

Leprosy Diagnosis: An Update on the Use of Molecular Tools

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Abstract

Leprosy is a chronic infectious disease caused by an obligatory intracellular mycobacteria *Mycobacterium leprae*, which presents tropism for Schwann cells and skin macrophages. Leprosy is a public health problem and early diagnosis is essential to avoid incapacities. The disease's clinical presentation varies from few to widespread lesions and its diagnosis continues to be a challenge due to the low sensibility of the conventional methods, based on bacillary counts of skin smears and histopathology. Molecular techniques, especially the methods to identify *M. leprae* DNA based on polymerase chain reaction (PCR) have emerged as a support of the conventional methods for the analysis of clinical samples in difficult to diagnose cases, such as pure neural leprosy, indeterminate and paucibacillary leprosy. The technique has also proved useful in the study of leprosy transmission and monitoring resistance to the WHO recommended Multidrug treatment. Different biological samples can be analysed and there is no consensus in the molecular diagnostic techniques respect of the most efficient nucleic acid extraction method, most appropriate methodology and genetic target for PCR. These methods provide a very valuable option for confirmation of difficult clinical cases with scarce bacilli but requires a well-equipped laboratory and the high cost makes it inaccessible to be used as a routine diagnostic tool in most endemic countries.

Leprosy diagnosis

Leprosy is a chronic infectious disease caused by an obligatory intracellular mycobacteria *Mycobacterium leprae* [1], which presents tropism for Schwann cells and skin macrophages [2,3]. At present, leprosy remains a public health problem. The main strategy to control leprosy is early detection and treatment with multidrug therapy (MDT) [4]. Despite being an ancient disease, known since Biblical times [5], its diagnosis continues to be a challenge due to the low sensibility of the conventional methods and impossibility to culture the bacillus "in vitro". Leprosy develops after an estimated incubation period of 2 to 10 years and presents a complex spectrum of clinical forms [6]. In clinical practice, the diagnosis is mainly based on the observation of clinical symptoms and supported by bacteriological analysis (Zielh-Neelsen smear (ZNS) and histopathology).

A negative ZNS only indicates that the concentration of bacilli is below 10,000 bacilli/mL [7], and this does not necessarily mean that the person is not infected [8]. This issue is especially problematic for individuals with pure neural leprosy (PNL), indeterminate (IL) and paucibacillary (PB) disease, which harbour a low burden of bacteria. On the other hand, with microscopic visualization all mycobacteria are phenotypically indistinguishable. Moreover, the serological techniques commercially available are inconclusive [9-12].

Many of the methods used in the diagnosis of other mycobacterial infections are not available in leprosy [13]. Research for the development of new diagnostic tools is particularly complicated since the only sources of bacteria are leprosy patients and a natural reservoir, the nine-banded armadillo (*Dasypus novemcinctus*) [14,15]. Thus, studying defined infections in mouse and armadillo models can provide insights into the host-pathogen interactions involved in this complex disease [16,17].

In leprosy, early diagnosis is essential and molecular techniques have emerged as a support of the conventional methods for the analysis of clinical samples. They offer culture-independent methods more sensitive and specific for the identification, confirmation and treatment of the infection, interrupting the chain of transmission and preventing the onset of disabilities [18,19].

M. leprae detection in clinical samples

Definitive identification of *M. leprae* in clinical specimens using PCR will depend largely on the standardization and other related factors

such as the number of copies of the target, the product size and the PCR conditions. Several non-commercial protocols using techniques based on the amplification of different sequences and targets have been developed. The most frequently used for diagnosis are:

Amplifying the gene encoding an 18 kDa antigenic protein by conventional PCR (cPCR) [20-26], with a sensitivity limit of 100 bacilli/sample [22] or of approximately 30 bacilli/sample by nested PCR (nPCR) [27]. Another method targets a gene that encodes an antigenic 36 kDa protein known as proline-rich antigen (*pra* gene) by cPCR [28-33], and can detect up to a single bacteria in the sample [28]. The *pra* gene has also been amplified by multiplex PCR (mPCR) [34]. Plikaytis and coworkers, developed a nPCR that amplifies a heat shock protein 65 kDa called *groEL*, which can detect 3 fg of *M. leprae*-DNA (single bacteria) [35]. An 85-antigen complex has also been used as a target, which encodes an 85 kDa antigen of three structurally related components [36]; 85B intergenic region by cPCR [37] and for the 85A-C gene by cPCR [37] or quantitative PCR (qPCR) [37-39]. Amplification of specific regions of microsatellites, as well as an internal sequence of the high-affinity manganese transporter (*Ml MntH*; *ML2098*) gene of the bacillus can also be useful for detecting *M. leprae* [40].

Several authors have based their methods on the amplification of specific repetitive sequences of *M. leprae* (RLEP region, 32 repeats per genome) [26,41-43], this technique has undergone modifications and using nPCR can amplify one-tenth of a single bacterial genome [27,44-46]. Others have combined PCR and southern hybridization [47-49], mPCR [50] or qPCR [19,38,51-53].

M. leprae can be identified combining PCR of the 16S rDNA internal transcribed spacer (ITS) region with restriction digestion of

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the amplified product (restriction fragment length polymorphism (PCR-RFLP)) and its subsequent sequencing [54,55] or by PCR-RFLP amplifying the hsp65 gene [56].

Finally, there are three *M. leprae*-specific quantitative reverse transcription PCR (rtPCR) assays based on the expression levels of *esxA*, encoding the ESAT-6 protein [52] and *hsp18* [52,57] could enable monitoring of *M. leprae* viability and amplification of a region belonging to the RNA ribosomal 16S [19,38,58-62] which can detect up to 10 viable bacteria in the sample [59].

***M. leprae* DNA extraction**

To achieve good results, good sampling, sample processing and DNA extraction is essential [63-66]. The successful amplification of a target sequence depends largely on the amount and quality of the nucleic acids extracted from the biological samples [64]. DNA extraction from clinical samples can be a difficult task, when there are low levels of genomic DNA and/or it is degraded. The mycobacterial cell wall complex has a waterproof structure, which hinders their lysis and subsequent release of the genetic material [67-69]. The efficiency of cell lysis, the ratio of DNA/RNA extracted, and residual extraction reagents will influence this process [69-71]. Other problems that may occur are, contaminants and inhibitors of PCR, the partial degradation of the DNA molecules and the duration of the protocols [65,72].

Procedures commonly used for the isolation of nucleic acids do not obtain good results for *M. leprae* DNA extraction [68,73,74] and pre-treatment steps with: freezing/thawing [75], bead beater with pearls [27,35] proteinase K [8,30,66,76] minimum 16 hours incubation [33], tris-HCL or tris-EDTA [44], detergents such as triton X-100 [22,77], and SDS [8,66,76-78], alone or in combination should be included.

Additionally, DNA extraction with phenol-chloroform mixture [66,79] can be used to obtain good quality DNA although it has several steps of sample handling, that can reduce performance and increase cross contamination [80]. Several studies have validated the ability of commercial kits: QIAamp DNA Mini Kit and DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, CA) for obtaining both high concentration [79] and good quality DNA with the highest purity value [66] even in samples with highly degraded [72] or scarce DNA [46;79;46].

Suitable samples for *M. leprae* nucleic acid detection

The DNA extraction can be performed in different biological samples such as:

Ear lobe and/or lesion lymph: Amplification of *M. leprae* DNA in lymph and slit skin smears has been reported and shown to have advantage over conventional methods [8,60,66]. The DNA extraction using these samples can increase both the sensitivity and specificity of the diagnosis and it can also be applied on stained slit skin smears [27,50,61,76] and on a FTA[®] elute card (patented Whatman FTA technology) (Cat no. WB120401; Whatman Inc., Florham Park, N.J., USA) [81]. Patients with negative stained smears can still transmit the infection and molecular culture-independent systems offer techniques to identify and treat them interrupting the chain of transmission and preventing the onset of disabilities [8,60,66,82].

Nasal secretion: Although the route of infection in the human is unknown, upper respiratory tract are considered the main door of entrance and exit of the bacilli in the body [83-86]. Active infected patients (mainly untreated multibacillary patients (MB)) are considered the main source of transmission usually by airborne droplets from nasal and/or mouth [24,87]. The extraction and amplification of *M.*

leprae DNA from nasal secretions can be very useful in both screening of potential contacts and leprosy control [43,49,85,88]. On the other hand, it has also demonstrated the importance of identifying healthy and subclinical carriers [50,83,84,87,89-92]. In endemic countries, some studies have shown that there are no differences in being a nasal carrier of *M. leprae* DNA between contacts and non contacts (controls) of a leprosy patient [83,87]. But, none of these studies have consistently associated the presence of the *M. leprae* DNA in nasal secretion with further development of the disease: it only indicates nasal carriage, which may imply active leprosy transmission [43,92].

Saliva and oral mucosa: Positive qPCR results suggest the potential usefulness of these two sites for sample collection, especially in paucibacillary patients [86,39,93]. *M. leprae* is present in the oral mucosa at a high frequency, implicating this site as a potential means of leprosy transmission [93]. At the same time, some authors found free-living bacteria on the oral epithelial surface, with a predilection for the tongue but the results do not correlate with the PCR results [93].

Sputa: Acid-fast bacilli detected by ZNS in sputa and *M. tuberculosis* complex PCR-negative may need to be identified at species level and in some cases *M. leprae*-species can be detected [55].

Whole blood: The *M. leprae* nucleic acid amplification in blood has been conducted in household contacts [46,49], and in post treatment patients and PCR positivity may persist for a period of time (6-8 years) [32,33,88]. This type of sample doesn't seem to be a valuable tool for correlating the association of PCR positivity in subclinical stage individuals with the risk for developing late leprosy [49] and some reports have detected that the presence of *M. leprae* in the blood of untreated leprosy patients is infrequent [94,95]. Contacts of multibacillary index cases with a positive PCR in blood are at higher risk of developing leprosy [95]. Others authors, propose their usefulness in whole-blood samples for early detection of leprosy cases [46].

Urine: The use of urine as a diagnosis sample is currently being evaluated [34,96]. The possibility of amplifying the *M. leprae* DNA in a simple non-invasive sample can be useful and maybe improve the diagnosis mainly in the tuberculoid form or in inconclusive cases where the slit skin smear is usually negative (PNL, IL and PB patients) [34].

Skin biopsy samples: This type of sample increases the possibility of finding bacilli [48], and leprosy molecular diagnosis can be done in: fresh skin biopsies [61], frozen skin biopsies [19,30,33,38,40,44,45,50], in formalin (although is not the appropriate preservative for amplification) [30], paraffin embedded [33,37,53,97], in ethanol 70% [24], nerve biopsies [98], in post-biopsy swab [33] or filter paper biopsy imprints [40]. An internal control and DNA serial dilutions must be added [66], because this sample is more likely to contain inhibitors of polymerase as: crosslinks between proteins and DNA by formylation of nucleic acids, length of time of fixation in formalin, lengths time of fixation in formalin and impurities form DNA purification can inhibit the polymerase reaction [22,30,37].

Conclusions of leprosy molecular diagnosis

In clinical practice, the molecular methods are always recommended linked to cases where it is difficult to confirm the diagnosis of leprosy using conventional methods [19,38,48,87,99-103] or in cases where the expected response to treatment is not observed [23,45,104]. PCR diagnosis is initially not necessary for MB patients with a high bacillary load and a high number of lesions. cPCR assays can remain for long periods of time positive in leprosy patients that have successfully completed their treatment [88]. In these cases it's necessary to estimate bacilli viability using qPCR [38,57,61]. It has to consider the objective

and outcome of employing molecular methods and select the most appropriate and useful techniques for the work.

There is no consensus in the molecular diagnostic techniques respect of the type of specimen or most efficient nucleic acid extraction method, or in the most appropriate methodology and target for PCR. So, the reproducibility between methods and different laboratories cannot be compared, except for the protocols designed to detect *M. leprae* MDT resistance [105]. The sensitivity can be highly variable and the specificity may be compromised according to the handling and methodology employed. The lack of a gold standard test for leprosy makes leprosy diagnosis essentially based on clinical features.

Surveillance of leprosy households and contacts with molecular tools increases the detection rate of less severe clinical presentations with lower bacteriological index [95,103,106] and this is essential for the control of the disease. Although PCR could be a useful tool for the detection of subclinical infection, only a few studies have consistently associated the presence of the *M. leprae* DNA with further development of the disease among household contacts [95].

The definitive identification of *M. leprae* is possible through the development of methods for the extraction, amplification, and identification of *M. leprae* DNA in clinical specimens using PCR [106]. A few studies have compared several genetic targets [26,27,38,40,52] employing several protocols standardized in small groups of patients [21,33,58,92]. The use of a repetitive sequence (RLEP) as a PCR target, provides the advantage of higher sensitivity because it is present at multiple sites in the genomic DNA, especially, in clinical samples, with low concentration of bacilli and/or degraded genomic material (PNL, IL and PB patients) [27,38,46,50,51,86]. The sensitivity of the qPCR and the nPCR were similar for the detection of paucibacillary cases but the qPCR has the advantage that results can be obtained rapidly and the number of bacilli in the samples estimated [53]. Nevertheless, real-time detection (qPCR) of *M. leprae* did not increase the clinical sensitivity of PCR over that of conventional protocols [107]. The excellent sensitivity and specificity of PCR suggests the test may be useful for the early diagnosis of leprosy but has its limitations for detecting the viability of the bacilli [51].

Overall, the excellent sensitivity and specificity of PCR suggests the technique may be useful and presents an advantage over conventional methods for the early diagnosis of leprosy especially in difficult clinical cases with few bacilli, such as pure neural leprosy, indeterminate and paucibacillary leprosy but has its limitations for detecting the viability of the bacilli [51]. It already has proved useful in *M. leprae* transmission and resistance to MDT studies and holds promise for detecting *M. leprae* infection before signs of overt disease in high risk groups (household contacts). But it requires a well-equipped laboratory and the high cost makes it inaccessible to be used as a routine diagnostic tool in most endemic countries.

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