Introduction

PRINCIPLE

Estimation of the number of acid fast bacteria, together with the recording of the morphological and tinctorial appearance of the bacilli are essential requirements in the assessment of the type of the infection, the severity of the infection and the response of the infection to treatment.

SPECIMEN

The skin smear is a method that permits such estimation, but it is to be used only as a supplement to a biopsy, which is required for a definitive diagnosis and classification of the type of leprosy.

SAFETY

Leprosy is not highly contagious however the samples should be treated as biohazards.

NOTES

1. Skin smears are taken from appropriate locations on the body as designated by the patient's physician.

2. Smears are routinely taken from both earlobes of newly admitted patients.

3. To give a more reliable index of progress, repeat smears are obtains from sites previously tested.

4. Time intervals between repeat smears are determined by the physician. Suggested minimal intervals:
   (a) for active patients: every 3-6 months
   (b) for inactive patients: yearly
   (c) for discharge: as required by the laws of local government.

5. All microscopic slides on which skin smears are made should be pre-cleaned in 70% alcohol, acetone or alcohol-acetone to remove amorphous debris found on such slides. The slides are wiped dry with a clean hand towel. Blades used in smear-taking are likewise cleaned.

Procedure for Obtaining Smears

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. The patient is seated with his back to the technician to minimize his reaction to the procedure.

2. The skin area is cleaned with 70% alcohol, after which it is wiped dry with cotton. Zepharin tends to make the skin too slippery.

3. A fold of skin is made relatively avascular by pinching. If the skin cannot be grasped by pinching, it is instead compressed. A glove worn on that hand may aid in grasping.

4. The smear location is tested for anesthesia with a sharp pin. If there is inadequate decrease in sensation, local anesthesia with 1T Xylocaine or ethyl chloride spray be carefully applied. The compression of the skin by pinching aids in the anesthesia.

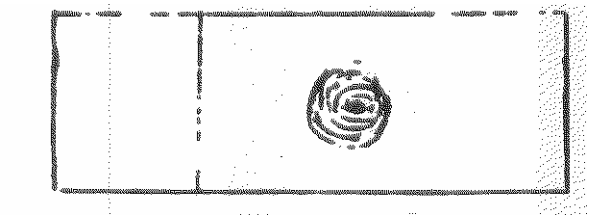
5. An incision 5 mm long and 4 mm deep is made with an alcohol-cleansed single-edge razor blade. (A scalpel may also be used.) Pressure is continuously applied to the area until an adequate smear has been taken.

6. If blood exudes, it is wiped away. Blood must be excluded to obtain a satisfactory representative smear. The hand of the operator which does the scraping contains a swab of sterile cotton in addition to the blade (see illustration below). This permits that hand to wipe away any blood, allowing the other hand to maintain relative avascularity.

Cotton is held between the third and fourth fingers and the blade between the thumb and second finger of the same hand. This hand is thus able to both scrape the area and wipe away any blood while the other hand maintains constant pressure and relative avascularity at the site.

7. The edges of the wound are scraped with the blade held at a right angle to the incision. Upon scraping, tissue juice and dermal tissue are obtained.

8. The material is transferred to a clean microscopic slide. A moderately thick smear, without gross blood, with a visible uniform opacity is made. The smear is made in a circular manner (see illustration below) on the slide encompassing an area of some 5-6 mm.



The smear is made in a circular manner on the slide ending in the center and leaving a central button which can be easily focused upon with the microscope.

9. The wound is treated and the edges are opposed to obtain minimal scarring.

10. A single technician takes all smears to provide for more uniform and meaningful results.

11. The smears are then fixed and stained. This is done on the same day that the smears are obtained (see method below).

12. The stained smears are examined under a quality microscope. Numbers and morphology of bacilli are reported per examination under the oil immersion objective. Koehler light illumination and best obtainable lens quality are essential to definitive examination. A single trained technician reads all smears.

Staining of Skin Smears

REAGENTS

10% formalin, carbol-fuchsin, acid-alcohol, alkaline methylene blue (see next page)

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Dry the slide with smear in air at room temperature or in an incubator.

2. Place slides in 10% formalin for 15 minutes for fixation.

3. Rinse in tap water.

4. Stain in Ziehl-Neelsen carbol-fuchsin for 20 minutes at 25□C, flooding the dye on the slide.

5. Wash off excess dye in tap water.

6. Rinse in acid-alcohol for one minute for decolouration.

7. Rinse slides thoroughly in tap water.

8. Counterstain with alkaline methylene blue for 10 seconds, rinse in tap water and dry in air. For permanent preparation, mount under coverslip in "Permount".

Z-N Carbol Fuchsin

Basic fuchsin	1.0 g
Phenol crystals	5.0 g
95% alcohol	10.0 cc
Water, to make	100.0 cc

Label as "Z-N Carbol Fuchsin" and date.

Acid Alcohol

Hydrochloric acid, concentrated	2.0 cc
95% alcohol, to make	100.0 cc

Label as "Acid Alcohol" and date.

Alkaline Methylene Blue

NaOH 10%	0.06 cc
Methylene Blue	0.35 g
95% alcohol	16.00 cc
Water, to make	100.00 cc

Label "Alkaline Methylene Blue" and date.

Microscopic Examination of Skin Smears

PRINCIPLE

The stained smears are examined under a quality microscope. Total numbers and morphology of bacilli are reported per examination under the oil immersion objective. Koehler illumination and best obtainable lens quality are essential for definitive examination. A single technician reads all smears. The smear will generally have similar numbers of bacilli throughout. However, four separate quadrants of the smear are examined for total numbers of bacteria (bacterial index). The morphology of the bacteria in each of the four quadrants is also noted. Results are averaged and reported.

Reporting of Total Numbers of Bacteria (Bacterial Index, BI)

Reporting is done using either a descriptive phrase or a numerical code on a semi-logarithmic scale.

Very numerous	-	over 1000 bacilli per oil immersion field
Numerous (5 plus)	-	100-1000 bacilli per oil immersion field
Moderate (4 plus)	-	10-100 bacilli per oil immersion field
Few (3 plus)	-	1-10 bacilli per oil immersion field
Very few (2 plus)	-	from 10-100 bacilli per entire slide (100 fields)
Rare (1 plus)	-	from 1-10 bacilli per entire slide

Reporting of Bacterial Morphology (Morphological Index, MI)

PRINCIPLE

Mycobacterium leprae is a rod-shaped bacterium usually having a length of 1-7 microns and a width of 0.3-0.5 microns. It is said to be **solid staining** only when there is dense uniform staining of the entire bacillus with even sides and rounded ends; the length of the solid bacillus is at least five times the diameter (width) of the bacillus. Only bacilli visualized in their entirety are counted as regards morphology; bacilli which overlap are counted for the bacterial index only.

A **non-solid** staining bacillus shows one or more of the following features: uniform dense staining short form (with rounded or fragmented ends) less than five times as long as in width, failure to stain uniformly, faint staining, the presence of beading and/or fragmentation. Odd shaped forms are considered non-solids.

Morphological Index is reported as the number of solid forms per 100 total bacilli examined. When there are less than 100 total bacilli per smear, they are reported as solid forms per total number of bacilli found. As with the bacterial index, all four quadrants of the smear are examined for the morphology of the bacilli. Results are averaged and reported.

A few bacillary forms are illustrated below.

A solid staining bacillus
B non-solid, at end of bacillus
C non-solid, at side of bacillus

D-E morphology cannot be determined due to overlap - counted only in regard to bacterial index
F shorter solid form than A
G short form - too short to be counted as solid - length is not 5x or more width
H fragmented bacillus (non-solid)
I beaded bacillus (non-solid)
J club form (non-solid)

Tissue Fixation

REAGENTS

Neutral Buffered Formaldehyde Solution (pH 7.0)

PROCEDURE

It is recommended that specimens be fixed in neutral buffered formalin. This solution lends itself to good fixation and use in different climates, as well as use in shipping tissue long distances. Formalin "neutralized" with calcium or magnesium carbonate added to excess becomes acid readily as tissue is fixed and is not recommended.

Neutral Buffered Formaldehyde Solution (pH 7.0)

37-40% Formaldehyde solution	100.0 ml
Distilled water	900.0 ml
Acid sodium phosphate-monohydrate ($NaH_2PO_4 \cdot H_2O$)	4.0 mg
Anhydrous disodium phosphate (Na_2HPO_4)	6.5 g

Label as "Neutral Buffered Formaldehyde Solution (pH 7.0)"

Staining Procedure for the Leprosy Bacillus Tissues
As used at the U.S.P.H.S. Hospital, Carville, LA

MATERIALS

Xylene	2 parts
Peanut or mineral oil	1 part
1% alcohol:	
Hydrochloric acid, concentrated	1.0 cc
Alcohol 70%	99.0 cc
Acid, carbolic, melted crystals	2.5 cc
Alcohol, absolute	5.0 cc
Basic fuchsin	0.5 g
Distilled water, to make	50.0 cc

Keeps well at room temperature.

Working Methylene Blue Solution:

Methylene blue	0.5 g
Glacial acetic acid	0.5 cc
Tap water	100.0 cc

PROCEDURE

This is considered to be a non-routine procedure therefore it should only be performed by experienced personnel.

1. Deparaffinize sections with Xylene-peanut oil or Xylene-mineral oil solution - two changes, six minutes each.

2. Rinse with running tap water for one minute. Then drain slides for some 45 seconds. **Do not remove all of the oil.** There should be a thin film of oil on the slide at all times during the procedure. The oil hastens acid-fast staining without the use of heat. Do not blot slides.

3. Stain with carbol fuchsin for 20 minutes at **room temperature**.

4. Wash in running tap water for 3 minutes.

5. Decolorize in 1% acid alcohol until the slide is faint pink. This takes approximately one minute for most slides and slightly longer for larger sections. The acid alcohol should be changed regularly and never used when it is more than a pink color. This is the most critical step.

6. Wash in tap water for 5 minutes.

7. Counterstain with methylene blue for 1-2 minutes according to desired background.

8. Wash in tap water for 20 seconds.

9. Allow to air dry. After the slide has air dried thoroughly, wipe excess stain with oil off the slide with a clean cloth dampened with water. Do not blot or wipe tissue section.

10. Mount in permount. This slide should be mounted in permount as soon as possible and never allowed to remain open to the air overnight.