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Agenda item 9

MOUSE FOOT PAD TECHNIQUE FOR VERIFICATION OF RESISTANCE TO MYCOBACTERIUM LEPRAE

by

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Introduction

The objectives and possibilities of bacteriological examination (BE) in leprosy are :

(a) To offer support to the clinical diagnosis of the disease : in paucibacillary (PB) leprosy the BE is negative or feebly positive, in untreated multibacillary (MB) forms of the disease, the BE is strongly positive.

(b) To make the distinction between PB and MB forms of the disease, since this has now important therapeutic implications, drug regimens for the two types of disease being different. The breaking point is a bacterial index (BI) of 2 or more at any site examined.

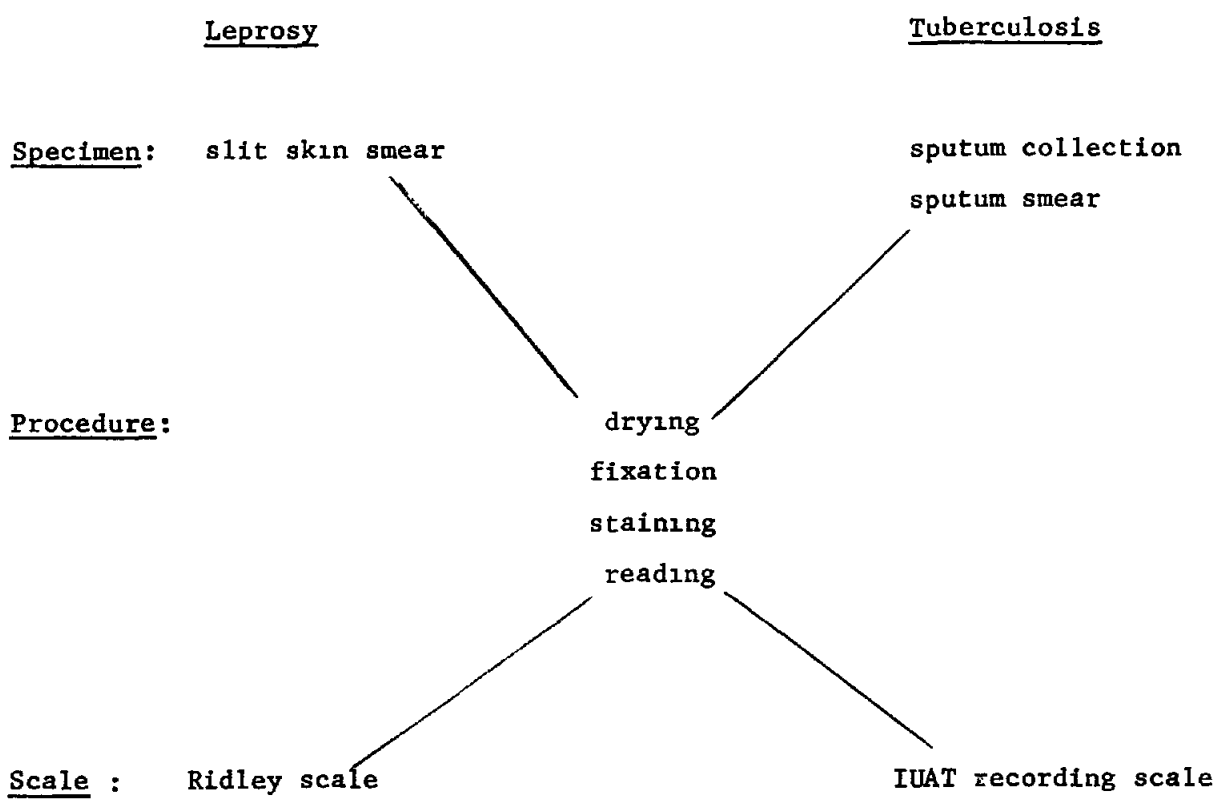
(c) To assist in the diagnosis of relapse or deterioration.

In the past the BE in leprosy has been largely neglected. This was somehow acceptable as long as therapy was based on a single drug to be administered to all patients, irrespective of the form of their disease. This has now changed and therefore the BE has become an indispensable part in leprosy control.

We have summarized in Table 1 the gradations of the different procedures for the diagnosis of leprosy. It appears that most information is obtained from the minimal investment in simple microscopy of slit-skin smears. Furthermore, the same staining method is applied in the diagnosis of tuberculosis. The latter constituting generally a more urgent health problem, it is all the more important that paramedical personnel be thoroughly trained to perform this technical procedure correctly.

The steps involved are shown in Table 2.

Table 2 : Steps in bacteriologic examination for leprosy and tuberculosis



Training should comprise the following headings :

(a) Fundamentals on the Ziehl-Neelsen (ZN) staining procedure.

(b) Collection and preparation of specimens, necessary material.

(c) Preparation of reagents, necessary material.

(d) Application of ZN technique.

(e) Reading.

(f) Interpretation - Action to be taken.

1. Fundamentals

The ZN procedure is a complex, regressive staining method comprising three essential steps :

(a) Overstaining with basic fuchsin. This is achieved by using carbol fuchsin containing phenol and applying heat or keeping the staining solution on the slide for a longer time at room temperature. Heating of the slides presents practical difficulties : it comsumes energy and frequently the slides are overheated, fried. Therefore we advocate the cold staining method, leaving the stain for 30 minutes.

(b) Decoloration (= regressive step) with either acidified alcohol or acid in water, all material, except Mycobacteria (in this case M. leprae or M. tuberculosis) loosing the red fuchsin stain. At this moment, leprosy bacilli in the preparation are stained red on a colourless background.

(c) Counterstaining of the background with methylene blue.

With these points in mind, one can understand why the composition of the staining solutions is as they are, and that many types of acids can be used as decolorizers : hydrochloric acid, sulfuric acid, nitric acid.

It is clear however that within an area and preferably a country, all procedures should be standardized.

2. Collection and Preparation of Specimens

The making of skin slit smears is clearly described in the THELEP Standard Protocol on the experimental treatment of multibacillary leprosy and should be thoroughly practised. At least three smears should be made :

- from one earlobe and two lesions.

If there is only one lesion :

- from two earlobes and one lesion.

More smears may be made, but this is a minimum. It is important that within a service, a region, a country, the smear-taking is standardized; if more smears are made, they should be done everywhere and always in the same way.

We do not advocate routine nose smears because the only way to perform them with minimum harm to the patient and maximum standardization is by examination of nose blow (See THELEP Standard Protocol).

We strongly advocate to mark the slides with a diamond pencil. This is a relatively expensive piece of equipment, but lasts for many decades. Personnel should be responsible for taking care of it.

Smears should be made from dermal rather than epidermal material and should neither be too thick nor too thin. Thick smears will produce cracks during drying or fixation; these will stain red, and may resist decoloration, less experienced personnel interpreting them as acid fast bacteria. Smears too thin may give false negative results and are difficult to read because it becomes difficult to focus the microscope correctly.

Smears should be dried for 15 to 30 minutes and fixed. Fixation can be done by passing slides through a flame, again with the danger of overheating or burning. It is preferable to fix the smears in formol vapours : 2 ml concentrated formaldehyde is introduced in a slide box, and the slides left for 15 minutes.

Preparation of Reagents

Each Health Centre should be equipped with the following material :

Table 3 : Material necessary for the preparation of staining reagents

	No.
- fixation containers each taking 5 slides	4
- 60 - 100 ml plastic staining bottles with pipettes (plastic)	3
- 1/4 L. bottle (plastic)	1
- 1 L. bottle (plastic)	4
- measuring cylinder 100 ml	1
- rinsing bottle	1
- plastic 10 ml pipet + bulb	1
- funnel (plastic)	2
- new fuchsin	25 g
- methylene blue	25 g
- phenol crystals	100 g
- HCl	1 L.

Table 3 (Cont'd)

	No.
- denaturated alcohol 1 L. bottles	10
- pincet = 18 cm long	1
- formol 40 %	1 L.
- instructions	

Instructions can read as follows. The advantage of the proposed technique is that no balance is necessary.

1 Stock fuchsin solution

Transfer contents of new fuchsin recipient into a 1 L. bottle, fill with denaturated alcohol. Agitate. Use next day. Refill bottle as long as there is a deposit.

2 5 % Phenol stock solution

Fill a 1 L. flask with water. With measuring cylinder take out 50 ml and pour away. Melt the phenol crystals in a water bath. With measuring cylinder add 50 ml of molten phenol to the water. Mix well. With measuring cylinder, take out 100 ml and pour away or store elsewhere.

3 Acid alcohol

Fill 1/4 L. bottle with denaturated alcohol. With pipet and bulb transfer 12 ml hydrochloric acid into the alcohol.

4 Stock methylene blue solution

Transfer contents of methylene blue powder recipient into a 1 L. bottle, fill up with denaturated alcohol. Agitate. Use next day. Refill bottle as long as there is a deposit.

(5) Working carbol fuchsin solution

Put filter paper in funnel. Through it, fill the 900 ml containing 5 % phenol solution flask (no. 2) with stock fuchsin solution (no. 1). Stopper flask. Mix. Rinse funnel. Using the filter paper containing funnel fill a staining bottle with working fuchsin solution. Rinse funnel.

(6) Working decolorizer

Using the funnel fill a second staining bottle with acid alcohol from flask no. 3. Rinse funnel with water.

(7) Working methylene blue solution

Fill 1 L. flask with water. Using the measuring cylinder take out 200 ml. Put filter paper in funnel. Through it fill the flask with methylene blue stock solution (no. 4). Rinse funnel. Using funnel with filter paper fill the third staining bottle with working methylene blue solution.

Put tape label on each flask. Indicate contents with black pencil or ballpoint.

Instructions should be put under some protective plastic.

Quality control evaluation should entail : inspection for the presence of a deposit in the stock solutions of basic fuchsin and methylene blue, readable labels, presence of material (pipet, bulb, measuring solution,diamond pencil), availability of instructions, general cleanliness, if possible preparation of stock or working solutions.

4. Application of Z.N. Technique

A staining rack may be made of any preferably rectangular recipient with approximate measures: length : 40-50 cm, width : 30-40 cm; height : 15 cm and up, supporting 2 or 4 metal or wooden sticks on which the slides may be deposited. Such material is locally available everywhere.

Cover smear with working carbol fuchsin solution. The use of staining bottles provided with pipettes and bulbs, containing pre-filtered staining solutions allows a considerable economy of staining solutions.

Leave basic fuchsin for 30 minutes, adding staining solution if slides dry up.

Using rinsing bottle, rinse slides with water.

Decolorize.

Rinse.

Counterstain with working methylene blue solution, 3 minutes.

Rinse.

Dry.

Opportunity should be given for training in all these procedures.

5. Reading

The Ridley Jopling scale should be adhered to everywhere.

The provision of some standardized slides is very useful.

We have prepared in our laboratory a series of slides each bearing three smears : the left one with a BI of 4, the middle and right ones with a BI of either 0, 1, 2 or 3, randomized.

These slides are fixed and numbered. They are accompanied by a list stating the slide number with the corresponding values of the BIs of the different smears.

These slides serve two purposes : quality testing of the staining procedure and of reading of the BI.

In the 4+ smear, there should be no difficulty in finding the acid-fast bacteria, provided the staining technique is satisfactory. By examining "blindly" the two other smears, the quality of the BI reading can be verified.

If a standard slide is stained and examined once every 4 - 6 months, and five slides are available, this should allow quality testing during 2 to 2 1/2 years. Furthermore if stained slides are kept, they later on will allow verification of BI reading.

6. Interpretation - Action to be taken

The interpretation of the BI reading can be summarized as follows :

New, untreated patients (All results refer to those at any one site.)

0 : no leprosy or BP leprosy

1 : bacteriologically proven PB leprosy

2 and more : MB leprosy

Old, previously treated leprosy.

0 : no leprosy, or treated PB or MB leprosy

1 : treated MB leprosy

≥ 2 : MB leprosy, keeping in mind that adequate treatment diminishes the BI by approximately 1 unit per year. Thus these results should be interpreted taking into consideration the kind and duration of previous treatment.

Significant increase of the BI is the result of either irregular drug intake or development of resistance.

We strongly discourage the generalized tendency to determine MIs. This is indeed a delicate procedure that can only be performed by trained people examining perfect smears with perfectly regulated research microscopes, and entails frequently a subjective factor.

Simple bacteriological examination of patients is on the whole of such a low quality and so much important, that every effort should be made to bring this technique to a high level in the first place.

SUMMARY

Fundamentals for training in bacterioscopy of leprosy are presented : the staining procedure, collection of specimens, necessary material, preparation of reagents, reading, quality control and interpretation.

Table 1 : Gradation of diagnostic procedures for the diagnosis of leprosy.

	Procedure	Necessity	Necessary infrastructure	degree of training of personnel	Information obtained
I	Clinical diagnosis skin and nerves	absolute	none	clinical training	clinical diagnosis and classification Diagnosis reactions
IIa	Bacteriologic diagnosis	absolute	minimal : microscope, slides, staining solutions	minimal : staining reading	Confirmation of MB leprosy. Distinction PB-MP forms BI Evolution under treatment Suspicion resistance
IIb	Histologic diagnosis	desirable	optimal microscopy	special for MI reading	Evolution under treatment Suspicion of resistance
III	Histologic diagnosis	desirable	histopathology lab	special in histopathology	Certain classification Idt, TT, BT BI and MI Differential diagnosis
IV	Mouse foot pad inoculation	exceptional	specialized research lab	specialized research	Proof of resistance Scientific research

I and IIb are fessible in the field and should be performed in all medical centers.
BI : bacteriological index, MI morphologic index