# PATHOGEN DETECTION

## Amanda Brum Fontes
Oswaldo Cruz Institute

## Flavio Alves Lara
Oswaldo Cruz Institute

## Adalberto Rezende Santos
Oswaldo Cruz Institute

## Philip Suffys
Oswaldo Cruz Institute

# Detection of *M. leprae* by Microscopy

Detection of acid-fast bacilli (AFB) by microscopy is recognized as the fastest, easiest, and least expensive tool for the rapid identification of leprosy cases. In many regions where leprosy is endemic, diagnosis is based purely on the detection of skin lesions and sensory loss. Although serology or PCR-based procedures have shown their value for leprosy diagnosis, bacilloscopy, which consists of the detection of AFB in lymph samples or in a microtome section of a skin biopsy, is still the basis for confirming clinically suspected leprosy (see Chapter 2.4). The preparation of microscopic slides from lymph samples is simple; however, the histopathologic analysis of skin biopsies is far more complex, as it demands personnel who are trained in the collection, fixation, preparation, and interpretation of skin and/or nerve biopsy slides. Although the specificity of acid-fast microscopy is excellent for *Mycobacterium* species and some related genera such as *Nocardia* and *Rhodococcus*, its sensitivity is less than that of other procedures.

The most commonly used staining technique to identify *Mycobacterium leprae* was first described by the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Neelsen (1854–1898) as a simple improvement of Robert Koch's complex staining method. While Ziehl was the first to

use carbolic acid (phenol) as the mordant in primary staining solution, Neelsen used basic fuchsin as the primary stain, resulting in the method known as Ziehl-Neelsen staining in the early to mid 1890s (1). Initially, the procedure applied heat so that the primary stain could penetrate the *Mycobacterium* waxy cell walls ("hot staining"). However, in 1915, Kinyoun published the "cold staining" variant, replacing the use of heat with the use of a higher concentration of carbolfuchsin in the primary stain (2). *Mycobacterium* species are stained bright red by carbolfuchsin and stand out clearly against a methylene blue background. Anderson was the first to relate the acid-fast property of *M. tuberculosis* to the presence of a membrane rich in a specific compound, mycolic acid. Mycolic acids confer resistance to decolorization by acids, including ethanol and hydrochloric acids, during staining procedures. Hence the term "acid-fast" (3).

When searching for the leprosy bacillus in smears or tissue samples, Ridley and Jopling established that a negative result should only be reported following the examination of at least 100 microscopic oil immersion fields, as recommended for tuberculosis (4). For that reason, the correct histological analysis is time-consuming and laborious. The number of bacilli identified by this method, together with the clinical and histopathological features, helps classify the disease form. The Ridley and Jopling classification of leprosy utilizes the bacilloscopic index (BI; see Chapter 2.4), varying from a score of 0 to 6, and is based on a logarithmic scale in which 0 represents the absence of bacillus; 1+ represents 1–10 bacilli in 100 fields; 2+, the presence of 1–10 bacilli in 10 fields; and 3, 4, 5, and 6+ represent the identification of 1–10, 10–100, 100–1000, and >1000 bacilli per field, respectively (4).

The Ziehl-Neelsen and Kinyoun methods remain reliable ways to visualize the presence of acid-fast bacteria in human exudates smears. However, a more recent adaptation of the Kinyoun staining method, the Fite-Faraco method, is currently the preferred staining procedure to identify *M. leprae* in human tissues (see Chapter 2.4). The main adaptation in the Fite-Faraco method is the dilution of the solvent xylene in the vegetable oils used during the deparaffinization step, because *M. leprae* is much less acid- and alcohol-fast than *M. tuberculosis* and thus can easily be missed in the examination of the slide.

The use of fluorochromes as alternatives for acid-fast staining was introduced with the auramine O-based method described by Hagemann (5) and the auramine-rhodamine–based method by Truant. Both stains result in strong orange fluorescently stained mycobacteria (6). A comparative study on staining procedures was performed using acid-fast sputum smear examinations for the detection of *M. tuberculosis* and demonstrated that the Truant method is the most sensitive, followed by Ziehl-Neelsen, and the Kinyoun method less sensitive (7), but there are no similar comparative data for *M. leprae*. In the last decade, the development of antibodies against *M. leprae* antigens and novel light microscopy-based techniques have become available, improving the imaging of *M. leprae* in human tissues and allowing observation at a higher resolution and with better sensitivity, with great impact on research but, unfortunately, with no practical contribution in diagnosis (8).
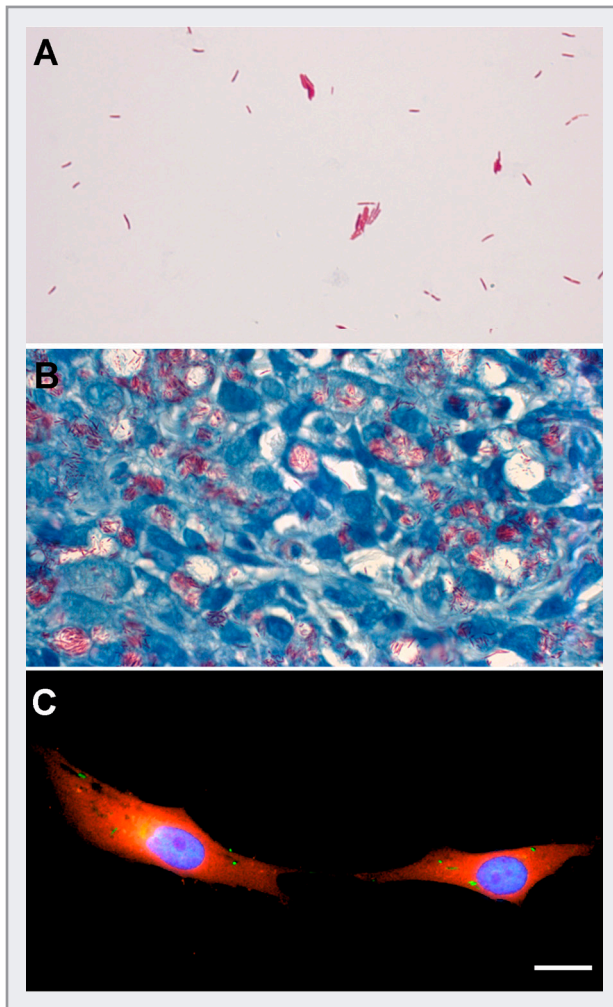
FIG 1 Staining of *M. leprae*.

A. Kinyoun staining of purified M. leprae harvested from a nude mouse footpad.

B. Fite-Faraco staining from a multibacillary leprosy skin lesion (courtesy of Dr. Sergio Antunes).

C. Confocal fluorescent immunocytochemistry of Schwann cells (red using antibody against S100) infected with M. leprae (green using antibody against PGL-1). Nuclei are blue with DAPI staining (courtesy of Dr. Victor Tulio Resende). Scale bar equals 13 µM in A; 20 µM in B and C.

**Highlights**

- Acid-fast microscopy is still the most frequently used detection tool for confirmation of leprosy.

- Fite-Faraco staining is the preferred method for staining *M. leprae*.

- Bacterial load (acid-fast bacilli counts) is associated with clinical forms of the disease.

- Microscopy in general provides low sensitivity in paucibacillary cases and the differentiation of *Mycobacterium leprae* from other *Mycobacterium* species is impossible.

- The bacterial index (BI) indicates the number of bacilli present in a specimen (see also Chapter 2.4 Appendix).

- Microscopy based on fluorochromes and antibodies against *M. leprae* provide higher sensitivity but are not used for routine diagnosis.

# Detection of *M. leprae* by Nucleic Acid-Based Tools

Although the detection of acid-fast bacilli (AFB) in tissue smears, lymph, or histological sections using various staining methods is satisfactory for confirming the diagnosis of more advanced leprosy, this procedure is less effective for diagnosing leprosy in the early stages, when clinical manifestations are not always clearly established, and in some paucibacillary (PB) cases. Because of the low specificity and sensitivity of serological tests (mainly for PB cases) (9), the low sensitivity of microscopy (1–3 x $10^4$ AFB/g), and the inability to differentiate *M. leprae* from other mycobacteria by microscopy, the use of nucleic acid-based methods for detecting *M. leprae* has been widely investigated.

The first report on the use of nucleic acid-based detection of *M. leprae* came from Clark-Curtiss and Docherty in 1989 (10). They described the use of a 2.2-kb *M. leprae* DNA fragment that allowed the specific detection of bacilli in material from multibacillary (MB), but not PB, patients by a hybridization-based procedure. In the same year, Woods and Cole (11) described the use of PCR for selective amplification of part of the *M. leprae*-specific repetitive sequence RLEP, describing agarose gel-based visualization of about 100 *M. leprae* cells present in armadillo liver, mouse footpads, and human biopsies. Using a heat-stable Taq DNA polymerase, Hartskeerl et al. (1989) (12) described specific amplification of the *M. leprae* gene encoding a 36 kDa protein, with a detection limit approximating one organism. Subsequently, several other PCR systems for *M. leprae* detection were developed, revealing detection limits ranging from one to 1000 bacilli (13, 14, 15, 16, 17, 18). Mostly agarose gel-based observations of amplicons have been reported, but some studies mention the use of hybridization to membranes (9), colorimetric assay in microtiter plates (19, 20, 21, 22), three primer systems (23), dot blot hybridization (24), nested PCR (25), peptide-nucleic-acid-ELISA (PNA-ELISA) (26), reverse line probe (27, 28), or high throughput reverse blotting (29) to detect amplicons.

Initially, purified *M. leprae* DNA, armadillo tissue, and fresh or paraffin-embedded skin biopsy samples were the most common specimens used for PCR, and several protocols for their treatment for PCR have been described with variable PCR yields (30, 31, 32, 33). With the development of procedures for DNA extraction of different types of tissues, PCR-based detection of *M. leprae* DNA in other clinical specimens such as skin smears, nerve biopsy, urine, oral and nasal swabs, blood, lymph node, hair bulbs, and ocular lesions became feasible.

Different PCR systems, targets, and applications for detecting *M. leprae* are summarized in Table 1. In 1993, Santos et al. (17) described the use of the PCR system described by Woods and Cole (11) with the inclusion of a hybridization step with an internal oligonucleotide, allowing the detection of 100 attograms of pure *M. leprae*-DNA, equivalent to one-tenth of a bacterial genome. Besides PCR-assay design, the quality of target DNA and the presence of PCR inhibitors can influence PCR yields. In addition, sample type and DNA extraction procedures are important for effec-

tive PCR assays. Santos et al. (17) evaluated extraction procedures on fresh skin biopsies, blood, and lymph fluid from the ear lobes of both MB and PB patients. Their results showed that simple freezing-boiling cycles in the presence of Triton X100 and pre-treatment with NaOH to eliminate PCR inhibitors was sufficient to yield amplification of bacterial DNA even in samples from PB patients. Simple methods for the extraction of DNA from clinical samples have been developed involving proteinase K-Tween 20 treatment without any further DNA purification (30). In that study, good PCR results were obtained using frozen or buffered formalin fixed biopsy samples. Negative results were obtained when samples were fixed with mercuric chloride or non-buffered formaldehyde in a study by Fiallo et al. (34). Pattyn et al. (35) reported an increase in PCR sensitivity when storing human biopsy or mouse footpad samples at room temperature for some weeks and the preferential use of 70% ethanol when compared to 10% formol for sample preservation. One elegant alternative for collecting and storing blood and lymph fluid from slit-skin smears is the use of Flinders Technology Associates (FTA) cards (36) as well as Ziehl-Neelsen slides for PCR detection of *M. leprae* (37) and genotyping (38). The use of commercial kits for sample processing has become routine practice; however, standardization is critical to avoid problems of PCR inhibition (39, 40).

When comparing PCR in skin biopsies of patients with different forms of the disease, Williams et al. (41) demonstrated that, besides confirmation of all MB cases, over 70% positivity was obtained in PB (smear negative) patients; a similar finding was described by Yoon et al. (42). In situ PCR of the skin lesion was later proposed for diagnosis in PB cases (43). Caleffi et al. (44) described a procedure amplifying a smaller fragment of the pra gene targeted by Parkash et al. (45) for the detection of *M. leprae* in urine samples of both tuberculoid (TT) and lepromatous (LL) leprosy patients. The procedure and results showed the potential for confirming the presence of *M. leprae* in TT cases that generally present negative AFB slit-skin smears. The influence of the size of the amplicon on PCR yield has also been described (46).

Very recently, Rosa et al. (47) investigated a loss of sensitivity in the oral cavities (see Chapter 2.4) of untreated leprosy patients by evaluating the presence of *M. leprae* in saliva using RT-PCR mediated amplification of the 85AC intergenic region. Although no direct relation between the presence of bacteria and loss of sensitivity was observed, positive saliva qPCR results from six out of 19 (31.6%) PB cases strongly suggested the potential of this clinical specimen for leprosy diagnosis. The presence of *M. leprae* in the oral epithelium was also confirmed by Morgado de Abreu et al. (48), suggesting that organisms shed from the oral cavity could be involved in disease transmission.

It has been shown that PCR can affect the diagnosis and treatment in cases of suspected early leprosy, even in the absence of confirmation by other diagnostic procedures (49). In such cases, however, extreme care and control of the PCR conditions should be practiced (50). Besides aiding the diagnosis of disease, nucleic acid amplification has been used to define populations at risk for disease development. De Wit et al. (51) investigated nasal swab specimens from clinically diagnosed leprosy patients to establish bacterial carriage among patients and evaluate the passive carriage and transfer to contacts of *M. leprae* in the nasal mucosa in endemic areas. Amplification products were found in 55% of untreated patients, 19% of the occupational contacts, 12% of

endemic controls, and none of the nonendemic controls, confirming the importance of the upper respiratory tract as a route of entrance, exit, and passive carriage in persons without symptoms of active disease. Pattyn et al. (52) found more positives in the nasal swabs of contacts from MB cases when compared to PB, but some of these MB cases had already been successfully treated. Santos et al. (53) confirmed the use of this sample for the detection of infected individuals at risk for developing the disease. But in 1994, van Beers et al. (54) reported that in an endemic region for leprosy, many individuals can carry *M. leprae* in their nose without symptoms of disease and, therefore, it is difficult to consider them at higher risk to develop leprosy. In addition, positive nasal swabs for *M. leprae* was not always related to the presence of disease (55, 56, 57). Preferentially, the evaluation of PCR data should be combined with the observation of other biomarkers for *M. leprae* and the use of different clinical samples (58). Monitoring transmission to contacts by PCR and, eventually, other diagnostic procedures has also been used in non-endemic countries such as Colombia (59, 60). In Brazil, PCR in nasal swabs together with anti-PGL-1 detection was used to detect subclinical infection in household contacts (61, 62). More data indicative that nasal carriage contributes to disease came from Bakker et al. (63), who demonstrated that patients with positive swabs have the highest transmission potential. Interestingly, Patrocinio et al. (64) demonstrated the invasion of nasal mucosa passing through the nasal inferior turbinate to reach peripheral blood.

Other types of samples were also investigated for the detection of subclinical infection, one of which was based on the microscopic observation of acid-fast bacilli in the hair follicles of leprosy patients, including PB cases, by Desikan et al. (65). Positive PCR was observed by Santos et al. (53) in hair bulbs from PB patients and from different areas of the body but not necessarily linked to the site of the lesion. However, Job et al. (66) demonstrated that both skin and nasal epithelia of untreated MB cases contribute to the shedding of *M. leprae* and cause a risk of infection for patient contacts. In 2004, Almeida and colleagues (67) performed RLEP PCR on blood and nasal swabs of healthy household contacts of leprosy patients and concluded that the test was not a valuable tool for defining individuals at risk. The data suggested a false association of PCR positivity and further development of leprosy. It should be emphasized that conventional PCR may not measure the viability of *M. leprae*, so the presence of *M. leprae* DNA may not predict infection and subsequent disease, or indicate the viability or death of bacilli during and after treatment. Nonetheless, positive PCR in the blood of index cases considerably increased the chance of household contacts developing the disease (68).

Conventional PCR has been evaluated for assessing viable bacillary load (30) and for follow up during and after treatment (69, 70). Interestingly, Santos et al. (71) observed that approximately half of the patients with indeterminate leprosy (I) were still PCR positive in one of their clinical samples analyzed by RLEP-PCR, four to eight years after they were discharged from MB or PB treatment schemes. The highest positivity rate was seen in blood samples, suggesting that live or dead bacilli are present and circulating much longer than expected (see Chapter 2.4). Conventional PCR is of limited value for indicating the efficacy of chemotherapy due to the amplification of DNA from dead bacilli (71). In addition, the reinfection of successfully treated individuals cannot be excluded, as observed in relapse cases in Brazil after therapy was completed (72) and as suggested by Rafi et al. (73).

In 1998, Kurabachew et al. (74) described the use of reverse transcriptase PCR (RT-PCR) targeting 16S rRNA for the detection of viable *M. leprae,* taking advantage of the high turnover rate of RNA. Jadhav et al. (75), Phetsuksiri et al. (76), Chae et al. (77), and Hirawati et al. (78) reported similar approaches for detecting the transcripts of the genes coding for the 18 kDa or 36 kDa proteins. This approach presents higher sensitivity and specificity than conventional PCR and, besides detection, allows for the quantification of *M. leprae* DNA (79, 80, 81, 82, 83, 84, 85, 86, 87, 88). Rudeeaneksin et al. (84) described the use of the *M. leprae* 16S rRNA gene as a target, allowing the detection of 20 fg of *M. leprae* DNA, equivalent to four bacilli, in skin biopsy specimens. Their results demonstrated 100% concordance with the clinical diagnosis of MB leprosy and 50% with that of PB. Martinez et al. (79) compared conventional and real-time PCR on frozen skin biopsy specimens from 69 leprosy patients using the antigen 85B-coding gene or the 85A-C intergenic region as a target for amplification. Using this approach, the detection and quantification of *M. leprae* DNA was possible in cases in which no bacilli were observed in conventional histological staining. Shamsi (89) performed both types of PCR on the 85 A-C intergenic region and confirmed the presence of *M. leprae* DNA by conventional PCR while estimating the copy number in ocular tissues from leprosy patients. Truman et al. (86) found excellent correlative results between RLEP TaqMan PCR and direct microscopic counting, permitting the detection of low numbers of bacilli and the rapid analysis of batch samples with high reproducibility. Martinez et al. (85) performed qPCR on frozen skin biopsy samples from untreated MB and PB leprosy patients as well as ten patients suffering from other dermatological diseases and five healthy donors. Interestingly, the PCR was positive in three cases not diagnosed as having leprosy and these patients developed leprosy 5–10 years after the collection of the biopsy. Four more non-leprosy cases were positive, suggesting that the patients had had the disease earlier or had subclinical leprosy. These results suggest that RLEP assay could be useful as a sensitive diagnostic test for detecting an *M. leprae* infection before major clinical manifestations.

Conventional and semi-quantitative PCR (using reverse transcription for comparison of RNA- and DNA-based targets) has been used for evaluating the efficiency of therapy. This use demonstrated that the monitoring of bacillary DNA and mRNA in lesions can be instructive with respect to disease progression and treatment regimen, as described in more detail in Chapter 5.3.

It is particularly difficult to diagnose the purely neural form of leprosy (PNL; see Chapter 2.5) (90). According to Ridley and Jopling (91), PNL occurs across the spectrum from the borderline lepromatous (BL) to the tuberculoid (TT) form. Because of the absence of skin lesions and clear histopathological features in the nerve, these patients cannot be classified as PB because they do not present bacilli in the skin smears (92). Thus, the diagnosis of PNL cases is always a challenge; however, PCR has been demonstrated to be a helpful tool for this purpose (90, 93, 94, 95). Bezerra da Cunha FM et al. (93) evaluated 58 patients believed to have PNL using RLEP-PCR on nerve biopsy. Fifty percent (50%) of the cases were positive, including 14 out of 38 cases that were negative for AFB. In the same year, Martinez et al. (79), using conventional and TaqMan RT-PCR assays, analyzed normal skin samples from six patients exhibiting PNL. Five of the patients were positive for the presence of *M. leprae* DNA, despite the absence of skin lesions. Related to the infection of the nerve system, Aung et al. (96) reported *M. leprae* by PCR in the spinal chord

and the cerebral cortex (97), while De et al. (98) reported a unique case of isolated tenosynovitis as the sole manifestation of PNL.

PCR also can help to differentiate leprosy from other diseases that present hypochromic or granulomatous lesions such as pityriasis alba, leishmaniosis, cutaneous tuberculosis (TB), sarcoidosis, and co-infections with other mycobacterioses as discussed in the chapter on the differential diagnosis of leprosy (see Chapter 2.3).

PCR-meditated detection of *M. leprae* has been used successfully with material from human remains (see Chapter 11.1) (99, 100). Such studies revealed that co-infection of *M. leprae* and M. tuberculosis could have led to the decline of leprosy (101) and that ancient *M. leprae* in medieval Europe was of the SNP genotype 3 (102) and in Japan was type 1 (103). Mendum et al. (104) reported that a shift from the type 2 strains to the later observed European and associated North American type 3 isolates may have occurred in Scandinavia and England during the 11th century. Interestingly, the use of PCR-mediated detection of *M. leprae* also demonstrated that leprosy cases were buried using the Nabe-kaburi method, indicating a social stigma against infectious diseases in 15th- to 18th-century Japan (105). A remarkable conservation of the *M. leprae* genome over time has been observed (106), and palaeomicrobiologic findings of leprosy have been reviewed by Donoghue (107).

Finally, the PCR-mediated detection of drug-resistant *M. leprae* (see Chapter 5.2) was first described by Williams et al. (108), who used a procedure for the simultaneous detection of *M. leprae* and resistance to dapsone. Later, direct PCR sequencing of genes associated with the resistance to rifampin and fluoroquinolones was added, allowing the detection of multi-drug resistant (MDR) leprosy. Although mostly limited to MB cases, MDR resistance has also been detected in PB leprosy (47). Other tools for drug detection such as RT-PCR, digital PCR, and whole genome sequencing can detect minor populations of resistant bacteria with much higher sensitivity and may become useful procedures for future studies.

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**Highlights**

- Nucleic acid-based detection techniques for *M. leprae* are becoming a standard of care for supporting the diagnosis of leprosy.

- Semi-quantitative PCR (qPCR) and reverse transcriptase-based PCR (RT-PCR) systems have been developed with enhanced sensitivity over conventional PCR, depending on the target gene or DNA sequence measured.

- The repetitive sequence RLEP is a promising target for PCR, allowing the detection of approximately one *M. leprae* genome.

- PCR-based detection is not positive in many PB biopsies (i.e., the sensitivity is not high in PB disease).

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Highlights (cont'd)

- Many clinical samples (e.g., skin, saliva, lymph, blood, hair bulb) can be examined for *M. leprae* DNA.

- PCR tests have helped diagnose the pure neural form of leprosy.

- PCR-based detection of *M. leprae* has been used to identify infected individuals at risk for developing leprosy. However, because of the high sensitivity of PCR-based systems, positive results can be due to passive carriage of bacilli.

- Nucleic acid-based analysis enables the differentiation of leprosy from other dermatoses.

- PCR can be used in paleomicrobiology (see Chapter 11.1), genotyping, and detection of drug-resistant strains of *M. leprae*.

# Detection of *M. leprae* in the Environment

Despite being studied for centuries, leprosy transmission has not been clearly elucidated. The main transmission source seems to be undiagnosed MB patients, most likely through spreading and absorption via the nasopharyngeal airway (109). Person-to-person transmission by direct contact or through respiratory secretions has not been proven; however, cases of leprosy have been described in the absence of contact with other leprosy cases. The presence of *M. leprae* in non-human sources and the environment could be partly responsible for the maintenance of leprosy endemicity in some parts of the world.

Numerous studies have identified the presence of *M. leprae* in rivers, wells, and soil samples. The identification of *M. leprae* has been based on a variety of detection procedures, including microscopy, mouse footpad assay, and identification by DNA/RNA amplification (110). Authors have suggested that soil (111, 112), water (113), insects (114), hemiptera (115), plants (116), armadillos (Chapter 10.2), and rodents (Chapter 10.3) could act as non-human sources of infection. Yet the notion of free-living *M. leprae* persisting in the environment is biologically improbable (117) due to its compromised genome (Chapter 8.2) resulting from reductive evolutionary changes.

Viability studies of *M. leprae* (see Chapter 5.3) by reverse transcriptase-PCR of 16S RNA has resulted in the detection of significantly more samples with viable bacilli in the soil in areas where leprosy patients resided (111, 118, 119, 120). Although these "live bacteria" were likely shed from infected humans, as demonstrated by characterizing SNPs that define the main *M. leprae* lineages, infectivity was not demonstrated. Nonetheless, Desikan et al. (121) showed that *M. lep-*

*rae* can survive in a diverse set of environmental conditions in soil or water for weeks and even months and maintain infectivity in the mouse footpad assay.

Unfortunately, there are several methodological barriers to identifying a non-cultivable bacterium from a complex sample such as soil that may contain many of the 150+ Mycobacterium species, including uncultured environmental isolates. Thus, recognizing that soils and aquifers are environments naturally rich in unknown and non-cultivable mycobacteria, care should be taken not to base our assumptions on the presence of a single marker for *M. leprae* without confirmation by sequencing a large number of *M. leprae*-specific genes.

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**Highlights**

- Doubts remain regarding the contribution of non-human sources of *M. leprae* to infection and disease transmission.

- Besides infected humans, possible sources of infection are the environment, insects, and other vectors and animals.

- Conventional PCR for detection can be supported by viability testing using RT-PCR.

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**TABLE 1 PCR assays for Mycobacterium leprae detection**

| DNA Target | PCR method | Material | Results | References |
|---|---|---|---|---|
| RLEP, rpoT, SodA, and 16S rRNA | PCR | Slit-skin smear (SSS), blood, soil samples of leprosy patients and their surroundings | The RLEP gene target was able to detect the presence of *M. leprae* in 83% of SSS, 100% of blood samples, and 36% of soil samples and was noted to be the best out of all other gene targets. | Turankar et al. 2015 (122) |
| ML0024 | qPCR | Peripheral blood | *M. leprae* DNA was detected in 22% of leprosy patients: 23.2% in PB and 21.4% in MB. Positivity among contacts was 1.2%. | Reis et al. 2014 (68) |
| RLEP | TaqMan real-time PCR | Paraffin embedded skin biopsy | The PCR detection rate for PB specimens was 74.5%. | Yan et al. 2014 (88) |

**TABLE 1 PCR assays for Mycobacterium leprae detection**

| DNA Target | PCR method | Material | Results | References |
|---|---|---|---|---|
| MntH | PCR | Paraffin blocks containing biopsy samples from tongue, buccal mucosa, and soft palate. | *M. leprae* DNA was detected in 78% of patients. | Morgado de Abreu et al. 2014 (48) |
| RLEP and TTC | Multiplex-PCR | Fine needle aspiration from the peripheral nerves of PNL. | *M. leprae* could be elicited in the nerve aspirates in 84.6% of the samples. | Reja et al. 2013 (123) |
| RLEP and TTC | Multiplex-PCR | Biopsy | PCR sensitivity was 87.8%, positive predictive value (PPV) was 95.6%, and negative predictive value (NPV) was 71.2%. | Reja et al. 2013 (124) |
| GroE-L (65-kDa) | Nested-PCR | Whole blood | *M. leprae* DNA was detected in 95.92% of MB, 70% of PB, and 6.25% of household contacts. | Wen et al. 2013 (125) |
| pra (36-kDa) | PCR | Urine samples | *M. leprae* DNA was detected in 46.6% of the cases. The positivity for patients with the TT form was 75%. In LL, the positivity was 52% and 30% for patients under treatment and non-treated patients, respectively. | Caleffi et al. 2012 (44) |
| RLEP | Multiplex PCR | Skin biopsy and slit-skin smear | PCR positivity was 82.3%. | Banerjee et al. 2011 (126) |
| ML0024 | qPCR | Lesion in the palate | Before treatment the PCR was positive ; however, after MDT, the result was negative. | Da Silva Martinez et al. 2011 (81) |
| RLEP | PCR | Slit skin smears preserved using FTA elute cards and 70% ethanol tubes | *M. leprae* DNA was detected in 60% and 58% of samples preserved in FTA elute cards and 70% ethanol, respectively. | Aye et al. 2011 (36) |
| 16S rRNA | PCR | Stained slit-skin smear negative slides | *M. leprae* DNA was detected in 32.6% of the samples. | Kamble et al. 2010 (37) |

**TABLE 1 PCR assays for Mycobacterium leprae detection**

| DNA Target | PCR method | Material | Results | References |
|---|---|---|---|---|
| 16S rRNA | PCR | Skin biopsy | The detection rate in MB and PB were 100% and 50%, respectively. | Bang et al. 2009 (127) |
| 16S rRNA | LightCycler real-time PCR | Skin biopsy | 100% of concordance with clinical diagnosis in cases of MB and 50% of PB leprosy. | Rudeeaneksinet al. 2008 (84) |
| 85 A-C intergenic region | Conventional and real-time PCR | Paraffin-embedded ocular tissue | *M. leprae* DNA can be detected using RT-PCR when acid-fast bacteria are seen in histopathological sections. | Shamsi et al. 2007 (89) |
| Antigen 85B-coding gene and 85A-C intergenic region | Conventional and TaqMan real-time PCR | Frozen skin biopsy specimens | The detection rate in MB was 100% and ranged from 62.5% to 79.2% in PB according to the assay used. | Martinez et al. 2006 (79) |
| RLEP | PCR | Nasal mucosa biopsies | Sensitivity of 69.2%, specificity of 89.9%, and an accuracy of 82.8% | Patrocínio et al. 2005 (64) |
| pra (36-kDa) | Real-time PCR | Skin biopsies | The sensitivity ranged from 33.3% to 88.9% in PB and MB patients, respectively. | Kramme et al. 2004 (82) |
| RLEP | PCR and southern hybridization | Blood and nasal swabs from healthy household contacts | Positivity in blood and nasal secretion was 1.7%. | Almeida et al. 2004 (67) |
| pra (36-kDa) | PCR | Urine | DNA of *M. leprae* was detected in 36.4% of lepromatous leprosy and in 40% of tuberculoid. The positivity among treated patients was 66.6%, while it was only 20% for untreated patients. | Parkash et al. 2004 (45) |
| 15kDa | PCR | Nasal mucus from asymptomatic household contacts | Bacillus was detected in 12.8% of the household contacts. | Guerrero et al. 2002 (128) |

**TABLE 1 PCR assays for Mycobacterium leprae detection**

| DNA Target | PCR method | Material | Results | References |
|---|---|---|---|---|
| RLEP | PCR | Blood, lymph, hair, nasal secretion, and skin biopsy | First report on detection of *M. leprae* DNA in PB patients, more than 5 years after completion of MDT. | Santos et al. 2001 (71) |
| RLEP | PCR | Blood, lymph, nasal secretion, and hair. | No conclusive diagnosis by traditional methods. However the individual was found to be infected with *M. leprae* after amplification of the bacterial DNA. | Santos et al. 1997 (129) |
| LSR/15kDA | PCR | Skin biopsy and slit-skin smears | PCR was specific and sensitive, with a detection level of 10 and 100 bacilli. Skin biopsies gave a higher detection rate than did slit-skin smears. | Misra et al. 1995 (24) |
| pra (36-kDa) | PCR | Fresh biopsy and slit-skin smear | In MB, 87.1% of biopsy specimens and 41.9% of slit-skin smears were positive. In PB, 36.4% of biopsy specimens and 18.2% of slit-skin smear specimens yielded detectable PCR amplification. | Wichitwechka et al. 1995 (130) |
| pra (36-kDa) | PCR | Sputum and slit-skin samples from treated patients | 25% of patients were found to be PCR positive. | Rafi et al. 1995 (73) |
| pra (36-kDa) | PCR | Nasal swab specimens | Among the total tested population, 7.8% were found to be PCR positive. | Klatser et al. 1993 (131) |
| pra (36-kDa) | PCR | Neutral formalin-fixed biopsy samples and frozen biopsy | Frozen sections: 100% positive in samples from untreated AFB-positive patients and 56% of the untreated AFB-negative. Fixed samples: 92% positive in samples from untreated AFB-positive and 61% of the samples from untreated AFB-negative. | de Wit et al. 1991 (30) |

# References

1.   **Bishop PJ, Neumann G.** 1970. The history of the Ziehl-Neelsen stain. Tubercle **51**(2)**:**196–206. PubMed PMID: 4099679. Epub 1970/06/01. eng.

2.   **Kinyoun JJ.** 1915. A note on uhlenhuths method for sputum examination, for tubercle bacilli. Am J Public Health (N Y) **5**(9)**:**867–870. PubMed PMID: 18009310. Pubmed Central PMCID: PMC1286695. Epub 1915/09/01. eng.

3.   **World Health Organization (WHO).** 2013. Laboratory services in tuberculosis control: microscopy. **258**(Part II). Epub 1998. eng.

4.   **Hagemann P.** 1938. Fluoreszensfärbung von Tuberkelbakterien mit Auramin. Münch Med Wochenschr **85:**1066–1068.

5.   **Truant JP, Brett WA, Thomas W Jr.** 1962. Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. Henry Ford Hosp Med Bull **10:**287–296. PubMed PMID: 13922644. Epub 1962/06/01. eng.

6.   **Somoskövi Á, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M.** 2001. Lessons from a proficiency testing event for acid-fast microscopy. Chest **120**(1)**:**250–257. PubMed PMID: 11451846. Pubmed Central PMCID: PMC2925666. Epub 2001/07/14. eng.

7.   **Hanscheid T.** 2008. The future looks bright: low-cost fluorescent microscopes for detection of Mycobacterium tuberculosis and Coccidiae. Trans R Soc Trop Med Hyg **102**(6)**:**520–521. PubMed PMID: 18405928. Epub 2008/04/15. eng.

8.   **Huang L, Fang W, Yu Y, Song H.** 2012. [Membrane protein characterization by photoactivatable localization microscopy]. Sheng Wu Gong Cheng Xue Bao **28**(11)**:**1378–1387. PubMed PMID: 23457790. Epub 2013/03/06. chi.

9.   **Burgess PJ, Fine PE, Ponninhaus JM, Draper C.** 1988. Serological tests in leprosy. The sensitivity, specificity and predictive value of ELISA tests based on phenolic glycolipid antigens, and the implications for their use in epidemiological studies. Epidemiol Infect **101**:159–171**.**

10.  **Clark-Curtiss JE, Docherty MA.** 1989**.** A species-specific repetitive sequence in Mycobacterium leprae DNA**.** J Infect Dis **159:**7–15.

11.  **Woods AS, Cole ST.** 1989. A rapid method for the detection of potentially viable Mycobacterium leprae in human biopsies: a novel application of PCR. FEMS Microbiol Lett **53**(3)**:**305–309.

12. **Hartskeerl RA, De Wit MY, Klatser PR.** 1989. Polymerase chain reaction for the detection of Mycobacterium leprae. J Gen Microbiol **135:**2357–2364.

13. **Williams DL, Gillis TP, Booth RJ, Looker D, Watson JD.** 1990. The use of a specific DNA probe and polymerase chain reaction for detection of Mycobacterium leprae. J Infect Dis **162**(1)**:**193–200.

14. **Plikaytis BB, Gelber RH, Shinnik TM.** 1990. Rapid and sensitive detection of Mycobacterium leprae using a nested-primer gene amplification assay. J Clin Microbiol **28:**1913–1917.

15. **Moudgil KD, Williams DL, Gillis TP.** 1992. DNA hybridization analysis of mycobacterial DNA using the 18-kDa protein gene of Mycobacterium leprae. FEMS Microbiol Immunol **4**(3)**:**165–174.

16. **Arnoldi J, Schlüter C, Duchrow M, Hübner L, Ernst M, Teske A, Flad HD, Gerdes J, Böttger EC.** 1992. Species-specific assessment of Mycobacterium leprae in skin biopsies by in situ hybridization and polymerase chain reaction. Lab Invest **66**(5)**:**618–623.

17. Santos **AR, De Miranda AB, Sarno EN, Suffys PN, Degrave WM.** 1993. Use of PCR-mediated amplification of Mycobacterium leprae DNA in different types of clinical samples for the diagnosis of leprosy. J Med Microbiol **39**(4)**:**298–304.

18. **Kang TJ, Kim SK, Lee SB, Chae GT, Kim JP.** 2003. Comparison of two different PCR amplification products (the 18-kDa protein gene vs. RLEP repetitive sequence) in the diagnosis of Mycobacterium leprae. Clin Exp Dermatol **28**(4)**:**420–424.

19. **van der Vliet GM, de Wit MY, Klatser PR.** 1993. A simple colorimetric assay for detection of amplified Mycobacterium leprae DNA. Mol Cell Probes **7**(1)**:**61–66.

20. **van der Vliet GM, Hermans CJ, Klatser PR.** 1993. Simple colorimetric microtiter plate hybridization assay for detection of amplified Mycobacterium leprae DNA. J Clin Microbiol **31**(3)**:**665–670.

21. **Jamil S, Wilson SM, Hacket M, Hussain R, Stoker NG.** 1994. A colorimetric PCR method for the detection of M. leprae in skin biopsies from leprosy patients. Int J Lepr Other Mycobact Dis **62**(4)**:**512–520.

22. **Haile Y, Ryon JJ.** 2004. Colorimetric microtitre plate hybridization assay for the detection of Mycobacterium leprae 16S rRNA in clinical specimens. Lepr Rev **75**(1)**:**40–49.

23. **Richter E, Duchrow M, Schlüter C, Hahn M, Flad HD, Gerdes J.** 1994. Detection of Mycobacterium leprae by three-primer PCR. Immunobiology **191**(4–5)**:**351–353.

24.   **Misra N, Ramesh V, Misra RS, Narayan NP, Colston MJ, Nath I.** 1995. Clinical utility of LSR/A15 gene for Mycobacterium leprae detection in leprosy tissues using the polymerase chain reaction. Int J Lepr Other Mycobact Dis **63**(1)**:**35–41.

25.   **Donoghue HD, Holton J, Spigelman M.** 2001. PCR primers that can detect low levels of Mycobacterium leprae DNA. J Med Microbiol **50**(2)**:**177–182.

26.   **Jadhav RS, Macdonald M, Bjune G, Oskam L; MILEP2 Study Group.** 2001. Simplified PCR detection method for nasal Mycobacterium leprae. Int J Lepr Other Mycobact Dis **69**(4)**:**299–307.

27.   **Sapkota BR, Ranjit C, Macdonald M.** 2006. Reverse line probe assay for the rapid detection of rifampicin resistance in Mycobacterium leprae. Nepal Med Coll J **8**(2)**:**122–127.

28.   **Xiong L, Kong F, Yang Y, Cheng J, Gilbert GL.** 2006. Use of PCR and reverse line blot hybridization macroarray based on 16S-23S rRNA gene internal transcribed spacer sequences for rapid identification of 34 mycobacterium species. J Clin Microbiol **44**(10)**:**3544–3550.

29.   **Wang HY, Kim H, Kim Y, Bang H, Kim JP, Hwang JH, Cho SN, Kim TU, Lee H.** 2015. Performance of PCR-reverse blot hybridization assay for detection of rifampicin-resistant Mycobacterium leprae. J Microbiol **53**(10)**:**686–693.

30.   **de Wit MY, Faber WR, Krieg SR, Douglas JT, Lucas SB, Montreewasuwat N, Pattyn SR, Hussain R, Ponnighaus JM, Hartskeerl RA, et al.** 1991. Application of a polymerase chain reaction for the detection of Mycobacterium leprae in skin tissues. J Clin Microbiol **29**(5)**:**906–910.

31.   **Sung KJ, Kim SB, Choi JH, Koh K, Na DS.** 1993. Detection of Mycobacterium leprae DNA in formalin-fixed, paraffin-embedded samples from multibacillary and paucibacillary leprosy patients by polymerase chain reaction. Int J Dermatol **32**(10)**:**710–713.

32.   **Nishimura M, Kwon KS, Shibuta K, Yoshikawa Y, Oh CK, Suzuki T, Chung TA, Hori Y.** 1994. Methods in pathology. An improved method for DNA diagnosis of leprosy using formaldehyde-fixed, paraffin-embedded skin biopsies. Mod Pathol **7**(2)**:**253–256.

33.   **Scollard DM, Gillis TP, Williams DL.** 1998. Polymerase chain reaction assay for the detection and identification of Mycobacterium leprae in patients in the United States. Am J Clin Pathol **109:**642–646.

34.   **Fiallo P, Williams DL, Chan GP, Gillis TP.** 1992. Effects of fixation on polymerase chain reaction detection of Mycobacterium leprae. J Clin Microbiol **30**(12)**:**3095–3098.

35. **Pattyn SR, Ursi D, Ieven M, Raes V, Jamet P.** 1992. Polymerase chain reaction amplifying DNA coding for species-specific rRNA of Mycobacterium leprae. Int J Lepr Other Mycobact Dis **60**(2)**:**234–243.

36. **Aye KS, Matsuoka M, Kai M, Kyaw K, Win AA, Shwe MM, Thein M, Htoo MM, Htoon MT.** 2011. FTA card utility for PCR detection of Mycobacterium leprae. Jpn J Infect Dis **64**(3)**:**246–248.

37. **Kamble RR, Shinde VS, Madhale SP, Kamble AA, Ravikumar BP, Jadhav RS.** 2010. Extraction and detection of Mycobacterium leprae DNA from ZNCF-stained skin smear slides for better identification of negative skin smears. Indian J Med Microbiol **28**(1)**:**57–59. doi: 10.4103/0255-0857.58732.

38. **Fontes AN, Gomes HM, Araujo MI, Albuquerque EC, Baptista IM, Moura MM, Rezende DS, Pessolani MC, Lara FA, Pontes MA, Gonçalves Hde S, Lucena-Silva N, Sarno EN, Vissa VD, Brennan PJ, Suffys PN.** 2012. Genotyping of Mycobacterium leprae present on Ziehl-Neelsen-stained microscopic slides and in skin biopsy samples from leprosy patients in different geographic regions of Brazil. Mem Inst Oswaldo Cruz **107**(Suppl 1)**:**143–149.

39. **da Silva Rocha A, Cunha Md, Diniz LM, Salgado C, Aires MA, Nery JA, Gallo EN, Miranda A, Magnanini MM, Matsuoka M, Samo EN, Suffys PN, de Oliveira ML.** 2012. Drug and multidrug resistance among Mycobacterium leprae isolates from Brazilian relapsed leprosy patients. J Clin Microbiol **50:**1912–1917. doi: 10.1128/jcm.06561-11.

40. **Fontes AN, Sakamuri RM, Baptista IM, Ura S, Moraes MO, Martinez AN, Samo EN, Brennan PJ, Vissa VD, Suffys PN.** 2009. Genetic diversity of Mycobacterium leprae isolates from Brazilian leprosy patients. Lepr Rev **80:**302–315.

41. **Williams DL, Gillis TP, Fiallo P, Job CK, Gelber RH, Hill C, Izumi S.** 1992. Detection of Mycobacterium leprae and the potential for monitoring antileprosy drug therapy directly from skin biopsies by PCR. Mol Cell Probes **6**(5)**:**401–410.

42. **Yoon KH, Cho SN, Lee MK, Abalos RM, Cellona RV, Fajardo TT Jr, Guido LS, Dela Cruz EC, Walsh GP, Kim JD.** 1993. Evaluation of polymerase chain reaction amplification of Mycobacterium leprae-specific repetitive sequence in biopsy specimens from leprosy patients. J Clin Microbiol **31**(4)**:**895–899.

43. **Natrajan M, Katoch K, Katoch VM, Das R, Sharma VD.** 2012. Histological diagnosis of early and suspicious leprosy by in situ PCR. Indian J Lepr **84**(3)**:**185–194.

44. **Caleffi KR, Hirata RD, Hirata MH, Caleffi ER, Siqueira VL, Cardoso RF.** 2012. Use of the polymerase chain reaction to detect Mycobacterium leprae in urine. Braz J Med Biol Res **45:**153–157. doi: 10.1590/s0100-879x2012007500011.

45. **Parkash O, Singh HB, Rai S, Pandey A, Katoch VM, Girdhar BK.** 2004. Detection of Mycobacterium leprae DNA for 36-kDa protein in urine from leprosy patients: a preliminary report. Rev Inst Med Trop São Paulo **46:**275–277.

46. **Goulart IM, Cardoso AM, Santos MS, Gonçalves MA, Pereira JE, Goulart LR.** 2007. Detection of Mycobacterium leprae DNA in skin lesions of leprosy patients by PCR may be affected by amplicon size. Arch Dermatol Res **299**(5–6)**:**267–271. Epub 2007 May 26.

47. **Rosa FB, Souza VC, Almeida TA, Nascimento VA, Vásquez FG, Cunha Mda G, Naveca FG.** 2013. Detection of Mycobacterium leprae in saliva and the evaluation of oral sensitivity in patients with leprosy. Mem Inst Oswaldo Cruz **108:**572–577. doi: 10.1590/s0074-02762013000500006.

48. **Morgado de Abreu MA, Roselino AM, Enokihara M, Nonogaki S, Prestes-Carneiro LE, Weckx LL, Alchorne MM.** 2014. Mycobacterium leprae is identified in the oral mucosa from paucibacillary and multibacillary leprosy patients. Clin Microbiol Infect **20**(1)**:**59–64. doi: 10.1111/1469-0691.12190. Epub 2013 Mar 8.

49. **Job CK, Jayakumar J, Williams DL, Gillis TP.** 1997. Role of polymerase chain reaction in the diagnosis of early leprosy. Int J Lepr Other Mycobact Dis **65**(4)**:**461–464.

50. **MILEP2 Study Group.** 2000. Approaches to studying the transmission of Mycobacterium leprae. Lepr Rev **71**(Suppl)**:**S26–S29.

51. **de Wit MY, Douglas JT, McFadden J, Klatser PR.** 1993. Polymerase chain reaction for detection of Mycobacterium leprae in nasal swabs specimens. J Clin Microbiol **31:**502–506.

52. **Pattyn SR, Ursi D, Ieven M, Grillone S, Raes V.** 1993. Detection of Mycobacterium leprae by the polymerase chain reaction in nasal swabs of leprosy patients and their contacts. Int J Lepr Other Mycobact Dis **61**(3)**:**389–393.

53. **Santos AR, Goes Filho JT, Nery JA, Duppre NC, Gallo ME, Suffys PN, Degrave WM.** 1995. Evaluation of PCR mediated DNA amplification in non-invasive biological specimens for subclinical detection of Mycobacterium leprae. FEMS Immunol Med Microbiol **11:**113–120.

54. **van Beers SM, Izumi S, Madjid B, Maeda Y, Day R, Klatser PR.** 1994. An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int J Lepr Other Mycobact Dis **62**(1)**:**1–9.

55. **Hatta M, van Beers SM, Madjid B, Djumadi A, de Wit MY, Klatser PR.** 1995. Distribution and persistence of Mycobacterium leprae nasal carriage among a population in which leprosy is endemic in Indonesia. Trans R Soc Trop Med Hyg **89**(4)**:**381–385.

56.  **Beyene D, Aseffa A, Harboe M, Kidane D, Macdonald M, Klatser PR, Bjune GA, Smith WC.** 2003. Nasal carriage of Mycobacterium leprae DNA in healthy individuals in Lega Robi village, Ethiopia. Epidemiol Infect **131**(2)**:**841–848.

57.  **Warndorff DK, Glynn JR, Fine PE, Jamil S, de Wit MY, Munthali MM, Stoker NG, Klatser PR.** 1996. Polymerase chain reaction of nasal swabs from tuberculosis patients and their contacts. Int J Lepr Other Mycobact Dis **64**(4)**:**404–408.

58.  **Torres P, Camarena JJ, Gomez JR, Nogueira JM, Gimeno V, Navarro JC, Olmos A.** 2003. Comparison of PCR mediated amplification of DNA and the classical methods for detection of Mycobacterium leprae in different types of clinical samples in leprosy patients and contacts. Lepr Rev **74**(1)**:**18–30.

59.  **Cardona-Castro N, Beltrán-Alzate JC, Manrique-Hernández R.** 2008. Survey to identify Mycobacterium leprae-infected household contacts of patients from prevalent regions of leprosy in Colombia. Mem Inst Oswaldo Cruz **103**(4)**:**332–336.

60.  **Cardona-Castro N, Beltrán-Alzate JC, Romero-Montoya M.** 2009. Clinical, bacteriological and immunological follow-up of household contacts of leprosy patients from a post-elimination area – Antioquia, Colombia. Mem Inst Oswaldo Cruz **104**(6)**:**935–936.

61.  **Araújo S, Lobato J, Reis Ede M, Souza DO, Gonçalves MA, Costa AV, Goulart LR, Goulart IM.** 2012. Unveiling healthy carriers and subclinical infections among household contacts of leprosy patients who play potential roles in the disease chain of transmission. Mem Inst Oswaldo Cruz **107**(Suppl 1)**:**55–59.

62.  **Brito e Cabral P, Júnior JE, de Macedo AC, Alves AR, Gonçalves TB, Brito e Cabral TC, Gondim AP, Pinto MI, Oseki KT, Camara LM, Rabenhorst SH, Nagao-Dias AT.** 2013. Anti-PGL1 salivary IgA/IgM, serum IgG/IgM, and nasal Mycobacterium leprae DNA in individuals with household contact with leprosy. Int J Infect Dis **17**(11)**:**e1005–e1010. doi: 10.1016/j.ijid. 2013.05.011. Epub 2013 Jul 16.

63.  **Bakker MI, Hatta M, Kwenang A, Van Mosseveld P, Faber WR, Klatser PR, Oskam L.** 2006. Risk factors for developing leprosy—a population-based cohort study in Indonesia. Lepr Rev **77**(1)**:**48–61.

64.  **Patrocínio LG, Goulart IM, Goulart LR, Patrocínio JA, Ferreira FR, Fleury RN.** 2005. Detection of Mycobacterium leprae in nasal mucosa biopsies by the polymerase chain reaction. FEMS Immunol Med Microbiol **44**(3)**:**311–316.

65.  **Desikan KV, Iyer CG.** 1972. The distribution of Mycobacterium leprae in different structures of the skin. Lepr Rev **43**(1)**:**30–37.

66.  **Job CK, Jayakumar J, Kearney M, Gillis TP.** 2008. Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. Am J Trop Med Hyg **78**(3)**:**518–521.

67.  **Almeida EC, Martinez AN, Maniero VC, Sales AM, Duppre NC, Sarno EN, Santos AR, Moraes MO.** 2004. Detection of Mycobacterium leprae DNA by polymerase chain reaction in the blood and nasal secretion of Brazilian household contacts. Mem Inst Oswaldo Cruz **99**(5)**:**509–511.

68.  **Reis EM, Araujo S, Lobato J, Neves AF, Costa AV, Gonçalves MA, Goulart LR, Goulart IM.** 2014. Mycobacterium leprae DNA in peripheral blood may indicate a bacilli migration route and high-risk for leprosy onset. Clin Microbiol Infect **20**(5)**:**447–452.

69.  **Kampirapap K, Singtham N, Klatser PR, Wiriyawipart S.** 1998. DNA amplification for detection of leprosy and assessment of efficacy of leprosy chemotherapy. Int J Lepr Other Mycobact Dis **66:**16–21.

70.  **Singh HB, Katoch K, Natrajan M, Sharma RK, Gupta UD, Sharma VD, Singh D, Chauhan DS, Srivastava K, Katoch VM.** 1999. Effect of treatment on PCR positivity in multibacillary leprosy patients treated with conventional and newer drugs ofloxacin and minocycline. Acta Leprol **11:**179–182.

71.  **Santos AR, Balassiano V, Oliveira ML, Pereira MA, Santos PB, Degrave WM, Suffys PN.** 2001. Detection of Mycobacterium leprae DNA by polymerase chain reaction in the blood of individuals, eight years after completion of anti-leprosy therapy. Mem Inst Oswaldo Cruz **96:**1129–1133.

72.  **da Silva Rocha A, Cunha Dos Santos AA, Pignataro P, Nery JA, de Miranda AB, Soares DF, Brum Fontes AN, Miranda A, Ferreira H, Boéchat N, Novisck Gallo ME, Sarno EN, De Oliveira ML, Suffys PN.** 2011. Genotyping of Mycobacterium leprae from Brazilian leprosy patients suggests the occurrence of reinfection or of bacterial population shift during disease relapse. J Med Microbiol **60**(Pt 10)**:**1441–1446.

73.  **Rafi A, Donoghue HD, Stanford JL.** 1995. Application of polymerase chain reaction for the detection of Mycobacterium leprae DNA in specimens from treated leprosy patients. Int J Lepr Other Mycobact Dis **63**(1)**:**42–47.

74.  **Kurabachew M, Wondimu A, Ryon JJ.** 1998. Reverse transcription-PCR detection of Mycobacterium leprae in clinical specimens. J Clin Microbiol **36**(5)**:**1352–1356.

75.  **Jadhav RS, Kamble RR, Shinde VS, Edward S, Edward VK.** 2005. Use of reverse transcription polymerase chain reaction for the detection of Mycobacterium leprae in the slit-skin smears of leprosy patients. Indian J Lepr **77:**116–127.

76.  **Phetsuksiri B, Rudeeaneksin J, Supapkul P, Wachapong S, Mahotarn K, Brennan PJ.** 2006. A simplified reverse transcriptase PCR for rapid detection of Mycobacterium leprae in skin specimens. FEMS Immunol Med Microbiol **48**(3)**:**319–328.

77.  **Chae GT, Kim MJ, Kang TJ, Lee SB, Shin HK, Kim JP, Ko YH, Kim SH, Kim NH.** 2002. DNA-PCR and RT-PCR for the 18-kDa gene of Mycobacterium leprae to assess the efficacy of multi-drug therapy for leprosy. J Med Microbiol **51**(5)**:**417–422.

78.  **Hirawati, Katoch K, Chauhan DS, Singh HB, Sharma VD, Singh M, Kashyap M, Katoch VM.** 2006. Detection of M. leprae by reverse transcription—PCR in biopsy specimens from leprosy cases: a preliminary study. J Commun Dis **38**(3)**:**280–287.

79.  **Martinez AN, Britto CF, Nery JA, Sampaio EP, Jardim MR, Sarno EN, Moraes MO.** 2006. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of Mycobacterium leprae DNA in skin biopsy samples from patients diagnosed with leprosy. J Clin Microbiol **44:**3154–3159.

80.  **Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, Williams DL.** 2009. Molecular determination of Mycobacterium leprae viability by use of real-time PCR. J Clin Microbiol **47:**2124–2130.

81.  **da Silva Martinez T, Nahas AA, Figueira MM, Costa AV, Gonçalves MA, Goulart LR, Goulart IM.** 2011. Oral lesion in leprosy: borderline tuberculoid diagnosis based on detection of Mycobacterium leprae DNA by qPCR. Acta Derm Venereol **91**(6)**:**704–707.

82.  **Kramme S, Bretzel G, Panning M, Kawuma J, Drosten C.** 2004. Detection and quantification of Mycobacterium leprae in tissue samples by real-time PCR. Med Microbiol Immunol **193:**189–193.

83.  **Sharma R, Lavania M, Katoch K, Chauhan DS, Gupta AK, Gupta UD, Yadav VS, Katoch VM.** 2008. Development and evaluation of real-time RT-PCR assay for quantitative estimation of viable Mycobacterium leprae in clinical samples. Indian J Lepr **80**(4)**:**315–321.

84.  **Rudeeaneksin J, Srisungngam S, Sawanpanyalert P, Sittiwakin T, Likanonsakul S, Pasadorn S, Palittapongarnpim P, Brennan PJ, Phetsuksiri B.** 2008. LightCycler real-time PCR for rapid detection and quantitation of Mycobacterium leprae in skin specimens. FEMS Immunol Med Microbiol **54:**263–270.

85.  **Martinez AN, Ribeiro-Alves M, Sarno EN, Moraes MO.** 2011. Evaluation of qPCR-based assays for leprosy diagnosis directly in clinical specimens. PLoS Negl Trop Dis **5:**e1354. doi: 10.1371/journal.pntd.0001354.

86. **Truman RW, Andrews PK, Robbins NY, Adams LB, Krahenbuhl JL, Gillis TP.** 2008. Enumeration of Mycobacterium leprae using real-time PCR. PLoS Negl Trop Dis **2:**e328. doi: 10.1371/journal.pntd.0000328.

87. **Lini N, Shankernarayan NP, Dharmalingam K.** 2009. Quantitative real-time PCR analysis of Mycobacterium leprae DNA and mRNA in human biopsy material from leprosy and reactional cases. J Med Microbiol **58**(Pt 6)**:**753–759.

88. **Yan W, Xing Y, Yuan LC, De Yang R, Tan FY, Zhang Y, Li HY.** 2014. Application of RLEP real-time PCR for detection of M. leprae DNA in paraffin-embedded skin biopsy specimens for diagnosis of paucibacillary leprosy. Am J Trop Med Hyg **90**(3)**:**524–529.

89. **Shamsi FA, Chaudhry IA, Moraes MO, Martinez AN, Riley FC.** 2007. Detection of Mycobacterium leprae in ocular tissues by histopathology and real-time polymerase chain reaction. Ophthalmic Res **39:**63–68.

90. **Jardim MR, Antunes SL, Santos AR, Nascimento OJ, Nery JA, Sales AM, Illarramendi X, Duppre NC, Chimelli L, Sampaio EP, Sarno EM.** 2003. Criteria for diagnosis of pure neural leprosy. J Neurol **250:**806–809.

91. **Ridley DS, Jopling WH.** 1966. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis **34:**255–273.

92. **World Health Organization (WHO).** 2013. Global leprosy situation 2012. Wkly Epidemiol Rec **87:**317–328.

93. **Bezerra Da Cunha FM, Werneck MC, Scola RH, Werneck LC.** 2006. Pure neural leprosy: diagnostic value of the polymerase chain reaction. Muscle Nerve **33:**409–414. doi: 10.1002/mus.20465.

94. **Rodriguez G, Pinto R, Gomez Y, Rengifo ML, Estrada OL, Sarmiento M, Lopez F, Beltran-Alzate JC, Cardona-Castro N.** 2013. Pure neuritic leprosy in patients from a high endemic region of Colombia. Lepr Rev **84**(1)**:**41–50.

95. **Ferreira Medeiros M, Jardim MR, Vital RT, Nery JA, Sales AM, de Moraes MO, Chimelli LM, Vidal Pessolani MC, Ferreira H, Sarno EN, Antunes SL.** 2014. An attempt to improve pure neural leprosy diagnosis using immunohistochemistry tests in peripheral nerve biopsy specimens. Appl Immunohistochem Mol Morphol **22**(3)**:**222–230.

96. **Aung T, Kitajima S, Nomoto M, En J, Yonezawa S, Arikawa I, Goto M.** 2007. Mycobacterium leprae in neurons of the medulla oblongata and spinal cord in leprosy. J Neuropathol Exp Neurol **66**(4)**:**284–294.

97. **Lee KH, Moon KS, Yun SJ, Won YH, Lee JH, Lee MC, Jung S.** 2014. Brain involvement by leprosy presenting as a frontal cystic lesion. J Neurosurg 121(1):184–188.

98.   **De A, Surana TV, Biswas S, Reja AH, Chatterjee G.** 2015. Isolated tenosynovitis as a sole manifestation: the great mimicker still continues to surprise us. Indian J Dermatol **60**(2)**:**213.

99.   **Rafi A, Spigelman M, Stanford J, Lemma E, Donoghue H, Zias J.** 1994. Mycobacterium leprae DNA from ancient bone detected by PCR. Lancet **343**(8909)**:**1360–1361.

100.  **Haas CJ, Zink A, Pálfi G, Szeimies U, Nerlich AG.** 2000. Detection of leprosy in ancient human skeletal remains by molecular identification of Mycobacterium leprae. Am J Clin Pathol **114**(3)**:**428–436. Erratum in Am J Clin Pathol 2000 **114**(6)**:**985.

101.  **Donoghue HD, Marcsik A, Matheson C, Vernon K, Nuorala E, Molto JE, Greenblatt CL, Spigelman M.** 2005. Co-infection of Mycobacterium tuberculosis and Mycobacterium leprae in human archaeological samples: a possible explanation for the historical decline of leprosy. Proc Biol Sci **272**(1561)**:**389–394.

102.  **Watson CL, Lockwood DN.** 2009. Single nucleotide polymorphism analysis of European archaeological M. leprae DNA. PLoS One **4**(10)**:**e7547. doi: 10.1371/journal.pone.0007547.

103.  **Suzuki K, Takigawa W, Tanigawa K, Nakamura K, Ishido Y, Kawashima A, Wu H, Akama T, Sue M, Yoshihara A, Mori S, Ishii N.** 2010. Detection of Mycobacterium leprae DNA from archaeological skeletal remains in Japan using whole genome amplification and polymerase chain reaction. PLoS One **5**(8)**:**e12422. doi: 10.1371/journal.pone.0012422.

104.  **Mendum TA, Schuenemann VJ, Roffey S, Taylor GM, Wu H, Singh P, Tucker K, Hinds J, Cole ST, Kierzek AM, Nieselt K, Krause J, Stewart GR.** 2014. Mycobacterium leprae genomes from a British medieval leprosy hospital: towards understanding an ancient epidemic. BMC Genomics **15:**270.

105.  **Suzuki K, Saso A, Hoshino K, Sakurai J, Tanigawa K, Luo Y, Ishido Y, Mori S, Hirata K, Ishii N.** 2014. Paleopathological evidence and detection of Mycobacterium leprae DNA from archaeological skeletal remains of Nabe-kaburi (head-covered with iron pots) burials in Japan. PLoS One **9**(2)**:**e88356. doi: 10.1371/journal.pone.0088356. eCollection 2014.

106.  **Schuenemann VJ, Singh P, Mendum TA, Krause-Kyora B, Jäger G, Bos KI, Herbig A, Economou C, Benjak A, Busso P, Nebel A, Boldsen JL, Kjellström A, Wu H, Stewart GR, Taylor GM, Bauer P, Lee OY, Wu HH, Minnikin DE, Besra GS, Tucker K, Roffey S, Sow SO, Cole ST, Nieselt K, Krause J.** 2013. Genome-wide comparison of medieval and modern Mycobacterium leprae. Science **341**(6142)**:**179–183. doi: 10.1126/science.1238286. Epub 2013 Jun 13.

107.  **Donoghue HD, Spigelman M, O'Grady J, Szikossy I, Pap I, Lee OY, Wu HH, Besra GS, Minnikin DE.** 2015. Ancient DNA analysis – An established technique in charting the evolution of tuberculosis and leprosy. Tuberculosis (Edinb) **95**(Suppl 1)**:**S140–S144. doi: 10.1016/j.tube.2015.02.020. Epub 2015 Feb 13.

108.  **Williams DL, Pittman TL, Gillis TP, Matsuoka M, Kashiwabara Y.** 2001. Simultaneous detection of Mycobacterium leprae and its susceptibility to dapsone using DNA heteroduplex analysis. J Clin Microbiol **39**(6)**:**2083–2088.

109.  **Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL.** 2006. The continuing challenges of leprosy. Clin Microbiol Rev **19**(2)**:**338–381.

110.  **Truman R, Fine PE.** 2010. 'Environmental' sources of Mycobacterium leprae: issues and evidence. Lepr Rev **81**(2)**:**89–95.

111.  **Lavania M, Katoch K, Katoch VM, Gupta AK, Chauhan DS, Sharma R, Gandhi R, Chauhan V, Bansal G, Sachan P, Sachan S, Yadav VS, Jadhav R.** 2008. Detection of viable Mycobacterium leprae in soil samples: insights into possible sources of transmission of leprosy. Infect Genet Evol **8**(5)**:**627–631.

112.  **Kazda J, Ganapati R, Revankar C, Buchanan TM, Young DB, Irgens LM.** 1986. Isolation of environment-derived Mycobacterium leprae from soil in Bombay. Lepr Rev **57**(Suppl 3)**:**201–208.

113.  **Matsuoka M, Izumi S, Budiawan T, Nakata N, Saeki K.** 1999. Mycobacterium leprae DNA in daily using water as a possible source of leprosy infection. Indian J Lepr **71**(1)**:**61–67.

114.  **Saha K, Jain M, Mukherjee MK, Chawla NM, Chaudhary DS, Prakash N.** 1985. Viability of Mycobacterium leprae within the gut of Aedes aegypti after they feed on multibacillary lepromatous patients: a study by fluorescent and electron microscopes. Lepr Rev **56**(4)**:**279–290.

115.  **Neumann Ada S, Dias Fde A, Ferreira Jda S, Fontes AN, Rosa PS, Macedo RE, Oliveira JH, Teixeira RL, Pessolani MC, Moraes MO, Suffys PN, Oliveira PL, Sorgine MH, Lara FA.** 2016. Experimental infection of Rhodnius prolixus (Hemiptera, Triatominae) with Mycobacterium leprae indicates potential for leprosy transmission. PLoS One **11**(5)**:**e0156037.

116.  **Kazda J, Irgens LM, Müller K.** 1980**.** Isolation of non-cultivable acid-fast bacilli in sphagnum and moss vegetation by foot pad technique in mice**.** Int J Lepr Other Mycobact Dis **48**(1)**:**1–6.

117.  **Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honoré N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T,**

Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. 2001. Massive gene decay in the leprosy bacillus. Nature **409**(6823)**:**1007–1011.

118. **Lavania M, Katoch K, Sachan P, Dubey A, Kapoor S, Kashyap M, Chauhan DS, Singh HB, Sharma VD, Jadhav RS, Katoch VM.** 2006. Detection of Mycobacterium leprae DNA from soil samples by PCR targeting RLEP sequences. J Commun Dis **38**(3)**:**269–273.

119. **Turankar RP, Lavania M, Chaitanya VS, Sengupta U, Darlong J, Darlong F, Siva Sai KS, Jadhav RS.** 2014. Single nucleotide polymorphism-based molecular typing of M. leprae from multicase families of leprosy patients and their surroundings to understand the transmission of leprosy. Clin Microbiol Infect **20**(3)**:**O142–O149.

120. **Mohanty PS, Naaz F, Katara D, Misba L, Kumar D, Dwivedi DK, Tiwari AK, Chauhan DS, Bansal AK, Tripathy SP, Katoch K.** 2016. Viability of Mycobacterium leprae in the environment and its role in leprosy dissemination. Indian J Dermatol Venereol Leprol 82(1):23–27.

121. **Desikan KV, Sreevatsa.** 1995. Extended studies on the viability of Mycobacterium leprae outside the human body. Lepr Rev **66**(4)**:**287–295.

122. **Turankar RP, Pandey S, Lavania M, Singh I, Nigam A, Darlong J, Darlong F, Sengupta U.** 2015. 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. Int J Mycobacteriol **4**(1)**:**54–59.

123. **Reja AH, De A, Biswas S, Chattopadhyay A, Chatterjee G, Bhattacharya B, Sarda A, Aggarwal I.** 2013. Use of fine needle aspirate from peripheral nerves of pure-neural leprosy for cytology and PCR to confirm the diagnosis: a pilot study. Indian J Dermatol Venereol Leprol **79**(6)**:**789–794.

124. **Reja AH, Biswas N, Biswas S, Dasgupta S, Chowdhury IH, Banerjee S, Chakraborty T, Dutta PK, Bhattacharya B.** 2013. Fite-Faraco staining in combination with multiplex polymerase chain reaction: a new approach to leprosy diagnosis. Indian J Dermatol Venereol Leprol **79**(5)**:**693–700.

125. **Wen Y, Xing Y, Yuan LC, Liu J, Zhang Y, Li HY.** 2013. Whole-blood nested-PCR amplification of M. leprae-specific DNA for early diagnosis of leprosy. Am J Trop Med Hyg **88**(5)**:**918–922.

126. **Banerjee S, Biswas N, Kanti Das N, Sil A, Ghosh P, Hasanoor Raja AH, Dasgupta S, Kanti Datta P, Bhattacharya B.** 2011. Diagnosing leprosy: revisiting the role of the slit-

skin smear with critical analysis of the applicability of polymerase chain reaction in diagnosis. Int J Dermatol **50**(12)**:**1522–1527.

127. **Bang PD, Suzuki K, Phuong le T, Chu TM, Ishii N, Khang TH.** 2009. Evaluation of polymerase chain reaction-based detection of Mycobacterium leprae for the diagnosis of leprosy. J Dermatol **36**(5)**:**269–276.

128. **Guerrero MI, Arias MT, Garcés MT, León CI.** 2002. [Developing and using a PCR test to detect subclinical Mycobacterium leprae infection]. Rev Panam Salud Publica **11**(4)**:**228–234.

129. **Santos AR, Nery JC, Duppre NC, Gallo ME, Filho JT, Suffys PN, Degrave WM.** 1997. Use of the polymerase chain reaction in the diagnosis of leprosy. J Med Microbiol **46**(2)**:**170–172.

130. **Wichitwechkarn J, Karnjan S, Shuntawuttisettee S, Sornprasit C, Kampirapap K, Peerapakorn S.** 1995. Detection of Mycobacterium leprae infection by PCR. J Clin Microbiol **33**(1)**:**45–49.

131. **Klatser PR, van Beers S, Madjid B, Day R, de Wit MY.** 1993. Detection of Mycobacterium leprae nasal carriers in populations for which leprosy is endemic. J Clin Microbiol **31**(11)**:**2947–2951.