with that of ZN staining and modified Fite-faraco technique in detecting Mycobacterium leprae in tissue sections.

AIMS AND OBJECTIVES

1. To compare the efficacy of auramine rhodamine with Ziehl- Neelsen and modified Fite-Faraco staining in the diagnosis of leprosy in tissue sections.
2. To assess the role of auramine rhodamine staining method in grading of Hansen's disease.

REVIEW OF LITERATURE

Historical Aspects:

Leprosy is the oldest disease known to mankind.[4] G.H. Armauer discovered Leprosy in 1873. He found that the cause of leprosy were the rods of bacilli in the lesion. He had initially observed them in skin, nerves and visceral lesions exhaustively in unstained tissue specimens and in due course he found that they could be better visualized if treated with dilute osmic acid. [5]

As the time progressed various methods were suggested and demonstrated by different people to identify lepra bacilli in slit skin smears and in tissue sections.

Conventional ZN method of staining is one of the oldest methods of detecting the organisms in tissue sections.[3]

The Fite methods are most commonly used for demonstrating lepra bacilli in tissue sections. [6] Wade fite and Fite-faraco stains can demonstrate acid fast organisms in tissue sections, which can also be demonstrated by fluorescence method and with PCR technique. [7]

In 1937 fluorescent microscopy for detecting Mycobacteria was first used by Hagemann. [8]

Following this in 1952, Gohar[9] described the advantages of fluorescent microscopy for detecting Mycobacterium leprae in smears.

In 1956, Khanolkar and Nerurkar[10] used fluorescent microscopy in the diagnosis of leprosy for smears.

In 1960, Kuper and May[11] introduced the fluorescent microscopy for the detection of acid fast organisms in tissue sections. They added rhodamine to older method of staining which improved contrast and better appreciation of bacilli by fluorescent microscope.

In 1966, Silver[12] et al, suggested various modifications in the procedure of fluorescent staining. These included variation in duration of exposure to stain and counterstain and mountant to be used.

Further in 1970, Mansifield[8] observed that with the use of fluorescent microscopy, the bacilli were easily and rapidly found. He also found that xylene peanut oil mixture for deparaffinisation produces much brighter staining of the bacilli in both fluorochrome and carbol-fuchsin stained tissue sections.

Jariwala and Kelkar [13] in 1979 observed that fluorescence method was superior to the modified Fite-faraco method in detecting Mycobacterium leprae in tissue sections particularly in paucibacillary cases. They also felt that the field covered was 16 times larger, so that an average section could, therefore, be scanned in two to three minutes.

In 1987, Bhatia[14] et al studied histopathological sections from cases of indeterminate, tuberculoid and BT cases by fluorescent method. They found in their study that fluorescent staining was superior to ZN staining and that it should supplement ZN staining when decision regarding negativity of smear arises.

In 1988, Bhatia[15] et al again conducted study on 84 skin smears and found that 75 smears showed AFB by auramine staining as compared to only 57 smears by ZN staining. Also the BI by auramine staining was higher as compared to BI by ZN

staining. The study also revealed a minimal interobserver variation by auramine method.

In 2002, Jain[16] et al conducted a study on 715 clinical specimens, which included sputum, fine needle aspirate, pus and body fluids and examined them by ZN and AR staining techniques, simultaneously. They found sensitivity of auramine rhodamine staining to be 98% as compared to 78.8% by ZN staining. Also fluorescent stain was more advantageous in paucibacillary cases.

Nayak and Shivarudrappa[17] in 2003, promoted fluorescent microscopy for leprosy diagnosis after a study conducted in Victoria Hospital and Bowring and Lady Curzon Hospital, Bangalore. They found a higher positivity rate with fluorescent staining as compared to modified Fite-Faraco. The speed of observation and the rapidity of finding the bacilli also reduce observer fatigue.

Conversely, Lacordaire[18] in 1972 found the modified Fite-faraco method to be superior compared with the fluorescent staining in detecting Mycobacterium leprae in tissue sections.

Also, in 1981, Hardas [19]and Lele, opined after their study on 117 smears and 69 biopsies that granular and dusty forms of the organisms were totally missed by fluorescent method. Also high frequency of artifacts makes it less advantageous.

In the mean time many authors have studied different histological patterns in tissue sections of skin biopsies of leprosy patients.

In a study conducted by Shenoi SD [20] et al 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[21] et al, 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%).

EPIDEMIOLOGY

Approximately 2,96,499 people live in areas where leprosy is an important problem. Leprosy continues to be a major public health problem in India with an annual new case detection rate of 1.43 per 10,000 population. In India the prevalence rate is 0.84 cases per 10,000 population. [22]

Leprosy is known to occur at all ages from early infancy to very old age. Age of onset is extremely variable with respect to time, place and country. [23]

Although leprosy affects both the sexes, M: F ratio is 2:1. This could be because leprosy workers are mostly men, the examination of women is less complete and less satisfactory particularly in certain cultural situations resulting in under detection of leprosy among females. It is also attributed to greater mobility and increased opportunities for contact among men.[22]

Where leprosy treatment facilities exist, inactivation or cure is an important mode of elimination from prevalence pool. Even in absence of specific treatment, a majority of patients particularly of tuberculoid and indeterminate leprosy tend to get cured spontaneously. A study in India has shown that over a period of 20 yrs, the

extent of spontaneous regression among children with tuberculoid leprosy was about 90%.

Bacterial Properties.

1. Taxonomical Classification

Class: Schizomycetes

Order: Actinomycetales

Family: Mycobacteriacae

Genus: Mycobacterium

Species: Leprae

2. Dimensions of the Bacilli

Length: 1-8 microns. They are slightly curved rod shaped bacilli with parallel sides and rounded ends.

Width: 0.3 microns

Thickness of bacterial wall: 20 nm

3. General Properties [24, 25]

It is acid fast, alcohol fast, gram positive, non-motile, non-spore forming, obligate intracellular bacilli. They are commonly seen in the cytoplasm of the macrophages and nerve bundles, either singly or in large bundles called as globi.

4. Biological Properties:

They cannot be cultured in laboratory media. In mouse foot pad, doubling time is 11-13 days. It divides by binary fission. Optimum temperature is minus 27-30^{o}C. Growth is decreased when tissue temperature is between 25^{o} to 36^{o}C.

5. Biochemical Properties:

Capsule is composed of lipids which are partly responsible for electron transparent zone. Cell wall consists of cross linked peptidoglycans covalently attached to an arabino galactam polymer.

6. Laboratory methods of detection :

By microscopic examination of

a) Slit skin smear.

b) Nasal scraping.

c) Nose-blow smears.

d) Skin biopsies.

Stains used:

- Haematoxylin and eosin.
- Ziehl – Neelsen staining with 5% H_2SO_4.[22]
- Modified Fite-faraco stain.[26]
- Auramine rhodamine fluorescent stain.[17]

Bacteriological index [24, 27] **(BI):**

Shows the density of lepra bacilli in a smear or tissue section. BI includes living, which are with solid staining and dead, which show fragmented or granular bacilli. According to Ridleys logarithmic scale, it is graded from zero to six +, which is based on the number of bacilli seen on an average microscopic field under 100 X objective.

Average number of acid fast Bacilli	**BI**
0 bacilli in 100 fields	O +
1-10 bacilli in 100 fields	1+
1-10 bacilli in 10 fields	2+
1-10 bacilli in a field	3 +
10 -100 bacilli in a field	4 +
100 -1000 bacilli in a field	5 +
> 1000 bacilli in a field	6 +

Paucibacillary BI 0-1 + Multi-bacillary BI >/= 2+

Auramine-Rhodamine staining technique and fluorescent microscopy. [17]

Fluorescent microscopy has been used by some for rapid screening and to reduce observer fatigue. There are few studies performed on tissue sections to detect M. leprae by fluorescent microscopy. There are differing views about the sensitivity of fluorescent microscopy in detecting M. leprae in tissue sections. In paucibacillary cases this method has advantages over the modified Fite-faraco method and also that it can be used as a supplementary tool when tissue sections stained by Fite-faraco method fail to detect the bacilli.

8. Polymerase Chain Reaction:

The use of DNA amplification based on PCR provides or exquisitely sensitive method for detecting M. leprae. This technique could be used to assess treatment of paucibacillary patients and to detect presence or persistence of bacteria in detecting sub-clinical infection. [25, 28]

Studies using this technique have detected M. leprae DNA on swabs from nasal mucosa of clinically normal individuals in a leprosy endemic population.

PATHOGENESIS AND IMMUNOLOGY OF LEPROSY[29]

Leprosy is characterized by well recognized pathological changes. These pathological characteristics are strikingly different from other infectious diseases because of unique features of M. leprae.

Commonly affected tissues are peripheral nerves and skin, rarely other tissues like respiratory mucosa, lymph nodes etc. can be affected.

Leprosy patients, especially lepromatous patients are the main source of infection. Bacilli are liberated into the environment through the oro-nasal sinuses and

skin ulcers of these patients. It is not absolutely certain how M. leprae enters the human host.

Lepra bacilli first infect the neural tissue. Primary target are schwann cells. Subsequently fate and type of lesion depend on immune states of the host. Bacilli multiply within the schwann cell and perineural cells, later the bacilli destroy them. Schwann cells liberate the bacilli, which enter the neighboring schwann cells and spread the infection intraneurally. When the intraneural infection is recognized, lymphocytes and macrophages infiltrate the nerve, later macrophages engulf the bacilli. The bacilli multiply within the macrophages and then are carried to other parts of the nerve and other nerves. Later they spread to other parts of the body through blood, lymph and tissue fluids. [31] Experimental studies have shown two portals of entry.[30]

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

b) Nasal mucosa.

Factors which influence the outcome of infection are age, skin, race, nutrition and intercurrent disease. The major factor which determines the outcome is the immune status of the host. [31]

The macrophages become foamy due to utilization of oxygen and nutrition from the cytoplasm, by the bacilli.

Later the macrophage ruptures, releasing the bacilli into the skin and other structures. These bacilli are picked up by fresh macrophages. The body responds by sending 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. In about 5% of cases the bacilli multiply in the macrophage probably by preventing the formation of phagolysosome.[31, 32]

Role of Immunology in Pathogenesis[32]:

There is involvement of cell mediated immunity and delayed hypersensitivity in the pathogenesis of leprosy. These are responsible for the development of leprosy, but it is the degree that determines the type of leprosy. This complete immune response involves mainly T-lymphocytes, macrophages, to some extent B Lymphocytes and the mediators.

The T-helper lymphocyte response to M. leprae determines whether an individual has tuberculoid or lepromatous leprosy.

Patients with tuberculoid leprosy have a defective TH1 response or a dominant TH2 response with production of IL-4, IL-5 and IL-10, which will suppress macrophage activation.

In tuberculoid leprosy there are good number 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, CD8+ T cells increase.

In lepromatous patients, CD4+ T helper 2 cells (TH2 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).

Classifications:

Classification of any disease, particularly leprosy, can be adjudged as satisfactory only if it can be applied without much difficulty by different groups of workers, that is clinicians, pathologists or immunologists. Between the different groups, there should be a correlation of the criteria and the understanding must be synchronized. [33]

The ancient Indian medicine by Sushrutha Samhita in 600 BC described three types – pure neuritic, non-lepromatous cutaneous lesions with sensory changes and lepromatous cutaneous in which sensory changes are not prominent features. [34]

Danielsen and Boeck (1848) divided leprosy into nodular and anesthetic types. Hansen and Looft changed the anesthetic to maculoanesthetic types in 1895. Neisler (1903) described three forms, namely - lepra tuberosa, lepra cutaneae and lepra-nervosum. Jadussohn (1905) for the first time described the leprosy as tuberculoid.[34]

In 1931, Leonard Wood Memorial, Manila, Phillipines, divided leprosy into 3 types namely cutaneous, neural and mixed. The International Leprosy Congress, Cairo (1938) adopted a classification in which the term 'Cutaneous' of the manila classification was replaced by 'Lepromatous'. [34]

The second Pan American leprosy congress (1946) classified leprosy based on histological grounds. Lepromatous was retained and neural was replaced by tuberculoid, and the third type was named uncharacteristic. [34]

WHO (1952) classified leprosy into lepromatous, tuberculoid, borderline and indeterminate. In 1955 Indian Association of leprologists classified leprosy into six classes: lepromatous, tuberculoid, maculo- anesthetic, borderline, polyneuritic and indeterminate. In revised Indian classification (1981) maculo--anesthetic type was removed and included in tuberculoid type.[35]

In the same year Job & Chacko classified leprosy into lepromatous leprosy, tuberculoid leprosy, borderline tuberculoid leprosy, borderline borderline leprosy, borderline lepromatous leprosy, indeterminate leprosy and polyneuritic. [34, 35]

Ridley and Jopling Immunological Classification (1966)

Ridley and Jopling (1966) suggested a classification system, which employed correlation of clinical and histopathological status. [36]

This classification is only for research purposes, according to Ridley and Jopling themselves.[37]

1. Tuberculoid (TT)
2. Borderline Tuberculoid (BT)
3. Borderline Borderline (BB)
4. Borderline Lepromatous (BL)
5. Lepromatous Leprosy (LL)

WHO Clinical Classification [34, 38]

This is a simple classification based on number of bacilli harbored in an individual.

1. Multibacillary [MB]

2. Paucibacillary [PB]

Bacteriological index if 2+ or more termed multibacillary and bacteriological index less than that are termed paucibacillary.

This was modified in 1988 as multibacillary with bacterial index 1+ or more, and paucibacillary with bacterial index 0+ [i.e., all smear negative]

Clinical Aspects of Leprosy Lesions

Leprosy, in some individuals, involves only one peripheral nerve (mononeuritic) or causes a single skin blemish which persists indefinitely or disappears on its own, while in others it produces multiple lesions or nodules, together with polyneuritis and damage to vital organs, such as eyes, larynx, testes and bones.[39]

WHO, has set guidelines based on at least two of the following [41]

1. Characteristic skin lesions.

2. Thickened nerves/AFB positive skin smear.

3. Sensory loss.

VARIOUS CLINICAL TYPES

1. Indeterminate Leprosy [31, 41, 42]

Indeterminate leprosy is the earliest form of leprosy which manifest as small one or few hypopigmented macules, about 1 cm or less than 5 cm in diameter, rarely erythematous. Nerve thickening is usually absent. Even skin smears are usually negative. The diagnosis is confirmed by histopathological examination. Indeterminate leprosy may heal or remain indeterminate for a long period of time. It may sooner or later progress to determinate forms of leprosy lesions.

2. Tuberculoid Leprosy [41, 42, 43]

Commonly seen on face, dorsum of extremities and lower back, and affects both skin and peripheral nerves. Lesions are usually single or two with well defined borders, and are macular or rarely plaques. They may be hypopigmented or erythematous with hair loss on their surface. Nerves are thickened with absence of tenderness. Sensations of touch may be preserved. Skin smears are usually negative for AFB.

3. Borderline Tuberculoid Leprosy [41, 42, 43]

Commonest type of leprosy. Lesions may be single or multiple with varying size and shape and are well defined, symmetrical with raised margins and hair loss. Hypopigmentation and dryness are less severe than tuberculoid type. Satellite lesions are seen near the edges of larger lesions.

Nerve thickening is present with asymmetry and there is loss of sensation over the lesions.

One of the striking features of borderline tuberculoid leprosy is tendency to present with type I reaction. Most cases present as reaction. Skin smears sometimes are positive for AFB.

4. Borderline Borderline Leprosy [Mid- Borderline] [41, 42]

Most unstable and rare form. It spans the spectrum between lepromatous and tuberculoid poles.

Multiple lesions of skin with varying size, shape and distribution are seen. They may be macules, papules or plaques with ill defined margins, having moderate hair loss. Nerve thickening is seen with asymmetry, with mild to moderate loss of sensation. Skin smears are positive with many bacilli.

5. Borderline Lepromatous Leprosy [41, 42, 43]

Classically the lesion start with macules, localized at first and later it is wide spread as seen in lepromatous type. These macules are wide spread over the extremities and lower back. Lesions are symmetrical and vary in size. Peripheral nerve thickening is present with impairment of sensation. Skin smears are positive with many bacilli often in clumps and globi.

6. Lepromatous Leprosy[32, 41, 42, 43, 44]

Lesions are seen all over the body with macules, papules or nodules and seen 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 hairloss [leprous alopecia]. Nodular lesions over the face coalesce together, with loss of eye lashes [madarosis] and depression of nasal bridge [leonine facies]. Trophic ulcer formations may be seen in the extremities.

Muscle weakness and wasting may be seen. There may be involvement of eyes, lymphadenopathy, testes or other systemic organs. Hands and feet may be swollen. Involvement of mucosa of upper respiratory tract is seen in 80% of new lepromatous cases.

Skin smears show plenty of AFB with multiple globi.

7. Pure Neuritic Leprosy [41]

Also called as pure neural, primary neural, primary neurotic, primary polyneuritic. Common in India. Presents with neurological deficit without any skin lesions. It may present as anesthesia in an extremity or present with gradual foot drop. Mono-neuritic is the most common form but multiple nerve involvement may be present.

Histological Features of skin

Structure of skin.

Skin consists of 3 layers: Epidermis, Dermis and Subcutis.

Epidermis:

The epidermis derived from ectoderm, is a keratinizing stratified squamous epithelium from which arises the cutaneous appendages, namely the pilosebaceous follicles, nails, apocrine and eccrine sweat glands.

In addition to keratinocytes there is a 'clear' cell population, which includes melanocytes and Langerhans' cells.

The epidermis comprises five layers or strata:

- Keratin cell layer (stratum corneum)
- Clear cell layer (stratum lucidum)
- Granular cell layer (stratum granulosum)
- Prickle cell layer (stratum spinosum)
- Basal cell layer (stratum basale)

The basal cells are tall columnar cells aligned perpendicular to the basement membrane and are the germinative cells of the epidermis and comprise stem cells and proliferative cells.

Prickle cells are polygonal in outline, have abundant eosinophilic cytoplasm, oval vesicular nuclei with conspicuous nucleoli.

Keratohyaline granules typify the granular cell layer. Further maturation leads to loss of nuclei and flattening of keratinocytes to form the plates of the keratin layer.

Adjacent cells are united at their free borders by intercellular bridges (prickles or desmosomes). It also unites the epidermis with the dermis is the basement membrane region.

Melanocytes, of neural crest origin are usually located along the basal layer of epidermis. The ratio of melanocytes to basal cells ranges from 1:4 on the cheek to 1:10 on limbs.

Langerhans cells are found within the supra basal layers of the epidermis and also in the dermis. They represent potent stimulators of a wide range of T cell mediated immune reactions.

Dermis:

The dermis or corium supports the epidermis and is composed of a fibrous connective tissue component, collagen and elastic fibres in intimate association with the ground substance.

Contained within the dermis are the epidermal appendages, blood vessels and nerves and a cellular component including mast cells, fibroblasts, myofibroblasts and macrophages. Smooth muscle is also represented in the erector pili muscles.

Sub cutis:

The sub cutaneous fat is divided into lobules by vascular fibrous septae and its cells are characterized by the presence of a large single globule of lipid which compress the cytoplasm and nucleus against the plasma membrane.[45]

Histological Features of Leprosy Lesions

Histological examination of all types of leprosy are done under following criteria.[41]

1. Cell Type:

Lymphocytes are present in varying numbers in all leprosy lesions and they are the predominant cell type in indeterminate leprosy.

Epithelioid cells and granulomas are found in tuberculoid types (BT and TT) whereas foamy macrophages are predominantly seen in Lepromatous types (LL & BL).

2. Bacterial Load:

Bacterial load is varied from almost absence of Mycobacterium leprae in tuberculoid types to bacilli packed macrophages in lepromatous types.

3. Nerves:

Involvement of nerves and the presence of bacilli inside the nerves is also a diagnostic feature.

I. Indeterminate Leprosy: [46, 47, 48]

Majority of the times, clinical diagnosis of indeterminate leprosy is varied. To make a definitive diagnosis, histopathological study is necessary.

Features are usually non-specific with epidermis showing no significant change. But the dermis show mild lymphocytic and macrophage accumulation around neurovascular bundles, superficial and deep dermal vessels, sweat glands and erector pili muscle. Focal lymphocytic invasion into the lower epidermis and into the dermal nerves may be observed. Schwann cell hyperplasia is a feature but it is highly subjective. The diagnosis hinges on finding one or more acid fast bacilli in the sites of predilection in nerve, in erector pili muscle, just under the epidermis or in a macrophage about a vessel. Without demonstrating bacilli the diagnosis can only be presumptive.

II. Tuberculoid Leprosy (TT): [43, 47, 48]

Epidermis shows atrophy, occasionally few areas of hypertrophy seen. Dermis is filled with granulomas containing aggregates of epithelioid cells even with langhans type of giant cells. Granulomas almost replace the nerves, sweat glands, hair follicles, erector pilorum muscles and sebaceous glands. These are surrounded by dense lymphocytic infiltrate. There is no clear zone (Grenz zone) and the granulomas are seen to hug the epidermis. Acid fast bacilli are rare and difficult to demonstrate.

III. Borderline Tuberculoid Leprosy (BT): [6, 46, 47]

Atrophy of the epidermis is minimal depending on the size and extensiveness of the granulomas. Dermis shows granulomas with peripheral lymphocytes that follow the neurovascular bundles and infiltrate sweat glands and erector pili muscles. Langhans giant cells are variable in number and are not large in size.

Granulomas along the superficial vascular plexus are frequent but they do not infiltrate up into the epidermis. Nerve erosion and obliteration are typical.

Acid fast bacilli are scanty and are most readily found in the Schwann cells of nerves.

IV. Borderline Borderline Leprosy (BB) Mid Borderline: [31, 46, 49]

Rare type and is unstable and has atrophic epidermis. Dermis shows grenz zone which is a clear zone which separates granulomas from the epidermis. Granulomas are ill defined composed of mixture of good number of epithelioid cells, scattered lymphocytes and few macrophages. Here the macrophages are uniformly activated to epithelioid cells but are not focalized into distinct granulomas. There are no langerhans giant cells.

Involvement of nerves also is seen with minimal destruction of the affected nerves but reactive proliferation and edema of the perineurium is seen.

Acid fast Bacilli can be seen in schwann cells and in scattered macrophages.

V. Borderline Lepromatous Leprosy (BL) [31, 46, 49]

Epidermis is always atrophic. Dermis shows mixture of many macrophages with large number of lymphocytes, which are separated from the epidermis by a clear zone (Grenz zone). Most of the macrophages are foamy with granular pink cytoplasm.

These inflammatory cells are also seen around hair follicles, sweat glands, sebaceous glands and erctor pilorum muscles which may damage them.

There is also marked infiltration around the nerves, which show proliferation of perineural cells with formation of onion skin perineurium (Concentric layers around the nerves) on cross section.

Plenty of Acid fast bacilli can be demonstrated, which are distributed in singles, clumps or occasionally in globi.

VI. Lepromatous Leprosy. [31, 46, 49]

Has definite atrophy of epidermis with flattening of the rete ridges. Dermis show band of cellular infiltration, consisting of majority of macrophages with few lymphocytes. This layer is separated by a grenz zone from the epidermis. Macrophages show vacuolated and foamy, pale cytoplasm. Few plasma cells may also be seen. The macrophages also infiltrate around the hair follicles, sebaceous glands and sweat glands. These structures appear atrophic.

Macrophages are also seen surrounding the nerves, but there is minimal proliferation of the perineurium.

Large number of acid fast bacilli arranged in clumps (Globi) are seen in macrophages, perineurium, schwann cells, sweat glands, sebaceous glands hair follicles, erector pilorum muscle and even in endothelial cells.

VII. Histoid Leprosy.[50]

Histoid leprosy is an unusual form of lepromatous leprosy. The epidermis is thinned by an expanding pseudoencapsulated dermal mass consisting of interlacing bands and whorls of spindle shaped histiocytes. In early lesions, predominant cells

may be polygonal or irregular histiocytes. The immediate sub-epidermal zone contains no infiltrate. The histoid masses contain unusually large numbers of acid fast bacilli packed tightly in bundles and groups without disturbing cellular detail.

The lesion can resemble a dermatofibroma and must be differentiated from other fibrohostiocytic and histiocytic skin tumors. It can be differentiated by demonstrating intracellular acid fast organisms.

Histopathology of leprosy reactions[6]

1. Type-I Reaction:

Immunopathologic spectrum of leprosy is a continuum, patients may move along it in both directions.

a) Upgrading Reaction: Shifts towards tuberculoid pole is called upgrading or reversal reaction. The granuloma becomes more epithelioid and activated and langhans giant cells are larger with increased lymphocytes. Important feature here is edema within and about the granulomas.

Bacilli are decreased in number or absent. There may be fibrinoid necrosis within granulomas & dermal nerves.

b) Down grading Reaction: Shifts toward the lepromatous pole is termed down grading reaction. Even here, edema is the most important feature, but the granulomas are disorganized with decrease in lymphocytic infiltration. There are good numbers of macrophages with persistence of giant cells. Fibrinoid necrosis within granulomas is less common. Over the time density of bacilli increases.

2. Type-II Reaction (Erythema Nodosum Leprosum- ENL)

Occur most commonly in lepromatous leprosy and less frequently in borderline lepromatous leprosy. May be observed not only in patients under treatment but also in untreated patients. Important feature here is dense infiltration of polymorphs in the dermis and subcutaneous tissue with microabscess formation. Damage to the elastic fibres and collagen is common. Rarely vasculitis or necrosis and ulceration of skin are seen.

Bacilli are reduced where as foamy macrophages containing fragmented bacilli are usual.

MATERIALS AND METHODS

Source of data:

Skin biopsies from patients clinically diagnosed as leprosy were received in the department of pathology, B.L.D.E.A's Shri B.M.Patil Medical College Hospital and Research Centre, Bijapur from August-2005 to July-2009.This included leprosy patients attending the dermatology clinics of the hospital. Skin biopsies were obtained after taking informed consent in all the cases.

Inclusion criteria

All cases clinically diagnosed as leprosy were included in the study.

Exclusion criteria:

1) Inadequate biopsy.
2) Bacillary fragments are not taken into consideration for diagnosis in case of fluorescent microscopy.

Sample size : 60 cases

Method of collection of data:

Pertinent clinical history like age, duration of the lesion, site of the lesion, significant family and personal history, history of associated diseases and any drug intake were taken and entered in the proforma. After detailed general and local examination, the site of the biopsy was selected. The selected patient's consent was taken after explaining the details of the biopsy procedure. The biopsy of the lesion is done along with the surrounding area. The biopsy area is cleaned & painted with an antiseptic solution and adequate amount of local anaesthetic (2% lidocaine) is injected to the skin and subcutaneous tissue.

Biopsy Technique:

Punch biopsy was used for obtaining samples of skin biopsy. It is important to select a proper site for biopsy. Biopsy was taken from the active lesion, after injection of local anaesthetic. The specimen obtained with a 4mm biopsy punch was used for histological study. A 3 mm punch was preferred for small lesions or biopsy from face for cosmetic reasons. After that the skin specimen was loosened with the biopsy punch instrument, dropped in a bottle containing 10% formalin, and sent to histopathology laboratory.

The biopsy specimen provided included history of previous biopsies, adequate clinical history and any special requests if required.

Gross examination of the skin biopsy:

The three dimensional size and shape of the skin biopsy was assessed including the circular or elliptical shape of the biopsy.

The entire skin biopsy was submitted for routine processing and embedded in paraffin wax. From each block, ribbons containing 4 serial sections each 5 microns were taken. One section were taken for routine haematoxylin and eosin staining and one each for ZN staining, fluorescent and Fite-faraco staining.

Details of the staining procedure.

5μm thick paraffin sections of the skin biopsy were stained with haematoxylin and eosin.

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.

ZIEHL- NEELSEN STAIN.

a) Carbol fuchsin

b) 1% acid alcohol.

c) Methylene blue.

d) Xylene.

Procedure.

1. Paraffin sections placed in xylene for 30 min two changes each.
2. Sections were hydrated by passing through 90%, 70% and 50% alcohol.
3. Sections were stained with carbol fuchsin for 10 minutes.
4. Sections were decolorized in 1% alcohol.
5. Sections were washed in running water.
6. Sections were counterstained with methylene blue.
7. Slides were dried, cleared in xylene and mounted.

Results

Acid fast bacilli- Red , Background – Light blue.

All stained sections were screened with 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.

MODIFIED FITE FARACO 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, sections were taken on clean scratch free glass slides without egg albumin or any other adhesive. These tissue sections were stained with fluorescent dye (Auramine-rhodamine) and examined under fluorescent microscope.

Procedure

Auramine rhodamine fluorescent stain as recommended by Kuper and May[11] was used.

Following procedure was used.

1) Deparaffinization was performed with 1 part peanut oil and 3 parts xylene mixture; two changes of 10 minutes each and then blotted carefully.
2) The slide was stained with filtered auramine rhodamine mixture at 65^0 C minutes.
3) The slide was washed in running water for 2 minutes.
4) Decolorization was performed in 0.5 % hydrochloric acid in 70 % ethanol for 2 minutes.
5) The slide was washed under running water for 2 minutes.
6) Counterstaining was performed with 0.5 % aqueous potassium permanganate for 2 minutes.
7) The slide was washed under running 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 glycerol using a scratch free cover slip.

Tissue sections were observed immediately under Carl Zeiss fluorescent microscope, which had HBO 50 high pressure mercury short-arc discharge. Excitation was with blue violet rays obtained with two BG 12 primary filters; an Abbe condenser was also used. Each time the sections were screened, auramine-rhodamine stained sections from a skin biopsy of a typical lepromatous leprosy patient and a skin biopsy from a normal individual were used as controls.

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. Bacteriological index was calculated under oil immersion field.

Method of Statistical Analysis:

The following methods of statistical analysis have been used in this study. The Excel and SPSS (SPSS Inc, Chicago, and Version 10.5) software packages were used for data entry and analysis.

The results were averaged (mean ± standard deviation) for each parameter for continuous data and numbers and percentage for categorical data presented in Tables and Figures.

Statistical analysis:

- Data represented by diagrammatic presentations and tabulations.
- Sensitivity, specificity, positive predictive value and negative predictive values calculated.
- Data collected analysed using Chi-Square test. 'p' value of < 0.05 is considered as statistically significant.
- Also data was analysed using Pearson Correlation ('r' value determined) for comparison between groups.

OBSERVATIONS

The present study was carried out on a total of 60 clinically diagnosed leprosy patients attending the department of Dermatology, venereology and leprology of Shri B M Patil Medical College , Bijapur from August 2007 to July 2009. The results obtained after staining the biopsy slides with ZN stain, Modified Fite-faraco and Fluorescent stain were analysed.

Table 1 : Age and sex distribution of patients.

Age in Years	Male		Female		Total	
	No	%	No	%	No	%
< 20	5	15.6	5	17.9	10	16.7
21-30	5	15.6	11	39.3	16	26.7
31-40	5	15.6	6	21.4	11	18.3
41-50	7	21.9	5	17.9	12	20
>50	10	31.3	1	3.6	11	18.3
Total	32	100	28	100	60	100
Mean ± SD	42.6 ±16.76		30 ±13.52		36.7 ± 16.3	

In the present study, patients in the age group of 21-30 years were affected most with 16 cases (26.7%). The least affected age groups are those < 20 years, comprising 10 cases (16.7%).

Age distribution of patients according to gender.

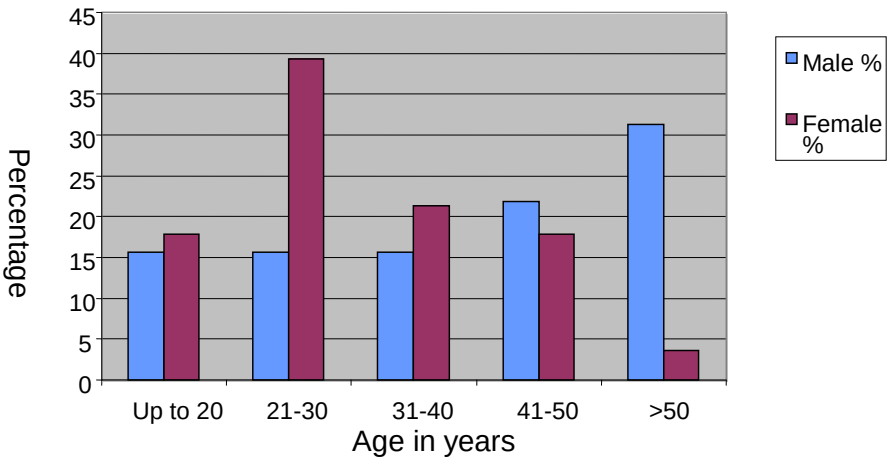


Table 2: Gender distribution of patients.

Gender	Number	%
Male	32	53.3
Female	28	46.7
Total	60	100

In the present study males were affected the most, with 32 cases (53.3%) and females being 28 cases (46.7%).

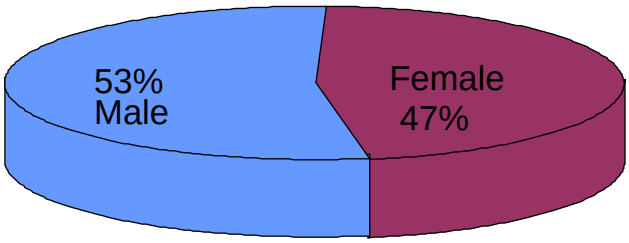


Table 3: Different histological patterns in present study.

HISTOPATHOLOGICAL DIAGNOSIS	Number (n=60)	%
Indeterminate Leprosy	30	50
Tuberculoid Leprosy	2	3.3
Borderline Tuberculoid leprosy.	14	23.3
Borderline Borderline Leprosy	0	0
Borderline Lepromatous Leprosy	2	3.3
Lepromatous Leprosy	12	20
Total	60	100

In our study indeterminate leprosy was the most common constituting 30(50%) cases, followed by borderline tuberculoid leprosy 14(23.3%), lepromatous leprosy 12(20%), borderline lepromatous 2(3.3%) and tuberculoid leprosy 2(3.3%). There was no borderline borderline case in our study.

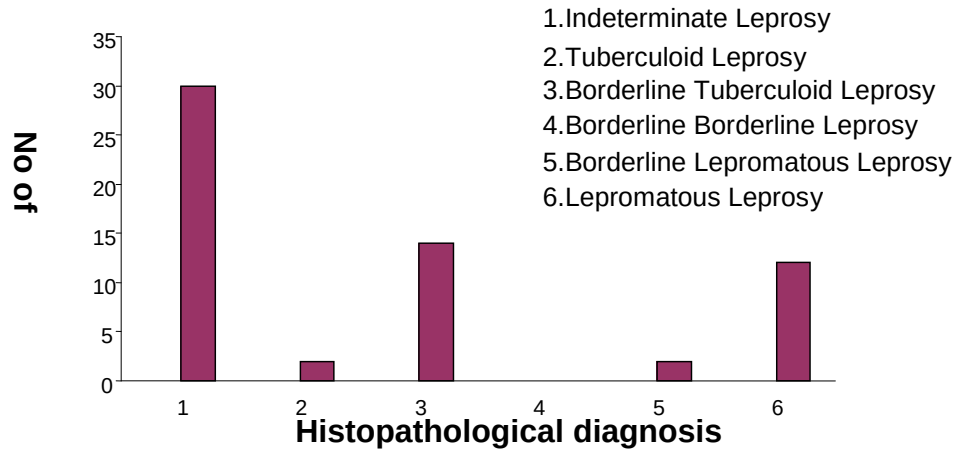


Table 4: Percentage of histological diagnosis positive for ZN Stain

Histopathological Diagnosis	Total No. of patients	No. of Postives	%
1.Indeterminate Leprosy	30	1	3.3
2.Tuberculoid Leprosy	2	0	0
3.Borderline Tuberculoid Leprosy	14	2	14.3
4.Borderline Borderline Leprosy	0	0	0
5.Borderline Lepromatous Leprosy	2	1	50
6.Lepromatous Leprosy	12	12	100
Total	**60**	**16**	**26.7**

In the present study various histological patterns showed varied positivity rates for ZN stain. 1(3.3%) out of 30 patients of indeterminate leprosy, 2(14.3%) out of 14 cases of borderline tuberculoid leprosy, 1(50%) out of 2 cases of borderline lepromatous leprosy and 12(100%) out of 12 cases of lepromatous leprosy were positive by ZN stain. None of the tuberculoid leprosy cases showed positivity with ZN stain.

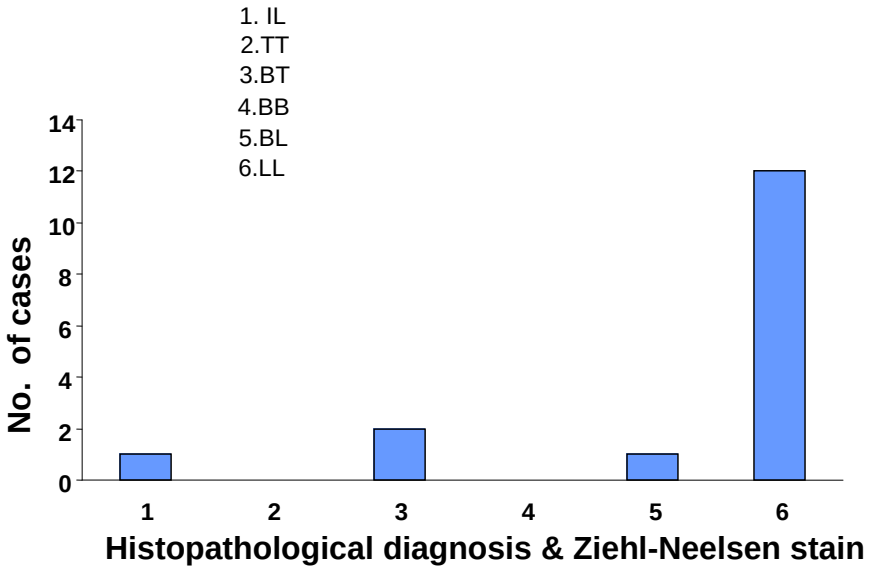


Table 5: Histological patterns and modified Fite-faraco stain

Histopathological Diagnosis	Total No. of patients	No. of Positives	%
1.Indeterminate Leprosy	30	1	3.3
2.Tuberculoid Leprosy	2	0	0
3.Borderline Tuberculoid Leprosy	14	4	28.6
4.Borderline Borderline Leprosy	0	0	0
5.Borderline Lepromatous Leprosy	2	2	100
6.Lepromatous Leprosy	12	12	100
Total	**60**	**19**	**31.7**

In the present study various histological patterns showed varied positivity for Modified fite faraco stain.

1(3.3%) out of 30 patients of indeterminate leprosy, 4(28.6%) out of 14 cases of borderline tuberculoid leprosy,2(100%) out of 2 cases of borderline lepromatous leprosy and 12(100%) out of 12 cases of lepromatous leprosy were positive by Fite-faraco stain. None of the tuberculoid leprosy cases showed positivity with Fite-faraco stain.

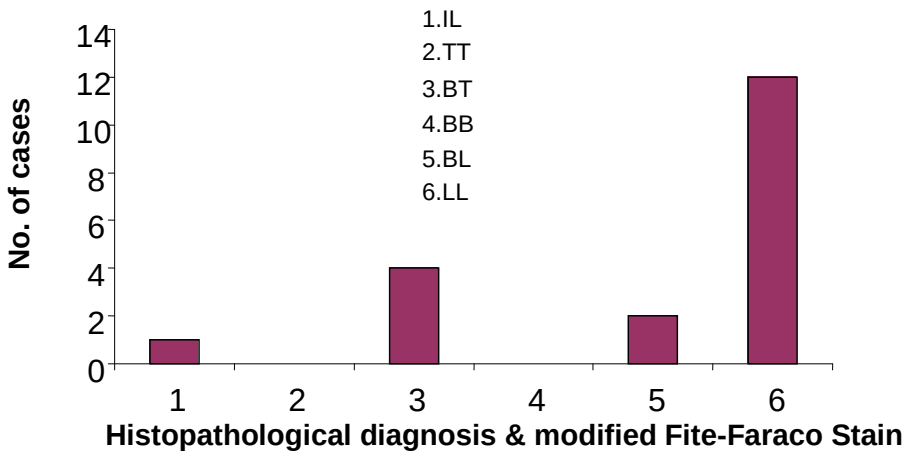


Table 6: Percentage of histological diagnosis positive for fluorescent Stain

Histopathological Diagnosis	Total No. of patients	No. of Postives	%
1.Indeterminate Leprosy	30	8	26.7
2.Tuberculoid Leprosy	2	0	0
3.Borderline Tuberculoid Leprosy	14	4	28.6
4.Borderline Borderline Leprosy	0	0	0
5.Borderline Lepromatous Leprosy	2	2	100
6.Lepromatous Leprosy	12	12	100
Total	60	26	43.3

In the present study various histological patterns showed varied positivity for fluorescent stain.

8(26.7%) out of 30 patients of indeterminate leprosy, 4(28.6%) out of 14 cases of borderline tuberculoid leprosy, 2(100%) out of 2 cases of borderline lepromatous leprosy and 12(100%) out of 12 cases of lepromatous leprosy were positive by fluorescent stain. None of the tuberculoid leprosy cases showed positivity with fluorescent stain.

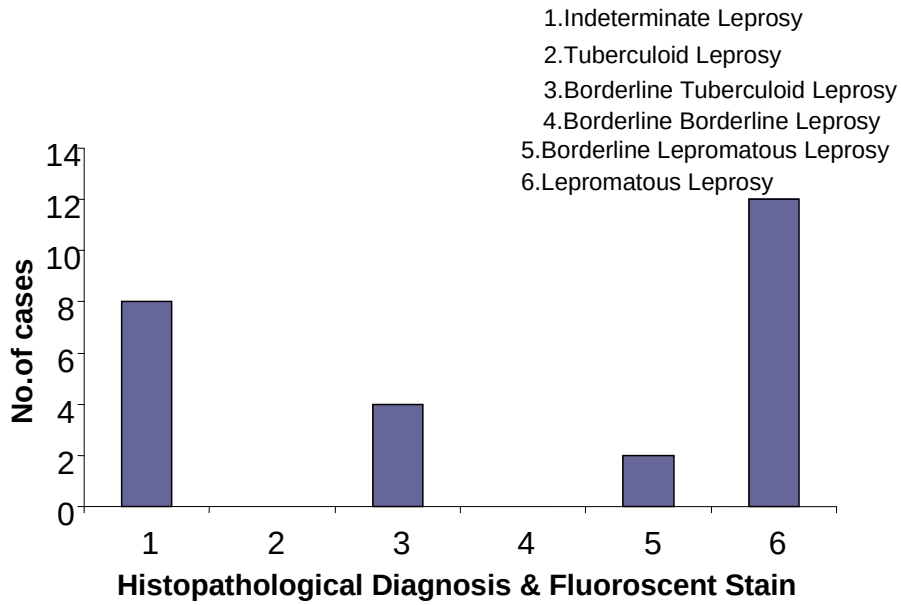


Table7: Comparison of positivity rates of ZN, modified Fite-faraco and fluorescent stains.

HISTOPATHOLOGICAL DIAGNOSIS	Total No. of patients	ZN Stain	Modified Fite-Faraco method	Fluorescent Method
	Positivity rate	*Positivity rate*	*Positivity rate*	
IL	30	3.3	3.3	26.7
TT	2	0	0	0
BT	14	14.3	28.6	28.6
BB	0	0	0	0
BL	2	50	100	100
LL	12	100	100	100
Total	60	26.7	31.7	43.3

Highest overall positivity rates were seen with FL (43.3%) compared to 31.7% and 26.7% with FF and ZN methods respectively.

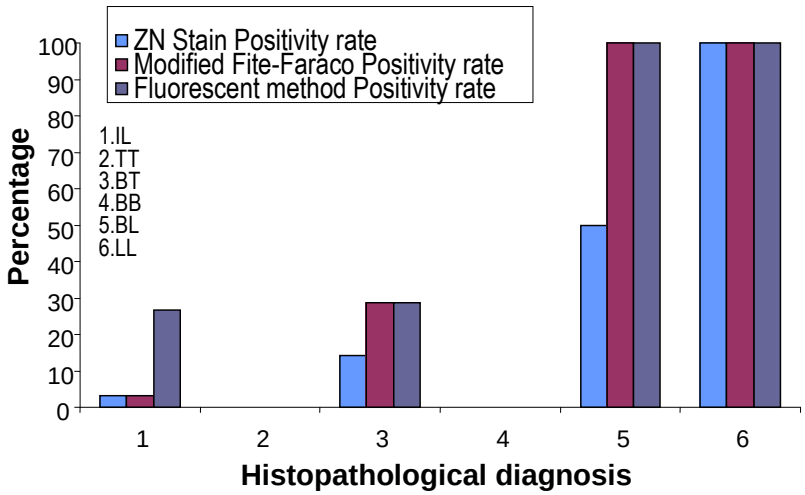


Table 8: Histological findings and correlation of modified Fite-faraco stain with Ziehl-Neelsen and fluorescent stain

Histopathological Diagnosis	Sensitivity		Specificity		PPV		NPV	
	ZN Stain	FL Stain	ZN Stain	FL Stain	ZN Stain	FL Stain	ZN Stain	FL Stain
1.IL	100	100	100	75.86	100	12.5	100	100
2.TL	-	-	100	100	-	-	100	100
3.BT	50	100	100	100	100	100	83.33	100
4.BB	-	-	-	-	-	-	-	-
5.BL	50	100	-	-	100	100	0	-
6.LL	100	100	-	-	100	100	-	-
Mean	75	100	100	91.95	100	78.125	70.8325	100

Considering Fite-faraco (FF) method to be the standard test, we compared the performance of ZN and FL stain methods. FL stain showed 100% sensitivity as against ZN which showed only 75% sensitivity compared to FF method. The apparent lower specificity of FL method is due to its higher sensitivity as reflected in its higher positivity rates compared to the FF stain (43.3% and 26.7% respectively), since we did not consider any artifacts and non-solid bacilli as positive in our results.

Table 9: Histological findings and correlation of mean bacillary index among modified Fite-Faraco stain with Zeihl-Neelsen and fluorescent stain

Histopathological Diagnosis	Total No. of patients	Mean Bacillay Index		
		FF Stain	ZN Stain	FL Stain
1.IL	30	1.1	0.8	1.2
2.TL	2	3	2.5	3.5
3.BT	14	1.2	1.2	1.4
4.BB	0	0	0	0
5.BL	2	3	4	4
6.LL	12	1.3	1.2	1.4
Total	60	9.6	9.7	11.5

Mean bacillary index of all the histological types with FF was 9.6, with ZN stain was 9.7 and with fluorescent stain was 11.5. This is in line with the higher sensitivity of fluorescent method.

Table 10: Correlation between Fite faraco v/s Ziehl – Neelsen and fluorescent method.

Method	Fite-Faraco	
	Pearson's 'r'	P Value
Zeihl-Neelsen	0.96	<0.0001
Fluorescent Method	0.98	<0.0001

As we can see from Table 10. overall both Zeihl-Neelsen and fluorescent methods show a statistically significant correlation with Fite-faraco method.

Table 11: Bacillary index correlation among patients with lower BI (BI<3) and higher BI (BI>3) between various staining types.

Method	Fite- Faraco	
	BI<3 , Pearson's 'r'	BI>3 , Pearson's 'r'
Ziehl Neelsen	-0.04 p=0.81	0.89 p<0.0001
Fluorescent method	0.73 (p<0.0001)	0.84 (p=0.0004)

Since BI is a continuous variable we divided the cases into two groups ie., those with BI<3 and those with BI>3, for comparison between groups. Ziehl-Neelsen method correlates well (r=0.89) with Fite- faraco method at higher BI (>3) but poorly and insignificantly (p=0.81) so with lower BI (<3). However fluorescent method retains good (r=0.73) and statistically significant correlation (P<0.0001) even at low bacillary loads. Thus fluorescent method is more sensitive in detecting lepra bacilli in cases with low bacillary load (BI <3).

Table 12: Cases showing upgrading of BI by fluorescent and ZN compared to FF among paucibacillary and multibacillary cases.

BI	***PB cases***	***MB cases***	***Total***
FL>FF	9	2	11
FL<FF	0	1	1
Net additional cases detected by FL	9	1	10
BI	***PB cases***	***MB cases***	***Total***
ZN>FF	1	0	1
ZN<FF	1	7	8
Net additional cases detected by FL	0	-7	-7

Among paucibacillary cases FL stain shows a higher BI compared to FF in 9 cases, while among multibacillary cases, only 1 additional case had a higher BI compared to FF.

No net additional case could be detected by ZN stain compared to FF. In fact ZN stain showed a lesser BI compared to FF among 7 multibacillary cases proving inferiority of ZN stain compared to FF stain.

Table 13 : Comparison of shift in Ridley's BI scale by FL stain and ZN stain with that of FF among paucibacillary and multibacillary cases.

BI	FF pauci to FLmulti	FF multi to FLpauci	Net upgradation of Ridley's scale.	FF pauci to ZNmulti	FF multi to ZNpauci	Net upgradation of Ridley's scale.
No. of cases	1	0	1	1	2	-1

Use of FL stain resulted in diagnosis of an additional case of multibacillary while ZN stain failed to correctly classify one case of MB as diagnosed by FF stain.

This ability of FL stain to diagnose additional cases of multibacillary cases has implications in therapy and follow-up.

DISCUSSION

Leprosy continues to be a major public health problem in India with a annual new case detection rate of 0.84 per 10, 000 population.[22] Leprosy affects skin, peripheral nerves and other organs directly or indirectly, leading to progressive and permanent deformities in the patients. Clinical presentations are varied with so many diversities between the clinical and histopathological features.

Histopathological examination is the keystone in the diagnosis and categorization of leprosy. Modified Fite-faraco technique is the routinely used method to demonstrate mycobacterium leprae in tissue sections. Detection of Mycobacterium leprae in tissue sections by modified Fite-faraco is tedious, time consuming and leads to observer fatigue. Hence fluorescent microscopy has been used by some for rapid screening, to reduce observer fatigue and to increase sensitivity.

There is an increasing need for evaluation of newer techniques for the detection of mycobacterium leprae to achieve rapid screening and reduce observer fatigue, while increasing sensitivity and specificity.

In the present study we compare the performance of fluorescent microscopy, modified fite faraco and ZN stains in detecting Mycobacterium leprae in tissue sections.

Skin biopsies of 60 patients (32 males and 28 females) clinically diagnosed as leprosy was studied.

Most patients (26.7%) were between 21 to 30 years. Indeterminate leprosy was the most common histological type (50%) followed by borderline tuberculoid leprosy(23.3%).

Table 14: Comparison of positivity rates of ZN staining, modified Fite-faraco and fluorescent stain with that of other studies.

Various studies	**ZN stain**	**Fite-Faraco procedure**	**Fluorescence method**
	No. of Positive cases	***No. of Positive cases***	***No. of Positive cases***
Present study	16(26.7%)	19 (31.7 %)	26 (43.3 %)
Mukkamil AS et al[17]	-	25 (44.64 %)	39 (69.64 %)
Jariwala et al[13]	-	20 (40.0 %)	22 (44.0 %)
Bhatia et al[15]	57(67.8%)	-	75(89.2%)
Lacordaire Lopes de Faria[18]	-	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 modified Fite-faraco which is comparable to the studies done by Mukkamil AS et al[17] and Jariwala et al.[13]

Also, in the present study, the positivity rate with ZN staining is lower as compared with that of fluorescent staining. A study done by Bhatia et al[15] which showed more positives cases by fluorescent method as compared to that by ZN stain though they did not use modified Fite-faraco in their study.

Thus our study shows that fluorescent stain is better than both modified Fite-faraco and ZN stain in detecting lepra bacilli in tissue sections.

In a study by Lacordaire Lopes de Faria[18] positivity rates with modified Fite-faraco is higher than that with fluorescent microscopy. In his study he used egg albumin as adhesive and phenol which produces considerable artifacts. He found the presence of artifacts from albumin and phenol to be a major problem. We did not face such problems because neither egg albumin nor any other adhesive was used. The bacilli however could be easily differentiated because the non specific artifact has pale yellow fluorescence, where as the bacilli have bright yellow fluorescence.

Table 15: Comparison of Positivity rates in various leprosy types by modified Fite- faraco and flourescent methods with that of other studies.

Leprosy types	*Mukkamil A.S et al*[17]	*Jariwala H. J et al*[13]	*Present Study*
	FF vs FL	**FF vs FL**	**FF vs FL**
	Positivity rate Difference	**Positivity rate Difference**	**Positivity rate Difference**
IT	32%	0%	23.4%
TT	33%	0%	0%
BT	17%	9%	0%
BB	*	**	0%
BL	*	0%	0%
LL	0%	0%	0%
	82%	13%	11.6%

* There were no BB and BL cases in the study by Mukkamil[17] et al.

**There were no BB cases in our study.

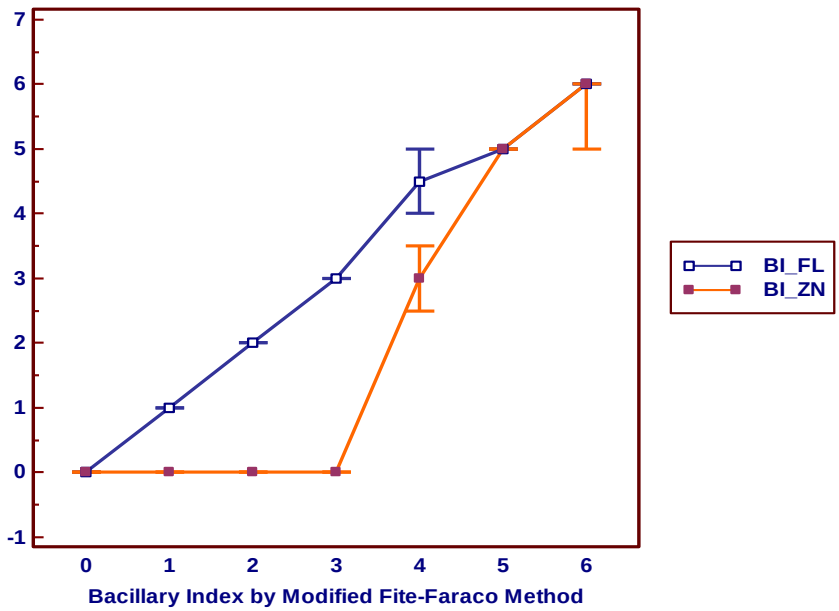
In the study done by Mukkamil[17] et al. positivity rate difference between FF and FL stains were higher in TT, whereas in our study the difference was higher in IT. This could be because of insufficient cases of TT in our study, leading to insufficient sample size for statistical evaluation.

However from the present as well as other studies it is evident that the positivity rate with fluorescent stain was more as compared to modified Fite Faraco. Furthermore the higher positivity rates with FL stain were seen in cases with lower bacillary load while the difference evened out with LL and BL cases where the difference in the positivity rates are nil. This highlights the superiority of fluorescent stain especially in cases with lower bacillary load.

Bacillary index correlation of Ziehl-Neelsen and fluorescent method with Fite – faraco.

Overall both Ziehl Neelsen method and fluorescent method correlate significantly (P<0.0001 in both and r= 0.96 and 0.98 respectively). However when we look at groups with lower (<3) and higher BI (>3), fluorescent method retains good (r=0.73) and statistically significant correlation (P<0.0001) even at low bacillary loads; However Ziehl Neelsen method shows poor (r=-0.03) and insignificant correlation with Fite- Faraco method (p=0.81) with lower BI (<3). This is similar to the observation seen with different histopathological types, where fluorescent method retains useful sensitivity even in histopathological types with lower bacillary load.

Hence fluorescent stain has an added advantage of its usefulness in assessing bacterial index needed to categorise leprosy especially at lower bacillary load, apart from its higher case pickup rate. This can have implications in catergorising a case as paucibacillary v/s multibacillary, having treatment implications.



Also among paucibacillary cases, in particular, FL stain shows a higher BI compared to FF stain. Hence FL stain is more useful in detecting Hansen's bacilli among paucibacillary cases.

Added to this, with FL, there is a shift of paucibacillary to additional multibacillary cases that has implications in therapy and followup.

Hence fluorescent stain has an added advantage of its usefulness in assessing bacterial index needed to categorise leprosy especially at lower bacillary load, apart from its higher case pickup rate.

CONCLUSION

- Fluorescent microscopy has higher case pick-up rates when compared to Ziehl –Neelsen and modified Fite-faraco stains as evident by its higher sensitivity.
- Fluorescent microscopy is more reliable for bacterial indexing as compared to modified Fite-faraco and ZN stain especially in low bacillary load (BI <3+)which is very important for precise categorization of leprosy and hence treatment.
- Fluorescent microscopy can be used as a supplementary tool when tissue sections stained by modified Fite- faraco method fail to detect the bacilli in tissue sections or categorise as paucibacillary cases.
- The procedure is valuable in cases where negativity of sections is to be certified.

SUMMARY

Skin biopsies from 60 leprosy patients were received in the department of pathology, B.L.D.E.A University Shri B. M. Patil Medical College Hospital, Bijapur from August-2005 to July-2009.

Each case was evaluated for the presence of acid fast bacilli and bacterial index, after staining with H&E, Ziehl-Neelsen , modified Fite-faraco and auramine-rhodamine stains.

Maximum number of patients were in 3rd decade, least affected being those < 20 years. Males were affected more compared to females. Indeterminate leprosy was the most common histological type, and borderline borderline least common.

Positivity rate with fluorescent stain was 43.3%, whereas with ZN and FF were 26.7% and 31.7% respectively. Also the mean bacillary index with FF was 11.5 which was higher than that of ZN and FF. Both FL and ZN correlated significantly ($p<0.005$) with the standard FF. However, FL did so at BI<3 which ZN failed to. FL stain showed a higher bacillary index in a net of 10 cases as compared to FF, whereas ZN showed a lower BI in 7 cases. Staining by fluorescent method detected an additional multibacillary case which was categorized as paucibacillary by FF.

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.

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PROFORMA

NAME **:** IP No. :

AGE **:** Date :

ADDRESS : Ref. by :

Chief complaints :

History of present illness :

Past history :

Treatment history :

Physical examination :

General physical examination:

Systemic examination :

Local examination :

Number :

Size :

Colour :

Sensation :

Nerves :

Investigations :

Hb%

ESR

TC

DC

Clinical diagnosis :

Histopathological diagnosis by :

1. Haematoxylin and eosin :

2. Zeihl-Neelsen stain :

3.Modified Fite-Faraco stain :

4. Auramine rhodamine stain :

B.L.D.E.A's SHRI. B.M. PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTER, BIJAPUR-586103

RESEARCH INFORMED CONSENT FORM

TITLE OF THE PROJECT : A COMPARATIVE STUDY OF ZIEHL-NEELSEN & MODIFIED FITE-FARACO WITH AURAMINE RHODAMINE STAINING IN DETECTION OF MYCOBACTERIUM LEPRAE IN TISSUE SECTIONS.

PRINCIPAL INVESTIGATOR : DR. DEEPA ADIGA.S.A.
P.G.DEPARTMENT OF PATHOLOGY

P.G. GUIDE : DR.SUREKHA.B.HIPPARGI

PURPOSE OF RESEARCH :

I have been informed that this study is done to know the diagnostic utility of auramine rhodamine stain in clinically diagnosed cases of leprosy.

PROCEDURE:

I understand that, I will undergo detailed history and clinical examination after which skin biopsy will be taken and will be subjected to pathological study.

RISK AND DISCOMFORTS:

I understand that, there is no risk involved in the procedure performed.

BENEFITS:

I understand that my participation in the study will help to know the diagnosis of the lesion within a short time after skin biopsy.

CONFIDENTIALITY:

I understand that the medical information produced by the study will become a part of hospital record and will be subjected to confidentiality and privacy regulations of the hospital. If the data is used for publications the identity of patient will not be revealed.

REQUST FOR MORE INFORMATION:

I understand that my participation is voluntary and I may refuse to participate or withdraw from the study at any time.

INJURY STATEMENT:

I understand that in the unlikely event of injury to me during the study I will get medical treatment but no further compensations.

I have read and fully understood this consent form. Therefore I agree to participate in the present study.

_____ _____

Participant / Guardian Date:

_____ _____

Signature of Witness Date:

I have explained the patient the purpose of the study, the procedure required and possible risk and benefit to the best of my ability in the vernacular language.

_____ _____

Investigator / P.G. Date:

_____ _____

Witness to Signature Date:

STUDY SUBJECT CONSENT STATEMENT:

I confirm that Dr. Deepa Adiga S A, has explained to me the purpose of research, the study procedure, that I will undergo and the possible discomforts as well as benefits that I may experience in my own language. I have been explained in my own language and I understand the same. Therefore I agree to give consent to participate as subject in this research project.

_____ _____

(Participant) Date

_____ _____

(Witness to signature) **Date**

MASTER CHART

Sl.No	Biopsy No.	Age	Sex	Histopathological diagnosis	FF	BI	ZN	BI	FL	BI
1	1/08	65	M	BT	POSITIVE	2+	NEGATIVE	0	POSITIVE	2+
2	513/08	68	M	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+
3	266/08	47	M	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
4	149/08	50	F	HL	POSITIVE	6	NEGATIVE	6	POSITIVE	6
5	482/08	15	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
6	516/08	65	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
7	15/08	30	F	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
8	344/08	38	M	HL	POSITIVE	6+	POSITIVE	6+	POSITIVE	6+
9	542/08	20	M	LL	POSITIVE	5+	POSITIVE	5+	POSITIVE	5+
10	868/08	14	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
11	1083/07	20	M	TT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
12	1491/07	60	M	HL	POSITIVE	6+	POSITIVE	5+	POSITIVE	6+
13	1069/08	25	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
14	0606/07	25	F	LL	POSITIVE	5+	POSITIVE	5+	POSITIVE	5+
15	1407/07	45	M	BL	POSITIVE	4+	POSITIVE	3+	POSITIVE	4+
16	704/08	10	F	IL	POSITIVE	0	POSITIVE	2+	POSITIVE	2+
17	988/08	30	M	BL	POSITIVE	3+	NEGATIVE	0	POSITIVE	3+
18	497/08	35	M	HL	POSITIVE	6+	POSITIVE	6+	POSITIVE	6+
19	1380/08	30	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
20	1295/08	13	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
21	1369/08	21	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
22	1389/08	35	F	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+
23	1447/08	30	M	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
24	1524/08	40	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
25	1485/9	35	M	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
26	219/08	40	F	LL	POSITIVE	5+	POSITIVE	5+	POSITIVE	5+
27	1882/08	20	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
28	1853/08	24	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
29	1860/08	43	M	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+
30	1906/08	70	M	LL	POSITIVE	4+	POSITIVE	2+	POSITIVE	5+

31	1966/08	74	M	HL	POSITIVE	6+	POSITIVE	6+	POSITIVE	6+
32	1968/08	28	F	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+
33	1772/08	45	F	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
34	608/08	43	M	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
35	1010/08	30	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
36	1022/08	30	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
37	1048/09	45	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
38	2088/08	25	F	HL	POSITIVE	5+	POSITIVE	5+	POSITIVE	5+
39	2090/08	50	M	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
40	2050/08	10	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
41	57/09	9	F	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
42	72/09	52	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
43	141/09	30	F	BT	POSITIVE	1+	NEGATIVE	0	POSITIVE	1+
44	130/09	42	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
45	346/09	22	F	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
46	355/09	15	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
47	356/09	38	F	BT	POSITIVE	4+	POSITIVE	3+	POSITIVE	4+
48	357/09	40	F	BT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
49	409/09	47	M	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+
50	506/09	40	M	LL	POSITIVE	6+	POSITIVE	5+	POSITIVE	5+
51	495/09	50	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
52	471/09	50	F	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+
53	624/09	35	F	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
54	653/09	52	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
55	645/09	60	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
56	745/09	52	M	IL	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
57	521/09	65	M	LL	POSITIVE	4+	POSITIVE	4+	POSITIVE	5+
58	873/09	26	F	BT	POSITIVE	0	POSITIVE	0	POSITIVE	1+
59	958/09	36	F	TT	NEGATIVE	0	NEGATIVE	0	NEGATIVE	0
60	1562/09	25	F	IL	NEGATIVE	0	NEGATIVE	0	POSITIVE	1+

Key to Master Chart :M - Male, F - Female