Lab. Support in leprosy

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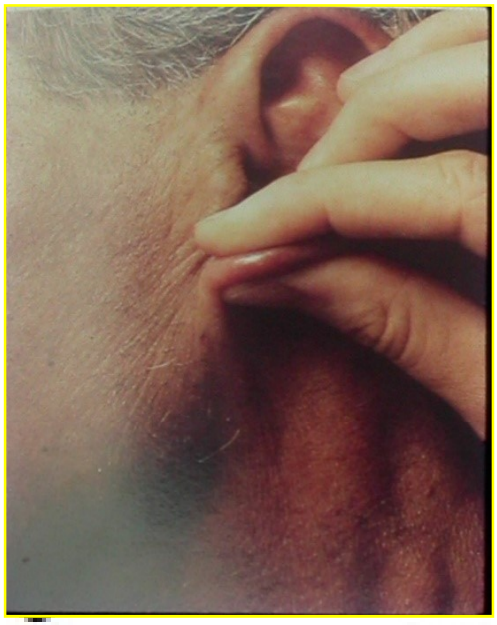
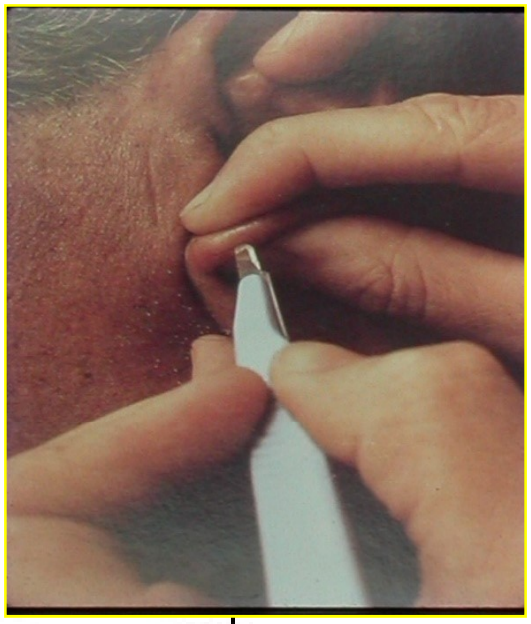
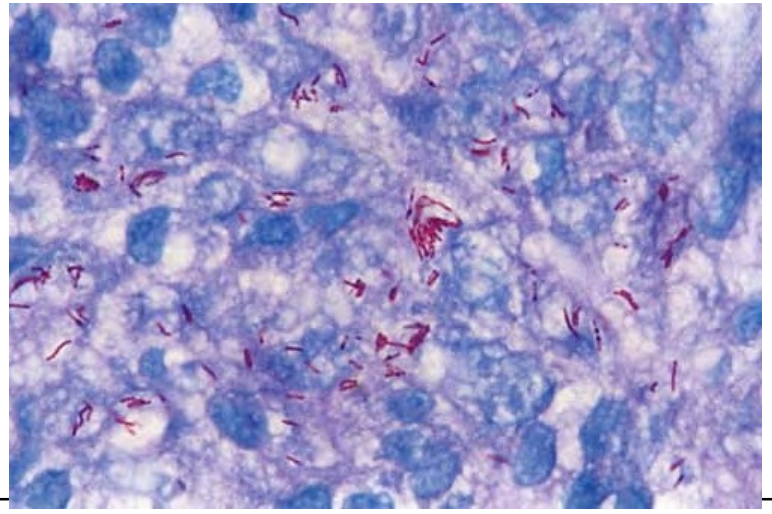
Available assays

- Slit Skin Smear-AFB stain (SSS) microscopy
- Histopathology
- Animal inoculation
- Serological assays
- Molecular assays

Slit skin smear preparation





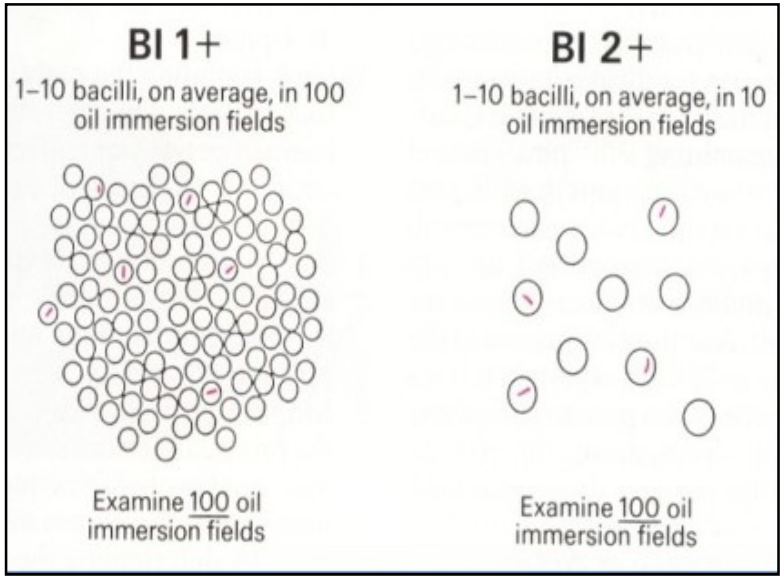


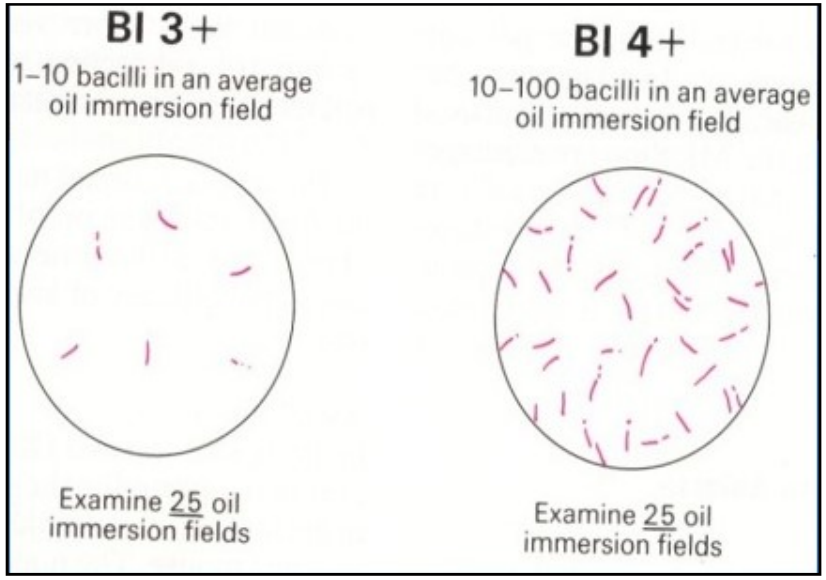
Grading of the smear

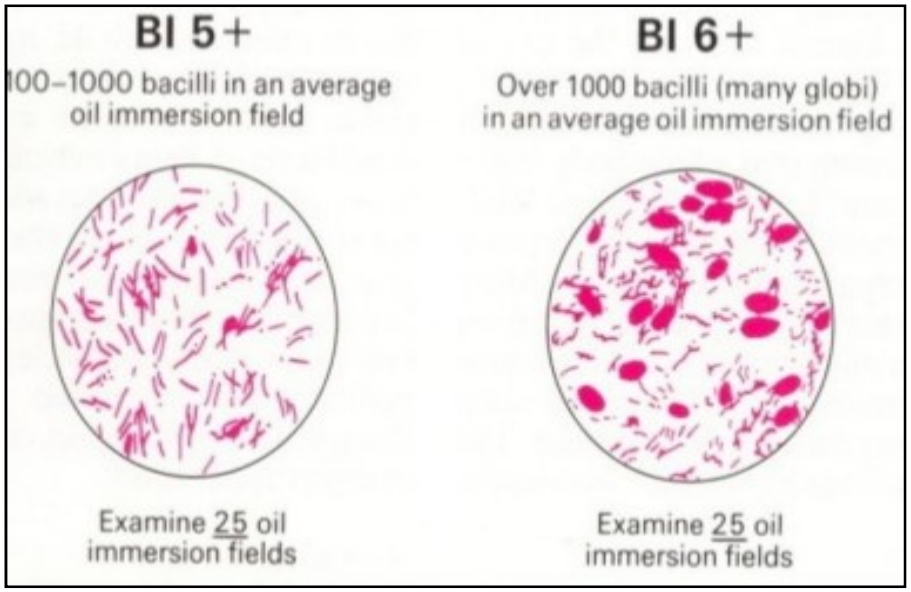
Description	Grade
1-10 bacilli in 100 OIF	1+
1-10 bacilli in 10 OIF	2+
1-10 bacilli per OIF	3+
10-100 bacilli per OIF	4+
100-1000 bacilli per OIF	5+
> 1000 bacilli / in clumps and globi in each OIF	6+

- Bacteriological index
- Morphological index

Bacteriological index



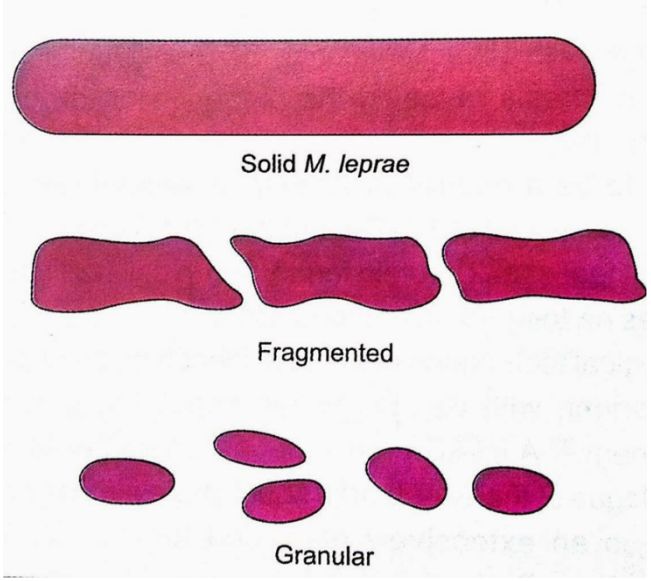




Bacteriological and morphological indices

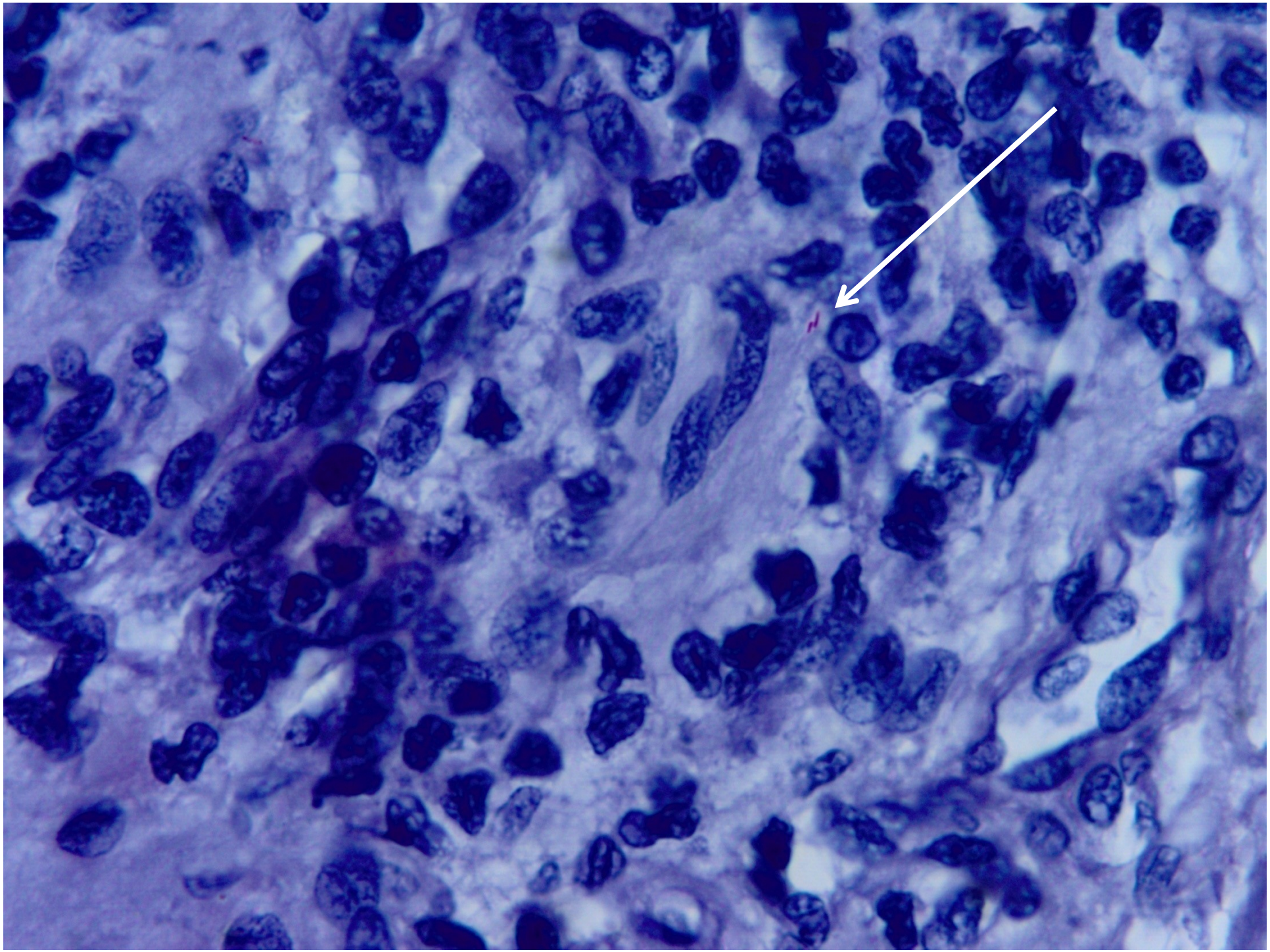
Bacteriological index- Indicates density of leprosy bacilli (live & dead) in the smears and ranges from 0 to 6+

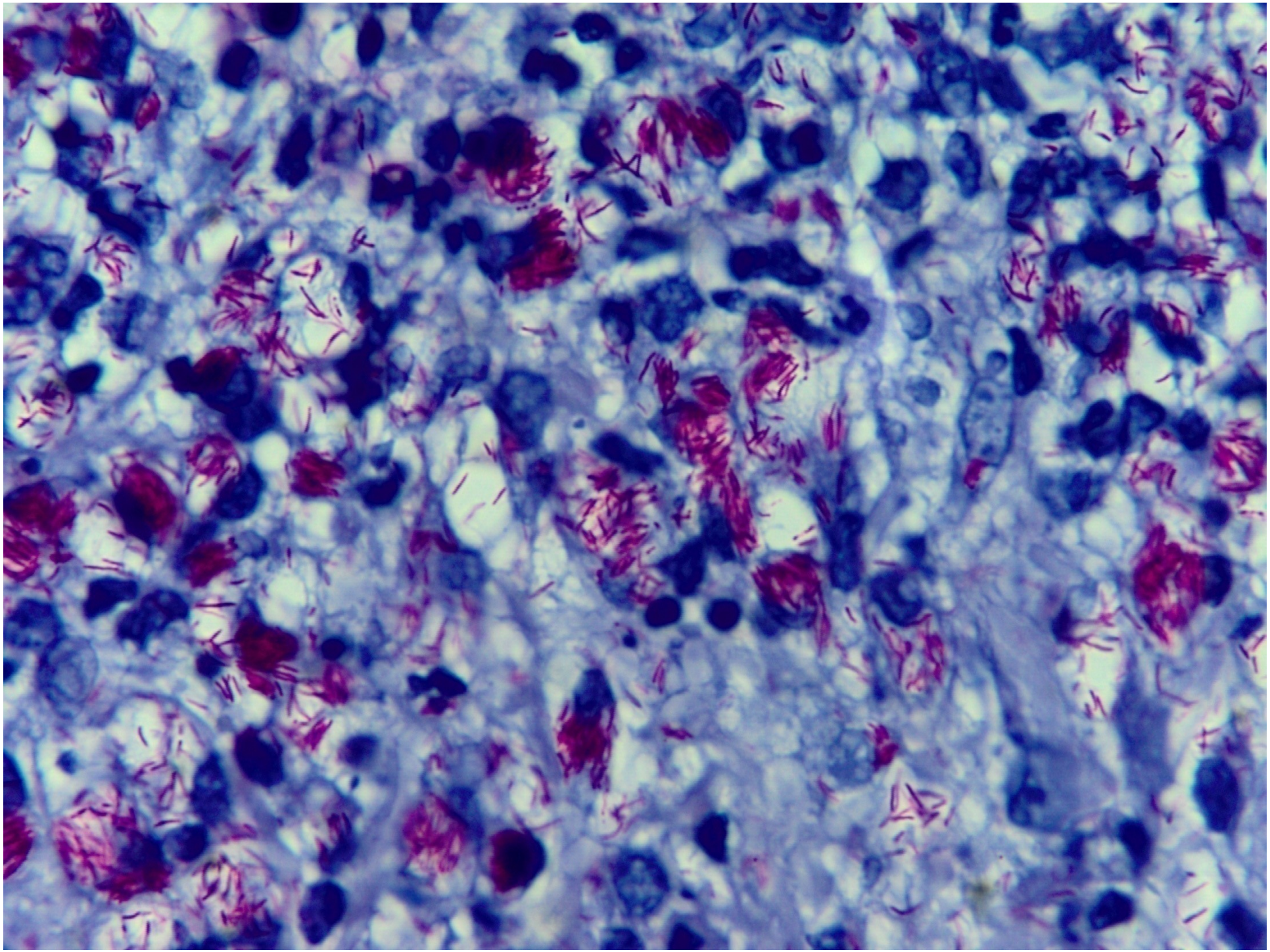
Morphological index- It is the percentage of presumably living bacilli in relation to total number of bacilli in the smear

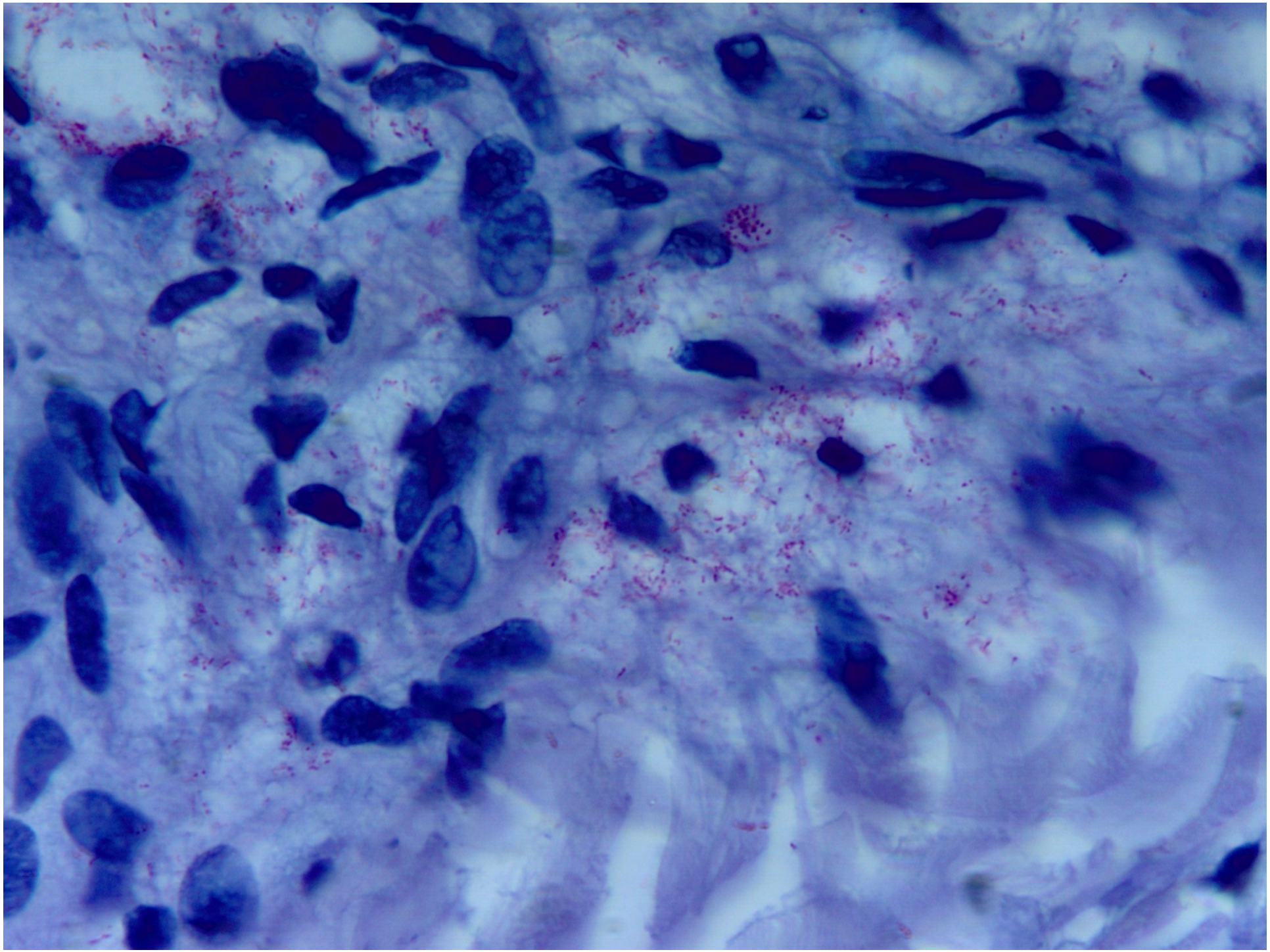


Histopathology

- Useful for classification of spectrum and follow-up





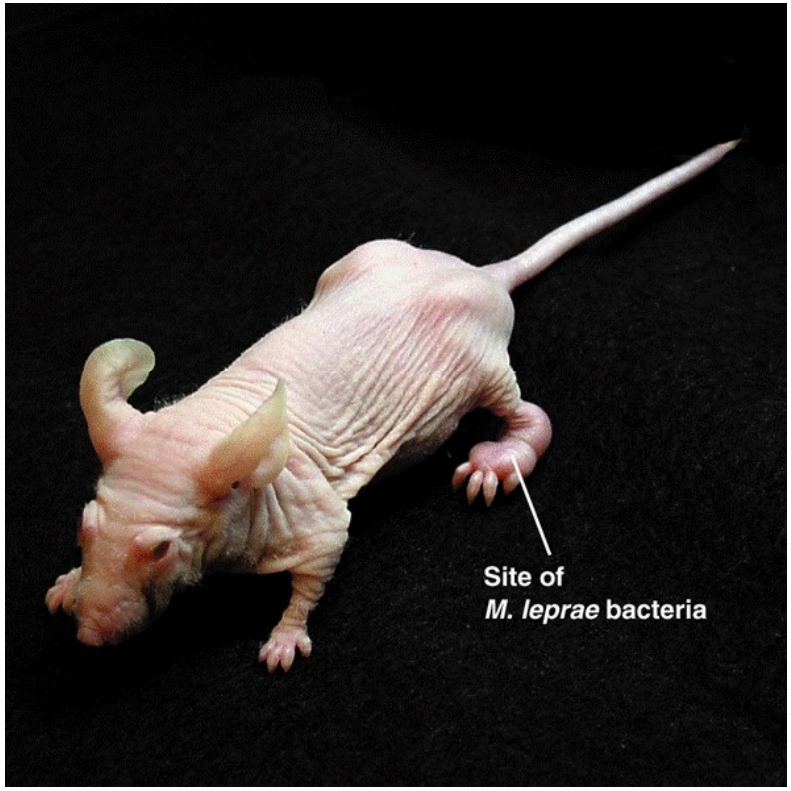


Slit skin smear examination

- Specificity 100 %
- Less sensitive (requires 10^4 AFB/ml)
- Negative in milder forms (PB)
- Repeated assessment of MI during the course of treatment helps to monitor the prognosis & identify those who are experiencing relapse

ANIMAL INOCULATION

Animal models



Athymic nude Mice

Dasypus novemcinctus

Non human reservoir

Slender loris

West African mangabey monkey

Indian pangolis

MOUSE FOOTPAD INOCULATION

- Swiss Albino Immuno-competent Mouse
- Thymectomized Immuno-deficient Mouse
- Congenital Immuno-deficient - Nude Mouse

Advantages of cultivation

- Sensitive than microscopy (10 times)
- Detection drug resistance
- Drug potency evaluation
- Detection of viability of bacilli

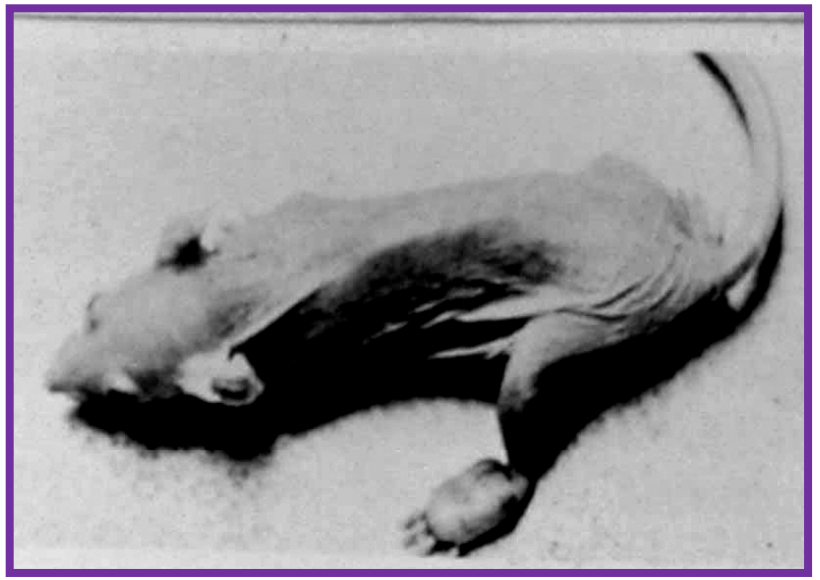
Disadvantages:

- Cumbersome & time consuming
- Ethical issues regarding use of animals

Limitations of Animal models

Immuno- competent Mouse

- Less multiplication
- High inconclusive reports



Immuno- deficient Nude mouse

- Difficult to maintain
- Susceptible for infection

Puzzles around *M.leprae*

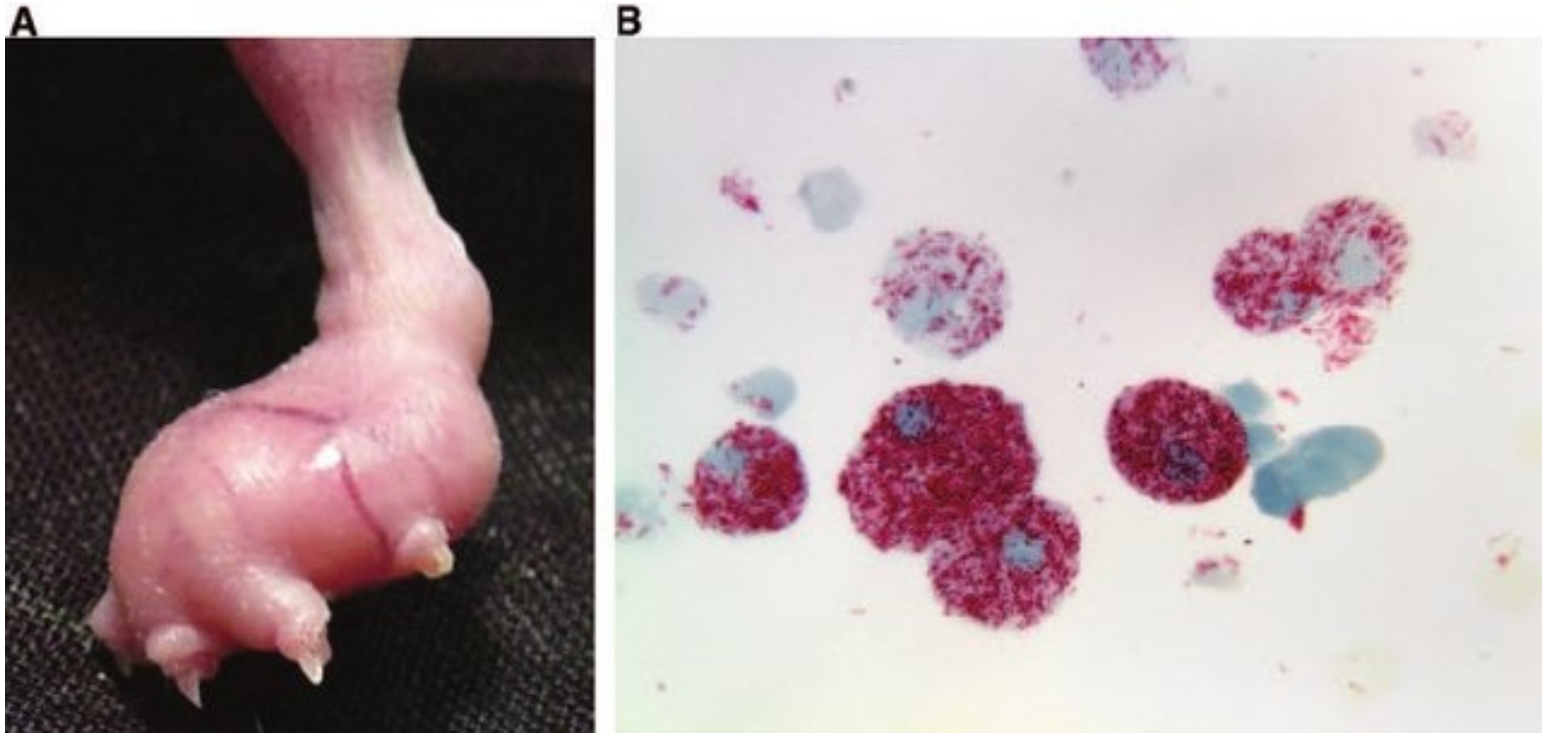
- Long generation time – 12 days
- Prefers cooler temperature for multiplication: 28-33°C
- Not able to be cultured in artificial medium

Limitations of Animal models

Nine Banded Armadillo

- *M.leprae* multiplies all over the body
- Selective availability – Southern America
- Does not breed in captivity

Mouse foot pad inoculation



Immunocompetent mice (modified Shepard technique)

Biopsy specimen is weighed

↓

Minced with scissors after addition of drops of HBSS

↓

Transferred to a cup of a Mickle tissue disintegrator

↓

Glass beads placed in the Mickle cup before sterilization

↓

Vibrated for 1 min, freq 50-60 cy/s

Tissue-homogenate is transferred to test-tube

↓

HBSS and BSA are added to a final volume of 2.5 ml

↓

Suspension is allowed to stand for 2 minutes

↓

Resulting supernatant is removed with a pipette

↓

Preparation of smears for counting

Preparation of smears and counting AFB

- Microscope slides with three fused ceramic circles, (1 cm in diameter)
- To prepare smears, 10 µl of formol-milk is applied and 10 µl of the suspension is added to the circle
- Liquids are immediately mixed and spread over the entire area

- Smears are stained by acid-fast stain, bacilli counted under oil-immersion objective

- 20 fields per circle or a total of 60 fields per slide are examined

- Calculation of No. of bacilli

Slide circle of diameter D, microscope field diameter d, the following formula is applied to calculate the number of bacilli in the suspension

$$\frac{\text{no. AFB}}{\text{ml sample}} = \frac{\text{no. AFB counted}}{\text{no. fields counted}} \times (D/d)^2 \times 100$$

Inoculation into Mice

- The suspension is diluted with HBSS to contain 3 x 10^3 AFB per 0.03 ml
- Suspension is injected subcutaneously into the mouse footpad with 1ml syringe and 27- or 30-gauge needle

One specimen - 66 animals

Group	No. of animals	Feed
Control	10	Normal
rifampicin I	8	0.03% rifampicin
rifampicin II	8	0.003% rifampicin
Dapsone I	8	0.01% dapsone
Dapsone II	8	0.001% dapsone
Dapsone III	8	0.0001% dapsone
Clofazamine I	8	0.01% clofazamine
Clofazamine II	8	0.001% clofazamine

Harvesting of M. leprae

- From 6 months after inoculation, mice sacrificed by cervical dislocation
- Inoculated foot is removed, fixed in 10% formalin for 3 days
- After fixation, foot placed in 5% formic acid in 70% ethanol for 7 days
- The decalcified foot is then processed for HPE and sections are stained for AFB

- Control group should be examined for increase in number of bacilli before examining drug fed animals
- Increase in number of bacilli upto or more than 2 log increase indicates multiplication of bacilli in mouse footpad
- Increase in same numbers in drug fed animals indicates resistance

Immunocompromised mice (Rees)

- Immunocompetent mice produces limited number of bacilli because of T-cell immune response

i. Thymectomy – 4-8 weeks

ii. Irradiated with 900 rads of gamma or X rays

SEROLOGICAL ASSAYS

Antibody detection

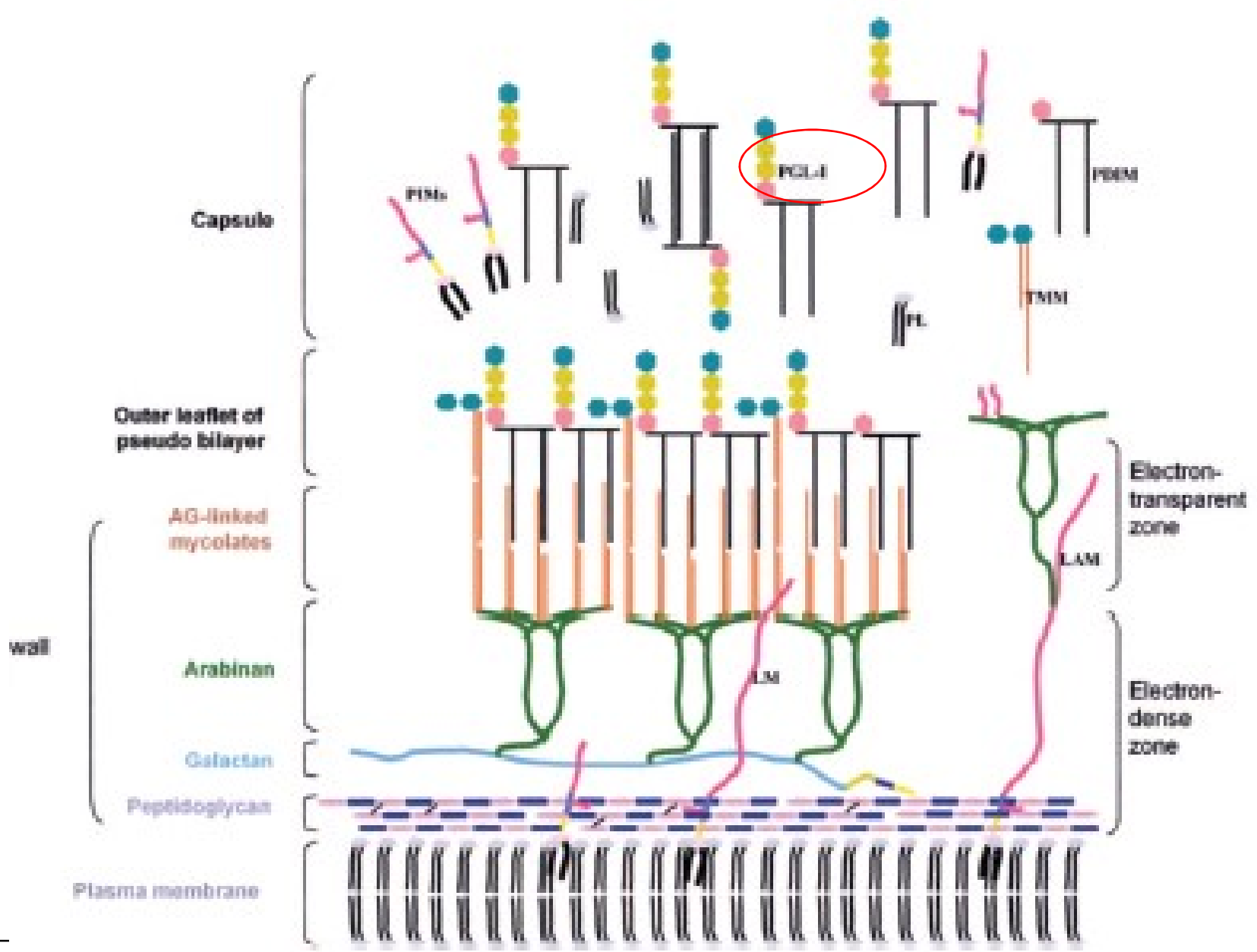


TABLE 1 - Use of serological tests as an auxiliary tool to diagnose leprosy.

Antigen	Major findings/comments	References
PGL-I native and/or mimetic	Helps detect early-stage leprosy	39
	Helps classify patients correctly	9, 10, 12, 13, 14, 15, 24
	Assists in differentiating between MB and PB forms	5, 15, 16, 17, 18, 19, 20, 21, 22, 24, 44
	Assists in differential diagnosis	23
	Assists in identifying patients with high bacterial load	11
	Helps diagnose MB leprosy	25, 43
ML0405	Improves performance of anti-PGL-I tests	25
ML2331	Assists in diagnosis and classification	5, 17, 26
LID-1	Assists in diagnosis	27
	Assists in diagnosis, specifically of MB leprosy	17, 25
	Assists in detecting early-stage leprosy	4
	Improves performance of anti-PGL-I tests	25
NDO-LID	Assists in rapid and consistent detection of MB leprosy	5, 28
	Assists in patient monitoring	29
	Increases sensitivity and specificity of anti-PGL-I tests	29

PGL-I: phenolic glycolipid-I**; ML:** *Mycobacterium leprae***; LID-1:** leprosy IDRI diagnostic-1**; NDO-LID:** natural disaccharide octyl-leprosy IDRI diagnostic-1**; MB:** multibacillary; **PB:** paucibacillary.

TABLE 2 - Use of serological tests in surveillance programs.

Antigen	Major findings/comments	References
PGL-I native and/or mimetic	Used to evaluate exposure to antigen	30
	Assists in identifying individuals with subclinical infection	18, 21, 32
	Assists in early diagnosis among household contacts	30, 43
	Can identify contacts at high risk of developing leprosy	11, 14, 21, 31, 33, 34, 36
	Can identify broad groups most at risk of becoming ill	37
	Can assist in monitoring household contacts	35
	Can indicate need for clinical examination	35
	Assists in identifying school-age children with higher risk of developing leprosy	38
	Assists in estimating potential of *M. leprae* transmission	37
ML0405 **ML2331**	Enhances PGL-I tests in identifying contacts at greater risk of developing leprosy	40
LID-1	Can be used to detect *M. leprae* infection	41
	Assists in identifying household contacts that require careful surveillance	35
	Can indicate need for clinical examination	35
NDO-LID	Allows detection of early-stage infection	29
	Can be used to monitor suspected cases of *M. leprae* infection	29

PGL-I: phenolic glycolipid-I; **ML:** *Mycobacterium leprae*; **LID-1:** leprosy IDRI diagnostic-1; **NDO-LID:** natural disaccharide octyl-leprosy IDRI diagnostic-1; ***M.***: *Mycobacterim.*

Fabri ACOC et al. Integrative literature review of the reported uses of serological tests in leprosy management. Rev Soc Bras Med Trop 49(2):158-164 Mar-Apr, 2016

TABLE 3 - Use of serological tests in therapy and neuritis.

Antigens	Major finding/comments
PGL-I native and/or mimetic	Assists in selecting the most appropriate multidrug therapy
	Can be considered as marker of re-infection and indicate long-term high risk of leprosy
	Allows evaluation of multidrug therapy
	Enables assessment of bacterial load after treatment
	Helps detect nerve damage
ML0405	Allows evaluation of multidrug therapy
ML2331	Indicates long-term high risk of leprosy
LID-1	Can be considered as markers of re-infection

PGL-I: phenolic glycolipid-I**; ML:** *Mycobacterium leprae***; LID-1:** leprosy IDRI diagnostic-1**.**

NDO-LID rapid test

- Smart phone based test reader platform
- Provides quantifiable data
- Can be applied for monitoring chemotherapy

Cardoso *et al*., Development of a quantitative rapid diagnostic test for multibacillary leprosy using smart phone Technology. BMC Infectious Diseases 2013, 13:497

- 35kDa based RIA/ELISA
- Monoclonal ab MLO4 identifies specific epitope on 35kDa antigen of M.leprae
- Serum antibody competition test (SACT) or inhibition assay
- 100 % sensitive in BL/LL; 50% in BT/TT

MOLECULAR METHODS

Molecular methods

- Specimen: skin biopsy, skin smear, nerves, urine, oral/nasal swabs & blood
- Application:
- To diagnose difficult cases (PNL, PB)
- To monitor the treatment
- To do surveillance of household contacts
- To assess the viability of M.leprae
- To quantitate the bacillary load

Molecular methods-Targets

DNA Targets	PCR Method	Results
Proline-rich antigen (pra-36 KDa)	PCR-Southern hybridization	87–100% MB, 36–60% PB
	TaqMan real-time PCR	89% BI^+, 33% BI^-.
18 kDa	PCR-Southern hybridization	99% MB, 74% PB.
		The specificity was 100%, and sensitivity ranged from 50% to 83%. A group of patients with other skin disorders was also tested.
RLEP	PCR-Southern hybridization	100% BI^+ or BI^-.
	PCR	100% MB, 73%, PB.
RLEP and TTC repeat	Multiplex-PCR	100% MB, 83% PB.
RLEP	TaqMan real-time PCR	The specificity was 73%, and sensitivity was 91%.
Ag85B	TaqMan real- time PCR	100% MB, 80% PB.
		The specificity was 100%, and sensitivity was 56%.
16S	Taqman real-time PCR	The specificity was 100%, and sensitivity was 51%.
16S	SyBr green real-time PCR	100% MB, 50% PB.

Molecular methods-Targets

DNA Targets	PCR Method	Sample	Population	Results
Proline-rich antigen (pra-36 KDa)	PCR-Southern hybridization	Nasal swab	Healthy	7.8%
	PCR-ELISA	Nasal swab	Healthy	7.8%
RLEP	PCR	Nasal swab	Healthy	31%
		Nasal swab	Household contacts	5.2% MB IC*, 3.8% PB IC.
		Nasal swab	Household contacts	10% MB IC, 6% PB IC.
RLEP and TTC repeat	Multiplex-PCR	Nasal swabs	Household contacts	11% MB IC, 1.3% PB IC.
ML0024	Real-time PCR	Blood	Household contacts	1.2%
RLEP	Nested PCR	Blood	Household contacts	6.25%

PCR

Extraction of DNA

↓

Amplification of targets

↓

Gel electrophoresis and documentation

(presence of 372 bp compared with 100bp ladder)

Very useful- reliability and rapidity in diagnosis and AMR detection

PCR Assay for *M.leprae* specific gene

Agarose showing *M.leprae* repetitive sequence PCR product

372bp →

RNA based PCR to demonstrate viability

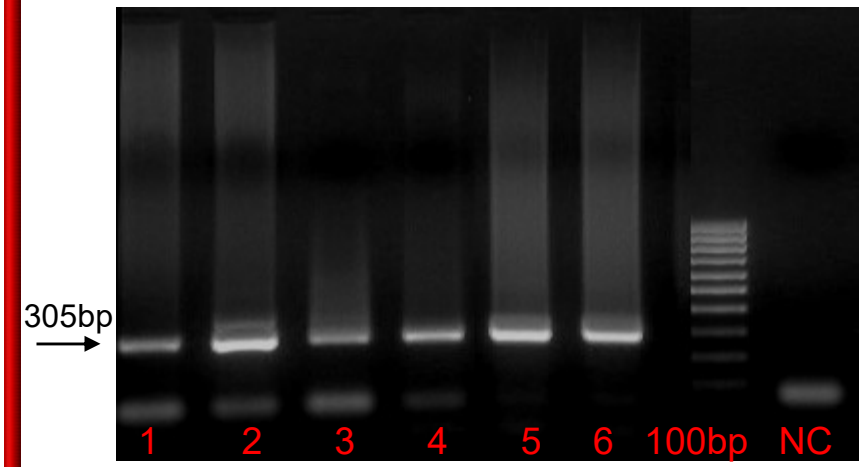
- RNA targeting amplification system is promising
- RNA more unstable than DNA; degrade faster after death
- Likely to correlate better with viable bacteria Messenger
- RNA (mRNA) has shorter half life-ideal for viability determination
- 16srRNA studied extensively and found useful

Drug resistance in *M.leprae*

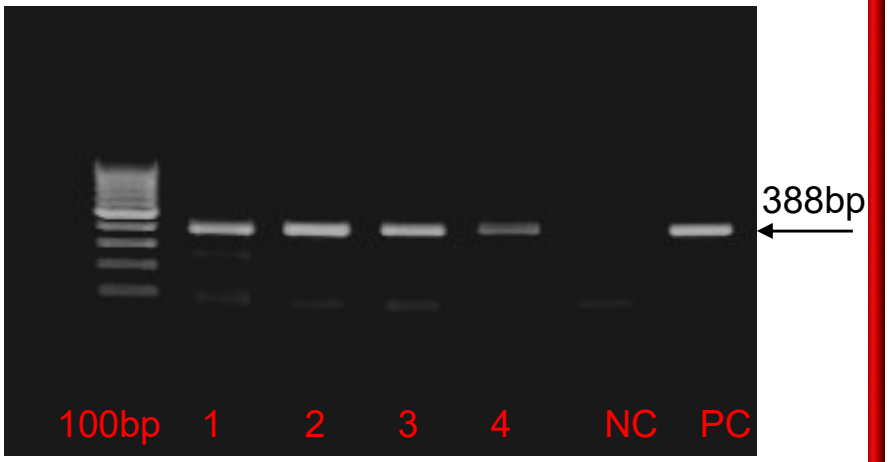
Drug	Target	Gene
Rifampicin	β-sub unit of DNA dependent RNA polymerase	*rpoB*
Dapsone	Targets dihydropteorate synthase (DHPS) enzyme	*folp1*
Quinolones	DNA gyrase	*gyrA*

Gel Documentation of PCR Products

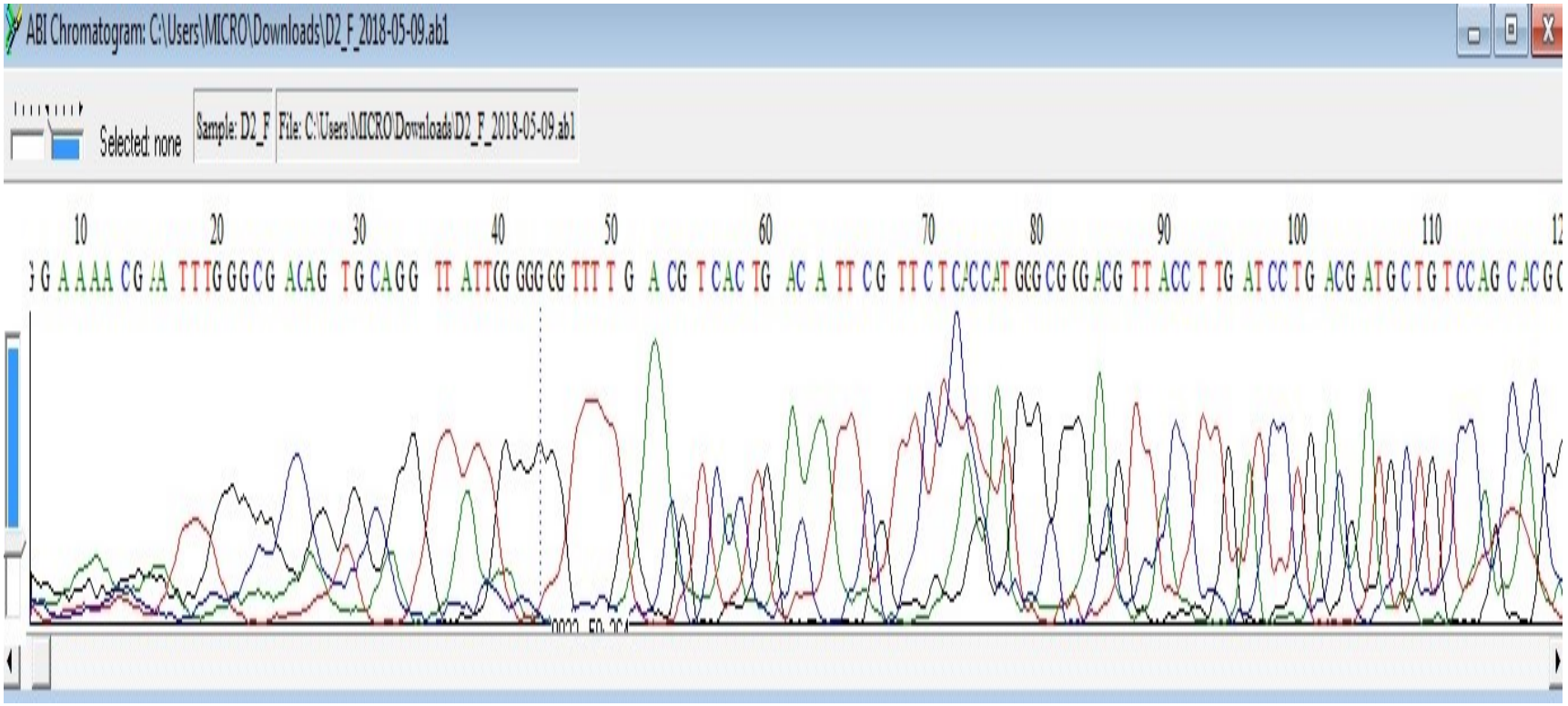
PCR Product of *rpoB* gene of *M.leprae(305bp)*



PCR Product of *folp1* gene of *M.leprae(388bp)*



Sequencing results



Sequencing results

Sequencing results

Partial sequence of *M. leprae* |ML0224|*folP1*

```
 91 - gct gtc cag cac ggc ctg gca atg gtc gcg gaa ggc gcg gcg att gtc gac gtc ggt ggc
 31 -  A   V   Q   H   G   L   A   M   V   A   E   G   A   A   I   V   D   V   G   G
151 - gaa tcg acc cgg ccc ggt gcc att agg acc gat cct cga gtt gaa ctc tct cgt atc gtt
 51 -  E   S   T   R   P   G   A   I   R   T   D   P   R   V   E   L   S   R   I   V
```

Partial sequence of *M. leprae* |ML1891c|*rpoB*

```
1261 - cgt ccg gtg gtc gcc gct atc aag gaa ttc ttc ggc acc agc cag ctg tcg cag ttc atg
 421 -  R   P   V   V   A   A   I   K   E   F   F   G   T   S   Q   L   S   Q   F   M
1321 - gat cag aac aac cct ctg tcg ggc ctg acc cac aag cgc cgg ctg tcg gcg ctg ggc ccg
 441 -  D   Q   N   N   P   L   S   G   L   T   H   K   R   R   L   S   A   L   G   P
1381 - ggt ggt ttg tcg cgt gag cgt gcc ggg cta gag gtc cgt gac gtg cac cct tcg cac tac
 461 -  G   G   L   S   R   E   R   A   G   L   E   V   R   D   V   H   P   S   H   Y
```

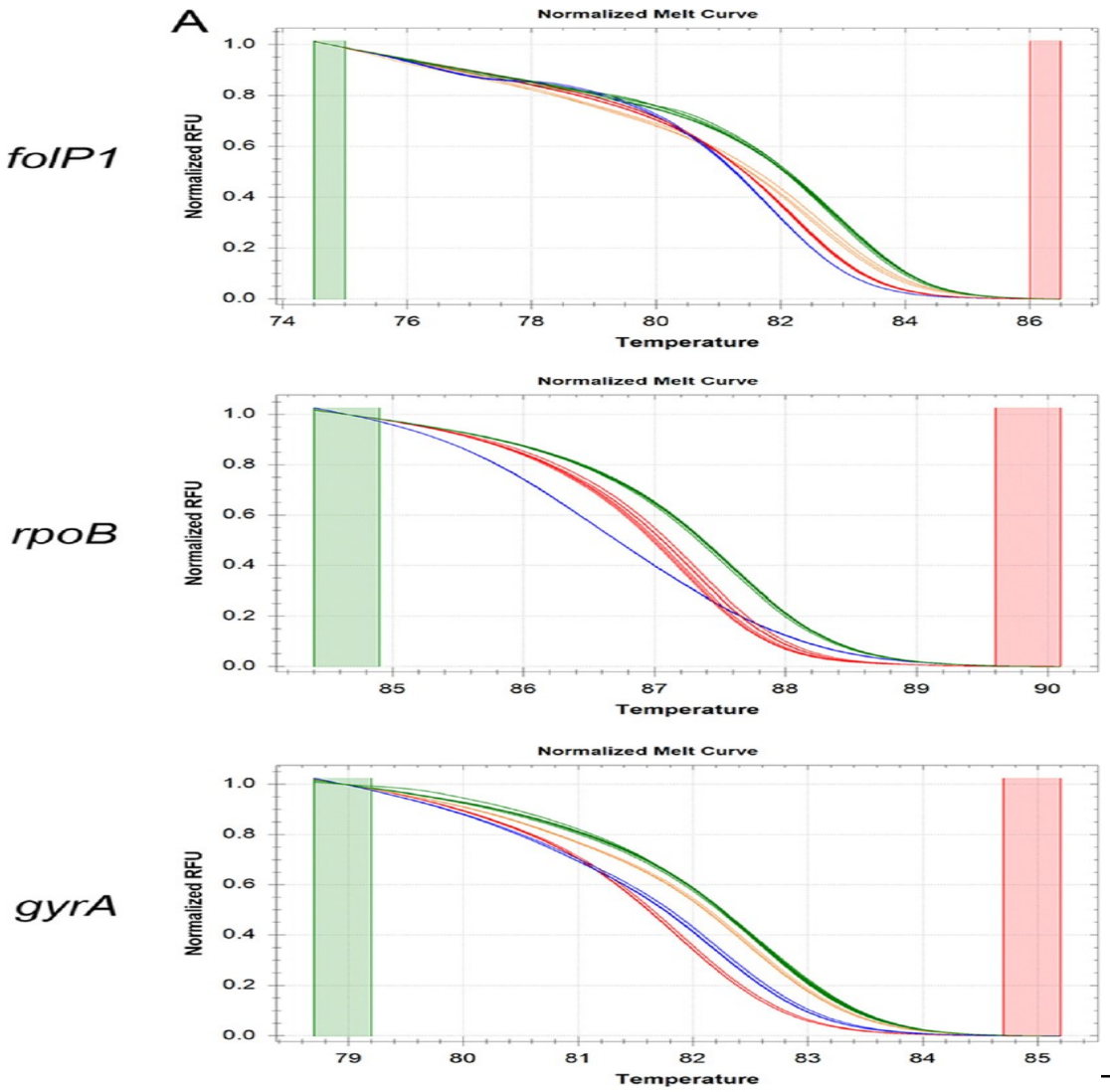
Partial sequence of *M. leprae* |ML0006|*gyrA*

```
181 - tta gac tcc ggt ttc cgc ccg gac cgt agc cac gct aag tca gca cgg tca gtc gct gag
 61 -  L   D   S   G   F   R   P   D   R   S   H   A   K   S   A   R   S   V   A   E
241 - acg atg ggc aat tac cat ccg cac ggc gac gca tcg att tat gac acg tta gtg cgc atg
 81 -  T   M   G   N   Y   H   P   H   G   D   A   S   I   Y   D   T   L   V   R   M
```

Real-Time PCR and High-Resolution Melt Analysis for Rapid Detection of *Mycobacterium leprae* Drug Resistance Mutations and Strain Types

Wei Li,[a] Masanori Matsuoka,[b] Masanori Kai,[b] Pratibha Thapa,[c] Saraswoti Khadge,[c] Deanna A. Hagge,[c] Patrick J. Brennan,[a] and Varalakshmi Vissa[a]

Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, USA[a]; Leprosy Research Center, National Institute of Infection Diseases, Tokyo, Japan[b]; and Mycobacterial Research Laboratory, Anandaban Hospital, Kathmandu, Nepal[c]



PCR-to study transmission & surveillance

Comparative evaluation of PCR amplification of RLEP, 16S rRNA, rpoT and Sod A gene targets for detection of *M. leprae* DNA from clinical and environmental samples

Ravindra P. Turankar [1], *Shradha Pandey* [1], *Mallika Lavania*, *Itu Singh*, *Astha Nigam*, *Joydeepa Darlong*, *Fam Darlong*, *Utpal Sengupta* [*]

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Guidelines for the Diagnosis, Treatment and Prevention of Leprosy

World Health Organization

2018

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Leprosy elimination

- Leprosy
- The disease
- Epidemiology
- The Global Leprosy Strategy
- Links and resources

Guidelines for the diagnosis, treatment and prevention of leprosy

Authors:
WHO SEARO/Department of Control of Neglected Tropical Diseases

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Downloads

- Guidelines for the diagnosis, treatment and prevention of leprosy
- Lignes directrices pour le diagnostic, le traitement et la prévention de la lèpre

Question 1a: Is there a diagnostic test for leprosy disease (PB and/or MB) that has sufficient sensitivity and specificity and whose use is feasible under programmatic conditions?

Population	Intervention	Comparator	Outcomes
Adults and children with suspected leprosy and leprosy patients (PB and MB) diagnosed clinically	• Tests that detect *M. leprae* nucleic acids (PCR), antigens or other components • Tests that detect host biomarkers such as antibodies (i.e. PGL-1 or NDO-LID) or chemokines and cytokines (i.e. IP-10, IL-10) or that detect antibodies together with chemokines and cytokines • Tests that detect "effects of the disease" such as nerve enlargement by ultrasound	Diagnosis of leprosy on the basis of having one or more of the following: • definite loss of sensation in a hypopigmented or reddish skin patch; • thickened or enlarged peripheral nerve, with loss of sensation and/or weakness of the muscles supplied by that nerve; • presence of acid-fast bacilli in a slit-skin smear or in a skin biopsy • histopathological diagnosis (skin/nerve-biopsy)	• sensitivity • specificity • predictive values

Question 1b: Is there a diagnostic test that has sufficient sensitivity and specificity to diagnose M. leprae infection (latent leprosy) among contacts and whose use is feasible under programmatic conditions?

Population	Intervention	Comparator	Outcomes
Contacts of patients with leprosy: • contacts of patients with PB leprosy • contacts of patients with MB leprosy • household contacts (of PB and MB) • social contacts (of PB and MB) • neighbours of patients with leprosy (PB and MB)	• Tests that detect host biomarkers such as antibodies (i.e. PGL-1 or NDO-LID) or chemokines and cytokines (i.e. IP-10, IL-10) or that detect antibodies together with chemokines and cytokines	Diagnosis of leprosy based on the basis of having one or more of the following: • definite loss of sensation in a hypopigmented or reddish skin patch; • thickened or enlarged peripheral nerve, with loss of sensation and/or weakness of the muscles supplied by that nerve; • presence of acid-fast bacilli in a slit-skin smear or in tissue/biopsy • histopathological diagnosis over a biopsy	• sensitivity • specificity • predictive values • adverse effects

Table 2. GRADE categories of the quality of evidence

Level of evidence	Definition
High	Further research is very unlikely to change our confidence in the estimate of effect
Moderate	Further research is likely to have an important impact on our confidence in the effect
Low	Further research is very likely to have an estimate of effect and is likely to change the estimate
Very low	Any estimate of effect is very uncertain

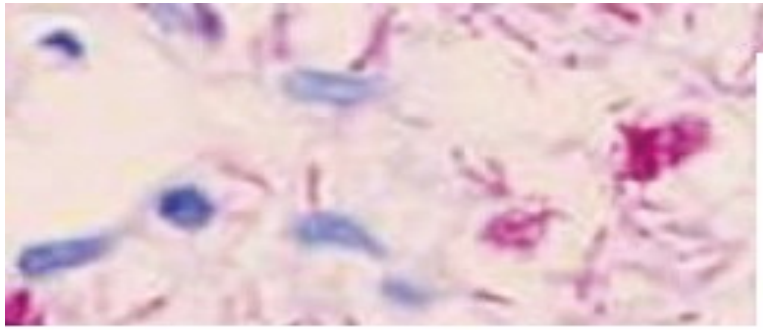
Area of the recommendation	Recommendation	Strength	Quality of evidence
Diagnosis			
Diagnosis of leprosy	The diagnosis of leprosy may be based on clinical examination, with or without slit-skin smears or pathological examination of biopsies.	Conditional	Low
Diagnosis of leprosy infection	There is currently no test recommended to diagnose leprosy infection (latent leprosy) among asymptomatic contacts.	Conditional	Low

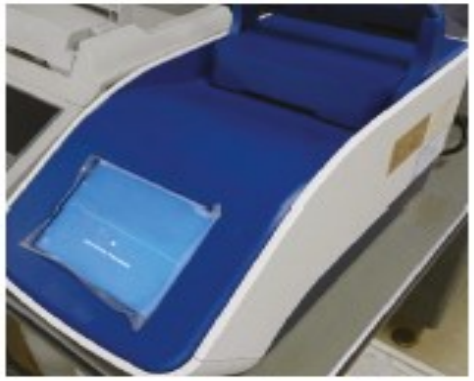
Therefore, based on currently available evidence, newer ELISA, lateral flow and PCR tests do not represent a clear advantage over current standard diagnostic methods (clinical diagnosis with or without confirmatory tests such as slit-skin smear or biopsy).

1. Leprosy diagnosis

Tests with promising results for higher diagnostic accuracy (e.g. PCR tests using tissue samples) should be assessed in larger, well-designed studies using assays that are standardized and feasible for use in field settings. Such studies should also evaluate their accuracy for predicting the development of leprosy in contacts. In addition, research is needed on the diagnostic utility of other tools, including ultrasound and other imaging tests, as possible aids to diagnosis.

New biomarkers are needed to identify persons with leprosy. Tests for these should be more accurate than previously evaluated ELISA and lateral flow tests. A test protocol study reported the utility of mixed assays that detect cell-mediated responses (cytokines and chemokines) as well as *M. leprae*-specific antibodies to detect both PB and MB leprosy *(94)*. More studies are needed to determine the use of identified biomarkers for diagnosis. Longitudinal studies are needed to assess how well these tests predict the development of overt leprosy in contacts of persons with leprosy.





A guide for surveillance of antimicrobial resistance in leprosy

2017 update

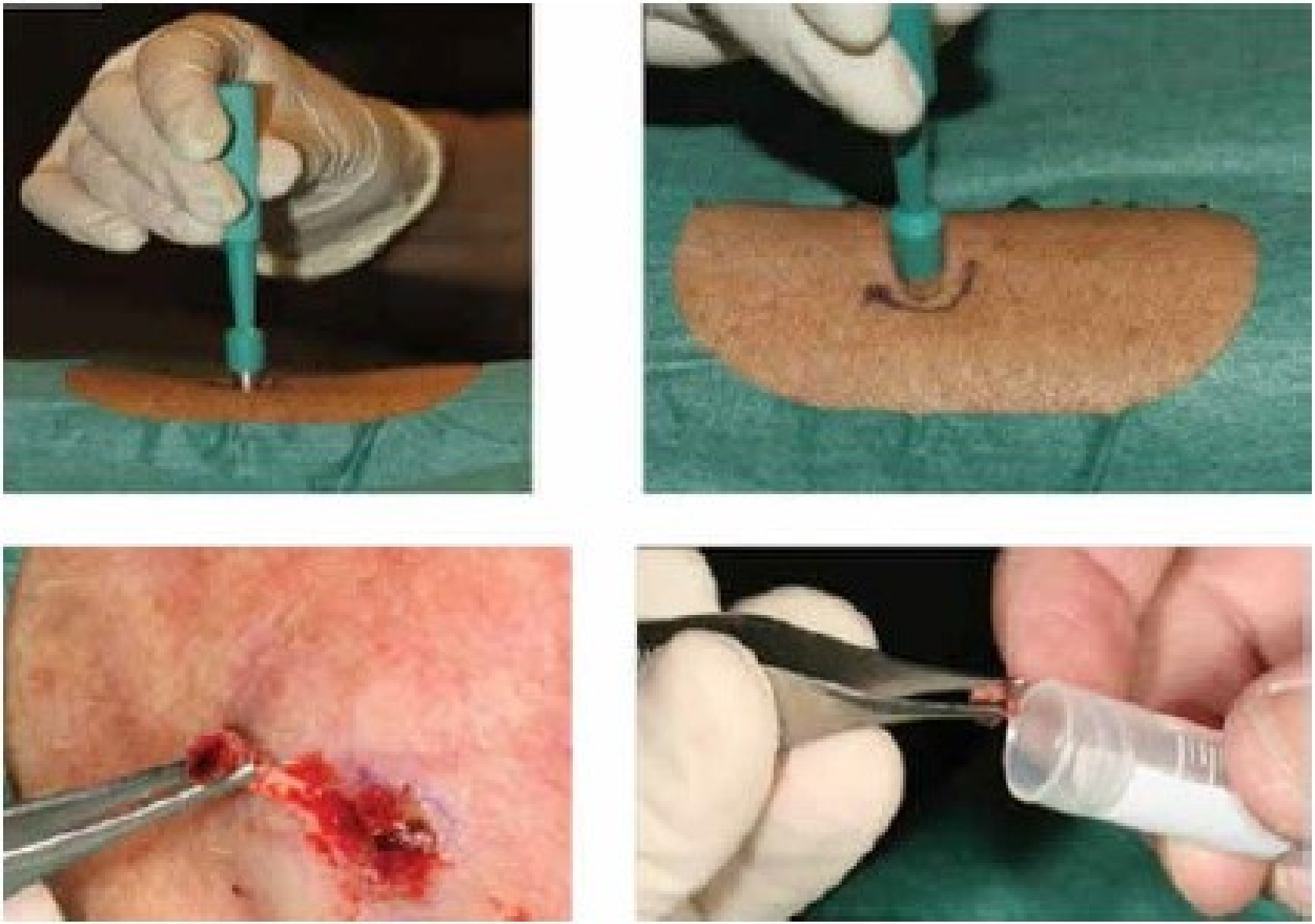
World Health Organization

- All data collected globally from 2010 to 2015 was synthesized during the global Consultation on Antimicrobial Resistance Surveillance in October 2016. Formal reports were received on a total of 1086 relapse cases and 776 new cases tested globally before the end of 2015, among which **resistance to rifampicin** was identified in 57 relapse cases (5.2% secondary resistance) and 16 new cases (2.1% primary resistance).

Fig. 2. Skin smear examination and centrifuge tube with 1 mL of 70% ethanol for collection of tissue specimens

Fig. 3. Skin punch biopsy sampling and tube for transportation

(photo courtesy: Dr Beatrice Flageul)



- A skin biopsy is collected preferably using a punch of 4 mm for new cases. For retreatment cases, a surgical biopsy of 6 mm is preferred, especially if the BI is close to 2+. The biopsy is then placed in a 1.8 mL centrifuge sterile tube (with screw cap), pre-filled with 1 mL of 70% ethanol (molecular biology grade absolute ethanol at 70% v/v + sterile deionized water
- from MilliQ or human injection quality 30% v/v, the mix being prepared in the laboratory) as described above. If this cannot be prepared at the health facility, biopsies can stay in an empty 1.8 mL sterile centrifuge tube (with screw cap) without any preservatives.
- Samples can be kept at room temperature until they are sent to the laboratory, possibly in batches, depending on the cost of transportation and on the number of samples per month.
- Bacilli are rapidly inactivated, which means that samples can be sent by routine transport without the need to control the temperature during transportation, or take additional precautions for biohazard control.

Take home message

Purpose	Method currently in use
For routine diagnosis	Clinical examination, SSS microscopy, HPE, PCR
To assess response to treatment	SSS, viability PCR
To assess drug resistance	PCR followed by sequencing; almost replaced animal inoculation
To detect *M.leprae* in environment	(Only for research)PCR
To detect latent infection	No test is currently recommended

THANK YOU