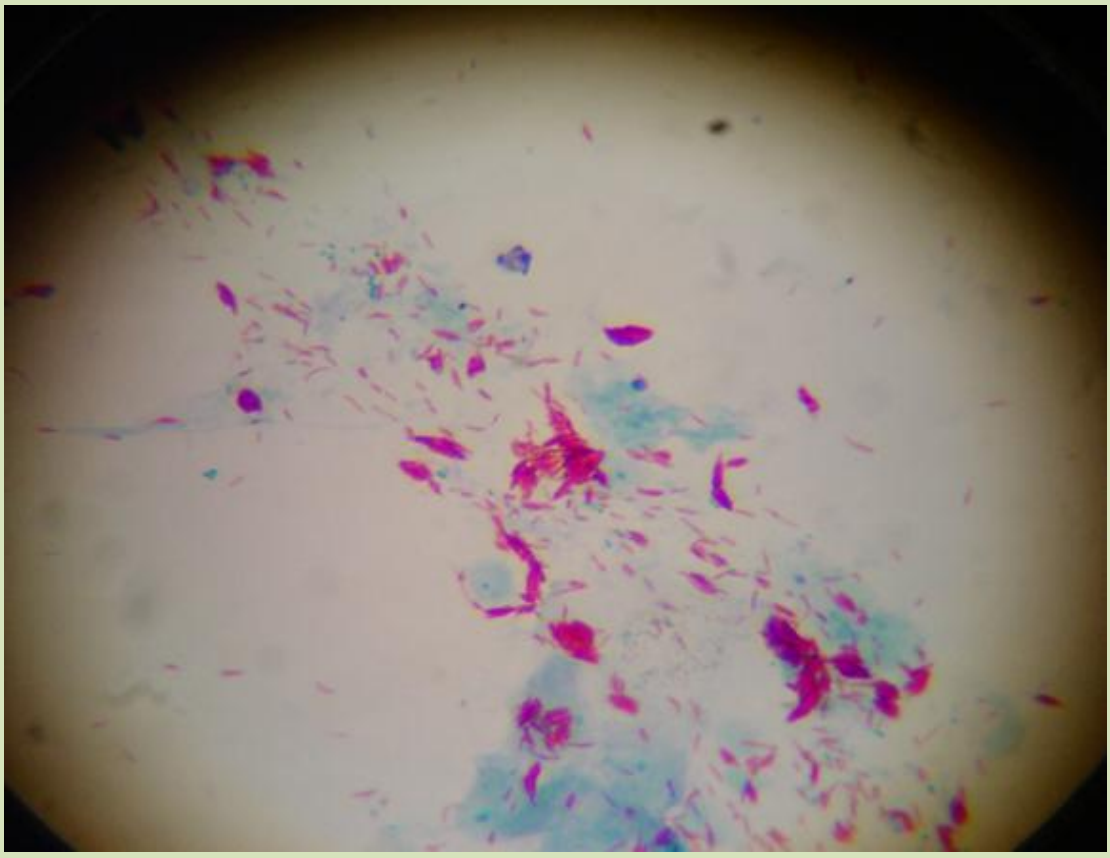
Module of Skin Smear Technique

for GHC Lab Technician



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Central Leprosy Teaching and Research Institute
Directorate General of Health Services
Ministry of Health & Family Welfare
Government of India
Tirumani, Chengalpattu, Tamil Nadu

Module for Skin Smear Technique for GHC Lab Technician

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Disclaimer

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Preface

It gives me immense pleasure and proud to publish Module for Skin Smear from premier Central Government Public Health Institution Central Leprosy Teaching and Research Institute engaged in service to the people affected with leprosy.

We all know that leprosy is a disabling disease, is still prevailing in the communities. Since there are no deaths and no epidemics, due to leprosy, it never comes in the limelight like other communicable diseases. We should be aware that the spread of leprosy is very slow, getting infection is difficult unlike many other infectious diseases and mycobacterium leprae, the causative agent of leprosy, is becoming weak genetically.

The leprosy can be completely cured with Multi Drug Therapy in 6 and 12 months which is available free of cost in all the Primary Health Centers, Dispensaries and Hospitals.

Our country contributes more than 50% of the leprosy cases detected globally. In 2013-14 India alone reported 1,26,0913 new cases, of which 5256 cases were with visible permanent disabilities. Every year about 5000 cases with disabilities are added to the community from which we can assess the cumulative figures and disabilities adjusted life years of disability burden due to leprosy.

We know that skin patch and loss of sensation is the main sign and symptom of leprosy. We have facilities to diagnose and treat cases of leprosy throughout India free of cost. This module designed to help the health professional about understanding and practicing skin smear techniques.

DIRECTOR
CLTRI

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Introduction

Leprosy is a chronic mycobacterial disease caused by the bacterium myco-bacterium leprae. It is a slow growing inter-cellular bacillus that infiltrates the skin, the peripheral nerves, the nasal mucosa and eye. The incubation period of leprosy is normally between two to ten years and may be as long as twenty years. The leprosy bacilli were discovered by Norwagian scientist, Armaeur Hansen in 1873. Hence, it is also called Hansen's disease. The bacilli measures about 3 to 5 micron in length and 0.2 to 0.5 micron in width and elongated forms can be seen sometime. Infection probably occurs when leprosy bacilli are discharged through the nose. Infection occurs when the bacilli enters through nasal mucosa or minor skin aberrations.

The main clinical features of leprosy are a variety of skin lesions and peripheral nerve trunk damage, which leads to anesthesia and paralysis causing deformities which are characteristic features of leprosy. Nerve damage occurs in untreated leprosy patients.

Classification

Previously leprosy is classified into Indeterminate leprosy (INDT), Tuberculoid Type (TT), Pure Neuritic leprosy (PN), Borderline leprosy (BT), Borderline Borderline (BB), Borderline Lepromatous (BL) & Lepromatous leprosy (LL). Now it is broadly classified into Paucibacillary (PB) and Multibacillary (MB).

Ridley's - Jopling Classification

Sl. No.	***Observations***	***TT***	***BT***	***BB-BL***	***LL***
1.	Number of skin lesions	Single usually	Single or few	Several or many	Many
2.	Size of Lesions	Variable	Variable	Variable	Small
3.	Surface of Lesions	Very dry, sometimes scaly	Dry	Shiny	Shiny
4.	Hair growth in (surface) lesions	Absent	Moderately diminished	Slightly diminished	Not affected
5.	AFB in nose blows or nasal scrappings	NIL	NIL	NIL (Scanty)	Many (globi)
6.	Lepromin Test	Strongly positive (+++)	Weakly +ve (+ or ++)	Negative	Negative

Practical Classification:

Sl. No.	***Observations***	***PB***	***MB***
1.	Number of skin lesions	1-5	>5
2.	Skin Smear	-ve	+ve

Skin Smear

A skin smear is a diagnostic & prognostic test in which a sample of Material is collected from a tiny cut in the skin and then stained for M. leprae. This test is done for:

1. To confirm clinical diagnosis of multi-bacillary leprosy in a suspect
2. To determine the state of infectivity
3. To help in diagnosis of relapse of leprosy
4. For classification and prognosis
5. To get negative certificate

Taking skin-smear is an invasive procedure and requires all aseptic precautions. Wash your hands, wear gloves and use sterilized equipment and a new blade for each patient.

Procedure

Materials Required:

1.Spirit Lamp

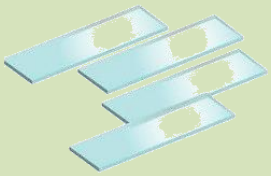
2. Tincture Benzoin

3. Scalpel

4. Sterile New surgical blade (No.15)

5. Match box

6. New glass slide (75mm X 25mm X 1.0cm)



7. Diamond Pencil

8. Sterile Cotton Balls

9. *Sterile Swab sticks*

10.Microscope

B. Selection of site

1. *Both ear lobes*
2. *Both arms*
3. *Both thighs*
4. *One Lesion - Recently Active*

C. Preparation of Slide

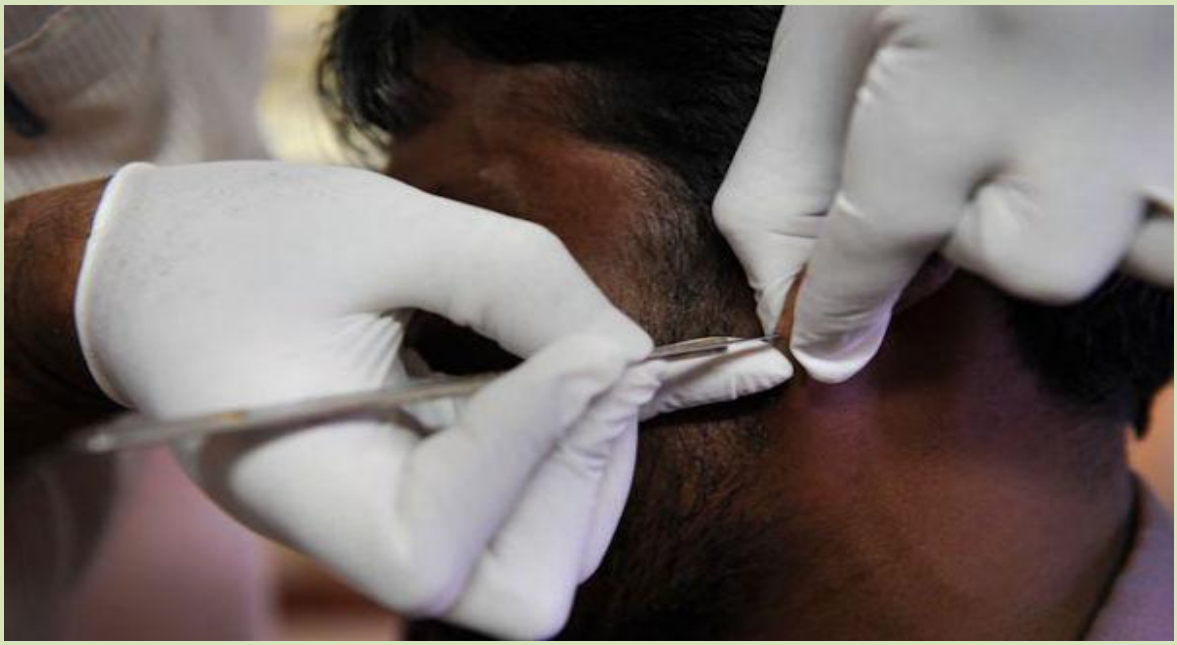
- *On a new slide, using a Diamond pencil write*
  Top - Lab No
  Bottom - Name of the patient and date
- *Gently warm the slide. After warming, clean with whatman filter paper No.3. Do not use cotton.*
- *Insert a No.15 surgical sterile blade in scalpel using a Artery forceps*
- *Do not touch the blade.*

D. Technique:

- *Thoroughly clean the selected site with Spirit or Ether to remove dirt and any saprophytes.*
- *Hold the skin pinched up and raise between the thumb and index finger of the left hand. This will squeeze out blood from the part and minimize bleeding when cut is made. Due to*

thickness of the skin, it is difficult to pinch up the skin, exert lateral compression with the two fingers stretching the bit of skin in between them.

- *With the point of sterile scalpel make an incision of 5mm long and 2-3mm deep; scrape the bottom and the sides of the slit to obtain sufficient material for a smear.*



- *Transfer the material from the point of scalpel to a clean new glass slide and a uniform and moderately thin smear of average 5-7mm diameter.*
- *Press the cut surface of the skin with a piece of cotton wool to stop bleeding and seal the part with tincture benzene etc.*
- *Allow the smear to air dry, fix it by passing the slide twice or thrice over the flame, the heat should be bearable to the skin. Do not over heat, which results in charring or cracking of smear. Too little heat may not fix the smear efficiently and it may wash out.*

Characteristics of Good Smear

- *Should be approximately 8-10 mm in diameter*
- *Should be uniform - should not be too thick in one area and too thin in other area. Rough estimate of acceptable thickness is that a print letter of hand on watch should be seen through, 80% of the area of the smear should be 80% transparent.*
- *The point where the scalpel first touched the side will always be less transparent than the other areas.*

Staining Technique

1. *Place the slides on a pair of staining rods over a sink or a basin after making sure that the rods are horizontal. 6-8 slides may be stained together at one time.*
2. *Fold a filter paper into the shape of a funnel and filter carbol fuchsin on to the smears*
3. *Heat gently with the flame of a spirit lamp or candle, until steam begins to rise.* ***DO NOT HEAT TO BOILING****. Keep flooding the slides with carbol fuchsin and gently warm thrice in a period of 5 minutes.* ***DO NOT ALLOW THE STAIN TO DRY****. This causes the deposition of stain particles on the smear, making microscopic examination difficult.*

4. *Wash gently in running tap water. If a tap is available, attach rubber tubing to the tap and open the tap gently. Use a mug if no tap is available, but pour water gently.*

5. *Cover the slides with decolourising agent – 5% sulphuric acid. Let it stand for 10 minutes. (If using acid-alcohol as decolourising agent, let it stand for 5-10 seconds only).*

6. *Wash gently with running tap water*

7. *Cover the slides with methylene blue solution for 1 minute.*

8. *Wash gently with running tap water.*

9. *Wipe the under surface of the slides clean with cotton dipped in acid alcohol in order to remove any dried up stain deposits or soot.*

10. *Stand the slides on the blotting paper of the slide rack to drain off water drops.*

11. *Wait till the smears are dry.*

The slides are now ready for examination

The lepra bacilli will be seen as pink rods. The bacilli may be seen in single, small groups or closely packed bunches called globi. Scattered among the bacilli, irregular blue stained structure are seen, they are the cells of various structures in dermis. The smears are graded for the purpose of BI (Bacterial Index) and MI (Morphological Index).

Bacterial Index (BI) – It is the average index of all the sites divided by number of sites

There are two methods commonly employed for this purpose:

I. *Dharmendra's method*

II. *Ridley's method*

Examination of Stained Slide

1. Put the slide on microscope stage

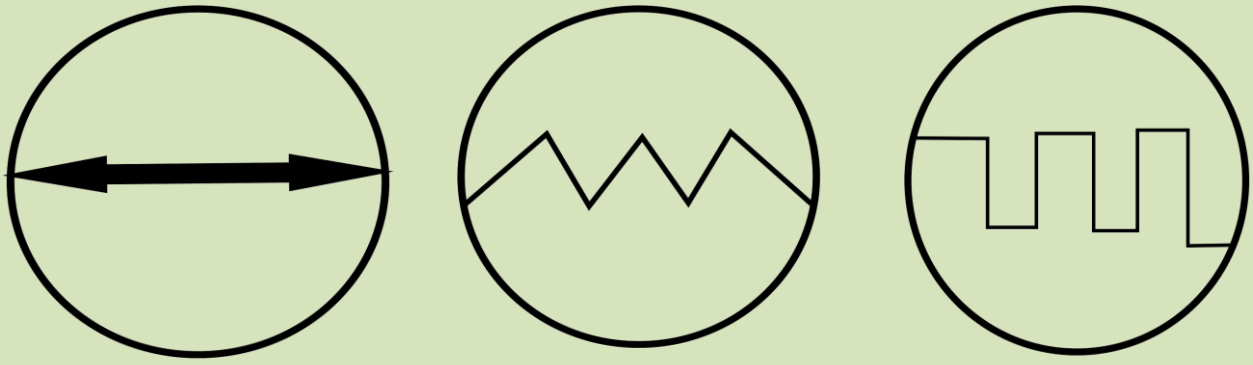
2. Focus the smear under 10x and observe uniformity/concentration of material. Select area which is not too thin or too thick.

3. Put oil on the smear and rotate objective to bring 100x in touch with oil.

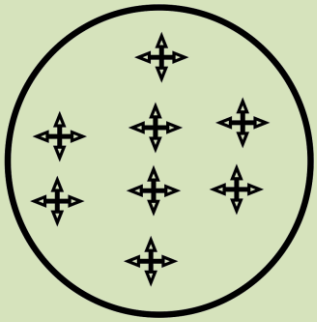
4. Focus using fine adjustment

5. Start examining smear

5 a. Smear can be examined in different ways



Examine smear using any one style of the above. After about 80 fields, it is beneficial to select randomly i.e.



Always ensure that you have examined the center of the smear. Never allow stain to dry on smear.

Precautions

- Avoid very fast movements of microscope stage.
- Avoid going to edge of the slide when using oil immersion objective.
- Clean the microscope stage and objectives before and after use.

Ridley's Method

1+ - *1-10 Bacilli an average in 100 microscopic fields*

2+ - *1-10 Bacilli an average in 10 microscopic fields*

3+ - *1-10 Bacilli an average in 1 microscopic field*

4+ - *10-100 Bacilli in an average microscopic field*

5+ - *100-1000 Bacilli in an average microscopic field*

6+ - *> 1000 Bacilli in an average microscopic field / innumerable bacilli*

ZIEHL - NEELSION'S
Acid fast method

ZIEHL - NEELSION'S acid fast method is a Technique for staining smears.

Equipment for the preparation of Stains

The equipment listed below is for a patient load of about 100 per week.

(a) Glassware

(b) Other equipment

(a) Glassware

Item	Capacity	Number
Conical flasks	1 L	**4**
Stock solution bottles with screw caps	1L	**4**
Dropper bottles	150-200 ml	**4**
Funnels	Large/Medium	**2**
Pipettes (for acid)	10ml	**2**
Stock solution bottle for distilled water	3-5 L	**1**
Beaker	50ml	**2**
Measuring cylinders		
for acid, phenol and rectified spirit	50ml	**2**
for preparing stock solutions	500ml	**4**
Glass rods		
for staining	to fit the mouth of the sink	**2**
for stirring		**4**

(b) Other Equipment:

Item	Capacity	Number
Common Balance	Upto 1 gm. Sensitivity	**1**
1 Hot plate	-	**1**
Filter paper	(available in sheets)	**2 or 3 boxes**
Spirit lamp/candles	-	**As needed**
Timer	-	**1**
Box of Matches	-	**As needed**

Stains

The reagents needed for the preparation of stains are:

- Basic Fuchsin
- Phenol
- 95% Ethyl alcohol
- Distilled water
- Concentrated sulphuric acid
- Concentrated hydrochloric acid
- Rectified spirit
- Methylene blue

WATER FOR PREPARATION OF STAINS

Use distilled water

If not available, use boiled, cooled and filtered water: If filter paper is not available, a doublefold of cloth freshly boiled, separately, may be used as a filter.

DO NOT USE TAP WATER

Three solutions have to be prepared for staining by the acid fast method

1. **The Primary stain***: 1% carbol fuchsin*
2. **The decolourising agent**: *3% acid alcohol solution*
3. **The counter-stain**: *1% Methylene blue*

PRIMARY STAIN

(1% CARBOL FUCHSIN: 1 LITRE)

Things Needed

a. Basic fuchsin

Weigh 10gms of Basic fuchsin in the balance

b. Melted Phenol

Place a few crystals of phenol in a small beaker. Place the beaker in a container with water and heat it on a Hot plate until the phenol melts. Measure out 50ml.

c. 95% Ethyl alcohol

Measure 100ml. of 95% alcohol

d. Distilled water

Measure 850 ml of distilled water.

e. Glass Rod, Conical flask , Funnel & Filter Paper

PROCEDURE:

1. Place basic fuchsin in a large conical flask
2. Add alcohol
3. Shake or stir with a glass rod to mix well
4. Add distilled water and shake or stir until all basic fuchsin is dissolved.
5. Add the remaining water and Mix well

6. Add phenol and stir to mix
7. Filter using a funnel with a filter paper placed in it. Place the funnel in a conical flask.
8. Collect the filtrate
9. Store in a screw-capped labeled bottle

DECOLOURISING AGENT:

(3% acid alcohol solution)

Concentrated Hydrochloric Acid – 3ml

70% alcohol - 97ml

COUNTER-STAIN

(1% Methylene blue)

a. Methylene blue - 10 gms.
b. Distilled water - 1000ml.

PROCEDURE:

1. Add methylene blue to distilled water and stir until dissolved.
2. Filter using a funnel with a filter paper placed in it. Place the funnel in a conical flask.
3. Store in a screw-capped labeled bottle
4. Pour into labeled dropper bottles and use as needed

All stock solutions may be stored for approximately one month. Wash and dry stock solution bottles once a month.

Morphological Index

Lepra bacilli when stained by Ziehl-Neelson's method take pink colour (stain). But most of the bacilli are stained irregularly and only a few are uniformly stained and the uniformly, intensely stained bacilli are viable and capable of multiplying and that all the other forms are dead or dying and are incapable of multiplying. The percentages of solid bacilli are called M.I.

Method:-

In a smear stained by Ziehl-Neelson's method for AFB 100 individually recognizable bacilli should be identified as showing uniform or irregular staining. Normally no difficulty will be experienced in dividing whether a bacillus in clumps and globi are not included. Four criteria given below are strictly fulfilled the label a bacillus as solid.

1. The whole length of the bacillus in stained uniformly and densely
2. The sides of the bacillus are parallel
3. The ends of the bacillus may be rounded, straight or pointed
4. The bacillus is at least four times as long as its width.

The changes in the morphology of human leprosy bacilli serve as useful indicators of progress during treatment. For example under the influence of a potent anti leprosy drug the bacilli lose their ability to stain intensely and uniformly with carbol fuchsion and show such features as irregular staining, beading and breaking up into fragments etc. Organisms which reveal such

characteristics in their morphology are considered to be non-viable at least in their ability to propagate in another living host.

Further, such morphological alterations occur in much shorter time than the reduction in number of organisms so that these changes are apparent much before the B.I. begins to fall. Therefore, we have in the MI a useful measure, which enables us to assess the suitability of any therapeutic regimens against leprosy in shorter period.

BI Depends Upon With:

1. Depth of the scrape
2. Amount of tissue fluid removed
3. Size and thickness of the smear etc.

M.I Depends Upon:

The availability of sufficient free stained bacilli.

NASAL SMEAR

METHOD:

A part of the lining of the nose is gently scraped and the fluid obtained is smeared on a microscopic slide. This is stained by Ziehl-Neelson method and examined under the microscope. The nasal smear is painful, therefore, the nasal mucosa has to be collected with care.

TECHNIQUE:

1. Seat the patient on a low stool
2. A sterile cotton swab stick dipped in normal saline inserted gently into the nostril
3. Scrape over the inferior turbinate bone
4. Smear the material on Microscopic glass slide (Oval shape)
5. Allow the slide to dry at room temperature
6. Fix the slide by gentle heat method
7. Stain the slide by Ziehl-Neelson cold method
8. Allow it dry at room temperature
9. Examine under 100x in a microscope

REPORT:

Positive / Negative

CENTRAL LEPROSY TEACHING AND RESEARCH INSTITUTE

CHENGALPATTU

DIVISION OF LABORATORIES

Laboratory Requisition Form

CLINICAL PATHOLOGY & SKIN SMEAR

Patient's Name : Age:

Hosp. No. Referred by Dr.

CLINICAL PATHOLOGY:

- ☐ Routine Urine Examination
- ☐ Routine Stool Examination

SKIN SMEAR:

- ☐ BI of Routine Sites
- ☐ MI
- ☐ Other Sites

(a) _____

(b) _____

(c) _____

(d) _____

Date: Signature of Medical Officer

Central Leprosy Teaching and Research Institute

CHENGALPATTU, Pin code 603 001,TAMILNADU,India
Laboratory Division, Clinical Pathology Lab

Skin Smear examination study report for Leprosy By (Direct Microscopy)

Method used: Wade's slit-scrape method employing *Ziehl–Neelsen AFB** staining technique
Grading method used: *Ridley's Logarithmic Scale grading**

Name of the Patient: Age/Sex OPD/ IP/ Hospital No:
Lab No Ward
Date of Investigation Referred by: Dr.
Type of Leprosy Duration of the disease

Skin Smear Site of examination		Results of Skin Smear sites (Direct Microscopy)			
			BI* ↓		MI* in % ↓
1.	Right Ear lobe	Result		MI in %	
2.	Left Ear Lobe	Result		MI in %	
3.	Right Arm	Result		MI in %	
4.	Left Arm	Result		MI in %	
5.	Right Thigh	Result		MI in %	
6.	Left Thigh	Result		MI in %	
Other Site:1	Fore head	Result		MI in %	
Other Site:2		Result		MI in %	
Other Site:3		Result		MI in %	
Other Site:4		Result		MI in %	
Average BI*/MI* from smear (site) →		BI =		MI % =	

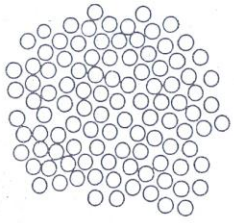
Ridley's logarithmic scale grading of AFB: Acid Fast Bacilli (M.leprae) in skin smear
Negative: No bacteria seen in100 oil-immersion field of microscope: When BI is 0.00 (i.e Negative), the %MI is NA (Not Applicable)
1+= 1-10 bacilli seen on an average ,in 100 oil- immersion field of microscope
2+ =1-10 bacilli on an average ,in 10 oil-immersion field of microscope
3+=1-10 bacilli seen on an average in each oil- immersion field of microscope
4+=10-100 bacilli seen on an average, in each oil- immersion field
5+=100-1000 bacilli seen on an average, in each oil- immersion field,
6+= More than 1000 bacilli seen on an average, in each oil- immersion field
*BI= Bacteriological Index,*MI: is the % score of Morphologically intact (uniform rod shaped) acid fast bacilli : *M.leprae*

Signature of the Lab Staff

Signature of the Officer in charge
(Laboratory Division)

LEPROSY SMEARS: BACTERIOLOGICAL INDEX - 'BI' (Ziehl-Neelsen stain)

BI = 0
No bacilli in 100 oil immersion fields

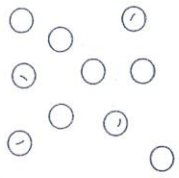


Examine 100 oil immersion fields

BI = 1
1-10 bacilli, on average, in 100 oil immersion fields

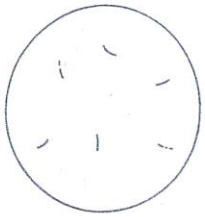
Examine 100 oil immersion fields

BI = 2
1-10 bacilli, on average, in 10 oil immersion fields



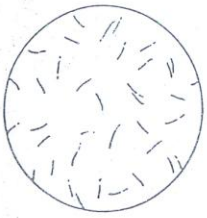
Examine 100 oil immersion fields

BI = 3
1-10 bacilli in an average oil immersion field



Examine 25 oil immersion fields

BI = 4
10-100 bacilli in an average oil immersion field



Examine 25 oil immersion fields

BI = 5
100-1000 bacilli in an average oil immersion field

Examine 25 oil immersion fields

BI = 6
1000 or more bacilli in an average oil immersion field

Examine 25 oil immersion fields

Original reference to the Bacterial (or Bacteriological) Index: Ridley DS, Bacterial Indices. In Cochrane, RG and Davey, TF (Editors), 'Leprosy in Theory and Practice' Bristol; John Wright and Sons Ltd, 1964, pages 620-22.

Details of taking, fixing, staining, and reading smears, including the BI, are given in 'Technical Guide for Smear Examination for Leprosy by Direct Microscopy' by Leiker, DL, and McDougall, AC. Published by Leprosy Documentation Service (INFOLEP), Wibautstraat 135, 1097 DN, Amsterdam, Netherlands.