Laboratory Investigations in Leprosy

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Andrea Clapasson and Silvia Canata

8.1 Laboratory Investigations

The most relevant problem in the fight against leprosy in its various forms is the delay in clinical recognition. This leads to the transmission of *M. leprae* or *M. lepromatosis*.

Therefore, it is very important to know the most suitable combination of laboratory investigations.

Until a few years ago, the diagnosis of leprosy was based on clinical evidence (presence of skin lesions with loss of sensitivity and/or thickened peripheral nerves) and on laboratory investigations such as search for acid-fast bacilli (AFB) in slit-skin smear (SSS) examination and histopathology. There has been an evolution in recent years, and different types of molecular biology analyses can help doctors during the differential diagnosis stage.

8.1.1 Tools for Laboratory Investigations

The tools for laboratory investigations are used for diagnosis, classification, and monitoring response to treatment. They may be divided into classical and modern assays. However, the "modern" assays must be used cum grano salis; they cannot

A. Clapasson (✉)
Ministry of Education, University and Research (MIUR), Rapallo, Liguria, Italy
e-mail: CLPNDR@yahoo.com

S. Canata
Department of Social Dermatology, University Hospital San Martino of Genoa, Genoa, Liguria, Italy
e-mail: CLPNDR@yahoo.com

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replace clinical examination and they are very important in the context of differential diagnosis.

The classical techniques are:

- Ziehl-Neelsen staining (ZN), auramine staining, and Kinyoun staining for the research of mycobacteria in SSS or in nasal swabs (NS).
- Fite-Faraco staining (FF) for research of mycobacteria in biopsy.
- Serological tests for research antibodies anti-PGLI and anti-35 kDa of *M. leprae*. These assays have a sensitivity which is in 90–100% of lepromatous patients (BL/LL) but only 40–60% in tuberculoid leprosy patients (BT/TT) [1].

The modern techniques are of molecular biology, based on polymerase chain reaction (PCR). Some of these are as follows:

- 16S rRNA real-time polymerase chain reaction.
- PCR targeting RLEP sequences [2].
- Microarray analysis and PCR-restriction fragment length polymorphism analysis (PCR-RFLP) for species typing of mycobacteria [3]. This typology of assay is very useful for the discrimination among *M. leprae*, *M. lepromatosis*, and other mycobacteria.
- Real-time PCR or limiting dilution PCR (LD-PCR) to monitor drug therapy [4].
- Reverse transcriptase PCR (RT-PCR) or real-time PCR to research viable bacteria [5].
- Single-strand conformation polymorphisms (SSCP) or sequencing techniques to identify relevant mutations in drug therapy [6, 7] or GenoType LepraeDR *test*. The last assay is commercial test and it is not certificated for *M. lepromatosis*, but only for MB patient.
- Sequencing techniques.

It is very important to remember that "the bacteria load of biological sample and previous drug assumption can lead a not true result." To minimize these falls it is necessary to choose the right *combination of techniques* of both molecular biology and histopathology. Each of these modern techniques has disadvantages and advantages. The main disadvantages are the need of expensive instrumentation, cold chain requirement, and qualified laboratory staff, while the advantages are sensibility and rapidity. An example of advantage is the rapidity in the research of resistant strain of *M. leprae*. If you use the mouse footpad technique, the result is obtained after 6–12 months, while the same result, by inverse hybridization or sequencing of specific gene, is obtained in 2 days [7].

The techniques based on molecular biology are used for the purpose of research, while ZN and FF techniques are used routinely for diagnosis of leprosy. However, the molecular biological methods are gaining importance and are indispensable for rapid determination of the species of mycobacteria, and for an accurate determination of the vitality of *M. leprae*.

8.1.2 Nasal Swabs and Slit-Skin Smear Examination

Rule not to be forgotten: *an accurate sampling produces a reliable result*.

The NS is a test where a sample of biological material, obtained by a dry swab of cotton, is smeared over the slide in a circular area. Looking for AFB in NS has only the purpose of determining the end of the contagiousness of multibacillary patients in treatment; it is not a diagnostic or classification criterion.

The SSS examination is a test where a sample of tissue fluid and pulp, obtained by a scalpel, is spread in onto the slide.

There is a difference between the morphology of bacteria in the nasal mucosa and the skin of the same patient.

In the nasal mucosa of untreated lepromatous leprosy patients, there is a higher percentage of solid-staining bacilli than that present in the skin.

The next step is ZN staining. It is performed for the diagnosis of new cases and classification of leprosy, for monitoring of therapy, or for identification case of relapsed of Hansen's disease.

The test is invasive and health personnel must wash his/her hands, wear gloves, and use sterilized equipment and a new blade for each patient.

The best sites for taking SSS are active edges of skin lesions and the cooler regions of the body (see Chap. 2, "Microbiology"). Samples should be obtained from three to six different sites (in BL-LL from both ear lobes and from the edge or just within the edge of four active lesions).

The SSS is useful for diagnosis, classification, choice, and monitoring of therapy and identification of relapse. The SSS test should be taken from:

- Patients suspected to have leprosy.
- Leprosy patients suspected of relapse.

8.1.3 Nasal Smear Technique [8]

We describe two phases in the technique of the nasal swab; these are sampling and staining.

The patient should sit at ease in good light with his head backward and his chin up; in this way, the nasal septum is easier to reach. The best time to collect the nasal secretion is the early morning. The specimen is obtained by rubbing the upper part of the septum using a small cotton swab mounted on a stick.

The biological sample is spread onto a slide. Leave the smears to dry in the open air; therefore, fix and stain as described for SSS.

8.1.4 Slit-Skin Smear Examination Technique [8]

The sites selected for SSS are cleaned with alcohol. Squeeze the skin between the thumb and forefinger, and maintain pressure to expel blood (Fig. 8.1a). Make an

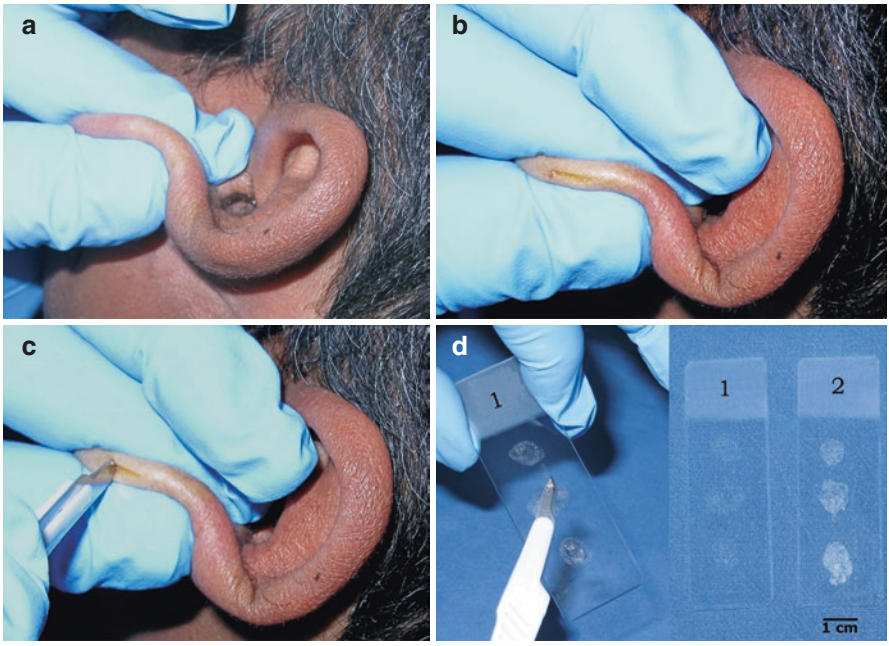

Fig. 8.1 (**a**–**c**) Slit-skin smear technique. (**d**) Slides with sample

incision in the skin about 5 mm wide and 2 mm deep. Continue pressing until the smear has been taken. If the wound continues bleeding, clean with a sterile gauze (Fig. 8.1b).

Turn the blade until it is at right angle to the incision and scrape, once or twice, some tissue material from the sides and bottom of the cut (Fig. 8.1c). Keep the sample bloodless because blood could interfere with the slide reading. Smear onto a slide in single layer (Fig. 8.1d), to cover an area of 5–10 mm diameter, by making circular movements with the flat side of the end of the blade. Slides are left in the open air until they are completely dry. Now they are fixed quickly passing the slides uppermost over the top of a Bunsen burner for three times. Heat fixation is critical because overheating affects the property of acid-fast staining and may crack the slide. Otherwise, a heating cabinet or a hot plate with temperature controlled is recommended. If this solution is used, an exposition for 5 min at 40–50 °C is necessary. Another method of fixation is exposure of the smear for 10 min in formaline fumes.

Limit of SSS technique is low sensitivity (it detects about a third of the AFB); the reading of the slide must be performed by trained personnel, using a microscope with 100× immersion objective.

The slides must not to be exposed to sunlight or dust [9]. After examination, all slides must to be kept for 3 months in a box closed to be re-examined, if the need comes.

The lack of sensitivity of ZN's method can be partly improved by using the auramine technique, but being a fluorescent method, the slides are only readable with a fluorescence microscope [10]. Smears that have been examined by fluorescence microscopy may be re-stained by Ziehl-Neelsen staining to confirm observations, but it is not possible and vice versa.

8.1.5 Cold Ziehl-Neelsen Technique for SSS and NS

1. Cover the sample (skin smear or nasal smear) with primary stain (*), for 20 min.
2. Rinse gently with indirect stream of tap water, until the water flows off clear.
3. Decolorize each slide separately with 2.5 mL of solution of hydrochloric acid and ethanol or sulfuric acid and alcohol (**). This step is more critical of all procedure, because *M. leprae* is more easily decolorized than other mycobacteria, for example, of *M. tuberculosis*. If duration of destaining is too long, there are false negatives, while if it is too short, there are false positives.
4. Rinse with indirect stream of tap water.
5. Counterstain with methylene blue 1% (***), for 30 s.
6. Rinse the stain with indirect stream of tap water until the water flows off clear.
7. Allow slides to dry, away from sunlight.
8. Observe the slides under oil immersion.

AFB appear red, while non-AFB organisms and cellular materials appear blue.
(*) Primary stain (1%):

(a) In a beaker previously weighed, dissolve 6.75 g of basic fuchsin in 67.5 g absolute alcohol.
(b) Add 37.5 g of 5% aqueous phenol [phenol solution: weight 5 g of phenol crystal and dissolve them in 100 mL distilled water (heating gently)].
(c) Add deionized water up to 675 g.
(d) Mix well and filter before use.

Prepare the solution with all components under the fume hood, using appropriate safety equipment (gloves, mask for dust and fumes). The prepared solution is transferred in dark glass bottle with screw cap (capacity 1 L). Label bottle with name of reagent as well as preparation and expiry dates. Store at room temperature for 6–12 months.

(**) Destaining reagent: 95 mL ethanol 96° and 1 mL hydrochloric acid 37% (fuming). *Important:* you must always add acid, drop by drop, to solvent, *not* vice versa.

In countries where the acquisition of alcohol may be problematic, an aqueous solution of 23.75% sulfuric acid and 3% alcohol may be used as decolorizing agent. This is prepared as follows: add 25 mL of 95% sulfuric acid *slowly* (*not* vice versa) to solution of 71.5 mL of distilled water and 3.3 mL of 90% denaturized alcohol.

(***) Counterstain: dissolve 1 g methylene blue in 100 mL distilled water.

Many mycobacteria can survive and grow in nutritionally poor environments such as water puddles and even chlorinated tap water. Environmental mycobacteria might be present in the tap water; boiled water does not solve the problem; you will kill them but they will appear again as AFB after staining. The water is a reagent and its quality is the most important thing; *use purified or distilled water for your solution, not tap water, rain water, or boiled water*.

If it is possible, include one positive and one negative control among the slides when you are staining, for the quality control of Ziehl-Neelsen reagents.

Wear personal protective equipment (respiratory, hand, eye, skin, and body protection) during preparation of solutions and during staining method, and you make the solution in a fume hood.

The *M. leprae* is more easy decolorized than other mycobacteria and its acid resistance is removed by treatment with pyridine. The AFB can be observed only if they are present at equal or higher concentration than 10^4/g of the skin [10].

M. leprae is not the only AFB; there are other microorganisms stained with ZN, namely, other mycobacteria, *Cyclospora*, *Cryptosporidium*, *Isospora*, *Nocardia*, *Rhodococcus* (partially acid fast), and some yeasts. Moreover, some substances are also stained by ZN like inclusions of lead and waxy substance.

8.1.6 Bacteriological Index (BI) and Morphological Index (MI)

Bacteriological index (BI) and morphological index (MI) provide complementary information.

The BI is a parameter directly related to the bacterial load; it is the estimated number of all bacteria (independently of their shape) present in the smear. It is obtained counting the bacilli in a number of oil immersion fields. The value is calibrated using the logarithmic scale of Ridley.

Grading of the BI of each smear

0→ = 0 AFB in any of 100 immersion fields (it is defined as negative)
1 + → = 1–10 AFB on average in 100 immersion fields
2 + → = 1–10 AFB on average in 10 immersion fields
3 + → = 1–10 AFB on average in each field
4 + → = 10–100 AFB on average in each field
5 + → = 100–1000 AFB on average in each field
6 + → = >1000 AFB on average in each field

The average score of the smears is the BI of patient.

The MI studies the shape of the bacilli. It is given by the percentage of the uniformly or solidly (S) stained bacilli. MI is the correlation between shape and vitality. The S-AFB are live bacilli. BI and MI should decrease during therapy.

8.1.7 Bacteriological Follow-Up

The assessment of BI suffers from a number of variables: depth of scrape, amount of tissue fluid removed, and size and thinness of the smear. These issues should not be underestimated, and in the follow-up, successive SSS are better performed by the same operator and samples should be taken at the same sites as previous ones. Finally fixation, destaining time, presence of blood, dust, dirty microscope slides, and direct sunlight are other parameters that may alter the final result. It would be useful to include one positive and one negative control among the slides when you are staining. Proper anti-leprosy therapy decreases patient's BI of about "1+" per year; therefore, bacteriological follow-up in multibacillary patients is carried out annually.

8.1.8 How to Read the Slide?

To check systematically every sample, run the slide with a movement similar to "zigzag" (Fig. 8.2). In this way, using a 100× oil immersion objective, examine 100 adjacent fields.

Depending on the type of stain taken from each AFB, you will have:

- "Solid" form (S). AFB have a homogeneous staining.
- "Fragmented" form (F). AFB have small gaps in the stain.
- "Granular" form (G). AFB are formed most degraded and they are formed by rosary beads. G-form are bacilli which show two or more unstained zones across the whole width of the bacillus [8].

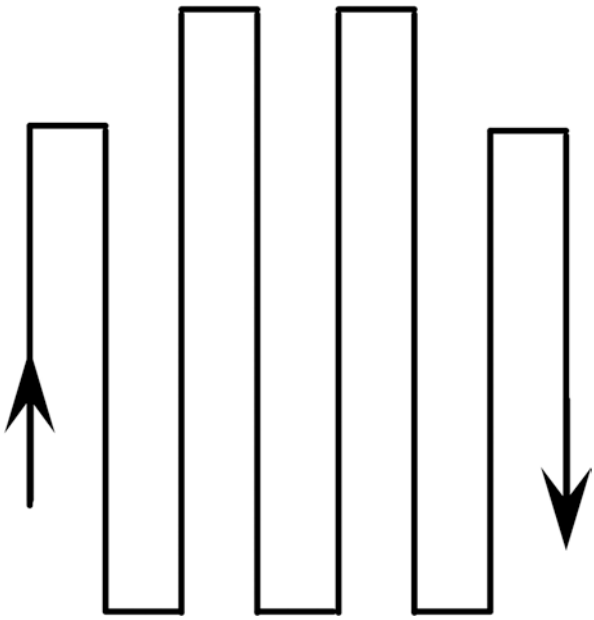

Fig. 8.2 Zigzag observation to scan smear

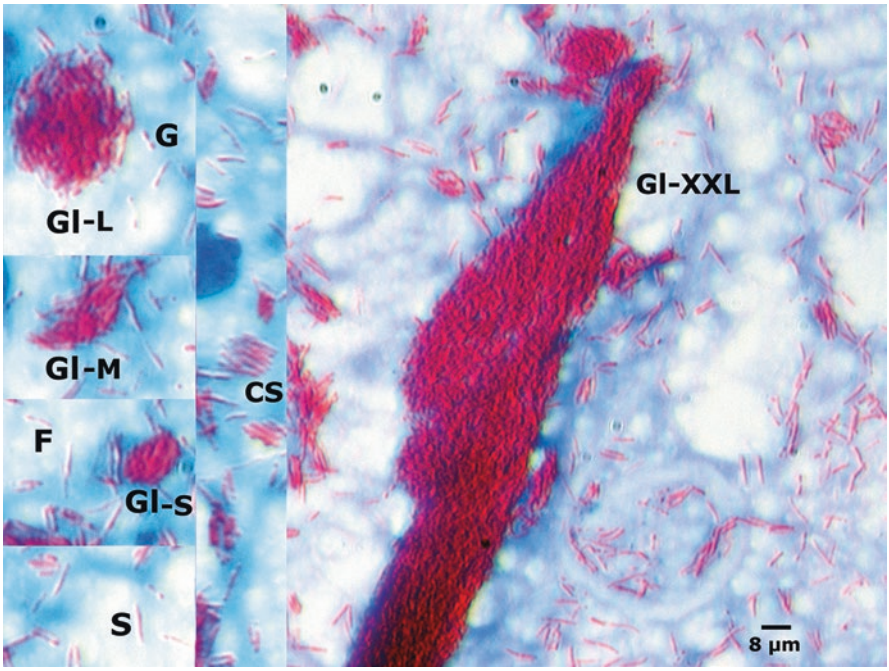

Fig. 8.3 Comparison of different globus: *Gl-L* large globus, *Gl-M* medium globus, *Gl-S* small globus, *Gl-XXL* extra-large globus, *CS* cigar-shaped cluster, *S* solid, *F* fragment, *G* granular

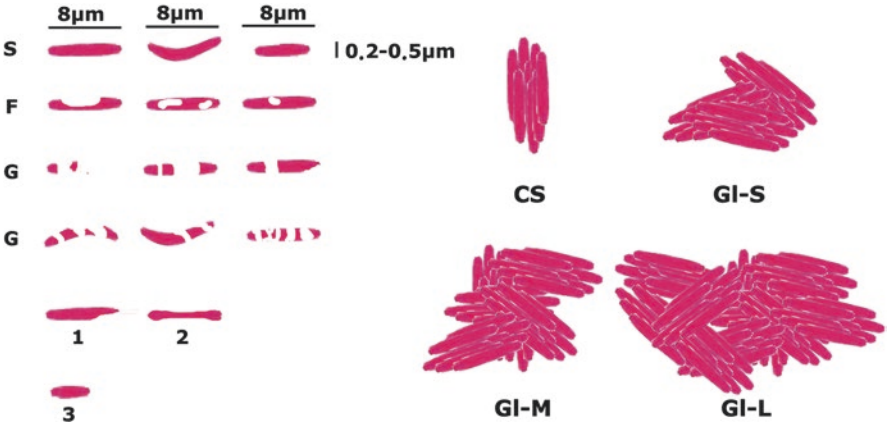

Fig. 8.4 Interpretation table of AFB: solid (S), fragment (F), and granular (G). (1, 2) Likely they are artifacts. These AFB should not be counted. (3) AFB is generally classified as F. Clusters in cigar-shaped (CS); globi: small (Gl-S), medium (Gl-M), and large (Gl-L)

It is also possible to find clusters of bacteria called "globi." During the therapy, each globus shows different affinity for the dye; it switches from a hyperchromic to a hypochromic staining.

The density of the bacteria decreases in the hypochromic globus; they are mainly F- and G-forms. The F and G are bacteria irregularly stained showing heavy degenerative changes.

The bacteria present in the globi cannot be counted accurately and must be estimated. A small globus may contain about 30 AFB, a medium 60, and a large about 100. Rarely in some lepromatous cases, it is possible to find extra-large globi which can contain more than 1000 AFB (Figs. 8.3 and 8.4).

The staining property of the AFB changes during the normal life cycle of the bacterium and during therapy.

In multibacillary cases at diagnosis, the "solids" are the minority, but during therapy they disappear first. Contemporarily, the percentage of fragmented and granular forms increases, and then remains only granular; these are the last to disappear (from the skin). This modification in the stain is an indirect parameter of viability of AFB and a measure of the patient's response to treatment.

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