Development and validation of a multiplex real-time qPCR assay using GMP-grade reagents for leprosy diagnosis

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OPEN ACCESS

Citation: Manta FSdN, Jacomasso T, Rampazzo RdCP, Moreira SJM, Zahra NM, Cole ST, et al. (2022) Development and validation of a multiplex real-time qPCR assay using GMP-grade reagents for leprosy diagnosis. PLoS Negl Trop Dis 16(2): e0009850. https://doi.org/10.1371/journal.pntd.0009850

Editor: Linda B. Adams, NHDP: National Hansen’s Disease Program, UNITED STATES

Received: September 27, 2021

Accepted: January 26, 2022

Published: February 18, 2022

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pntd.0009850

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Abstract

Leprosy is a chronic dermato-neurological disease caused by Mycobacterium leprae, an obligate intracellular bacterium. Timely detection is a challenge in leprosy diagnosis, relying on clinical examination and trained health professionals. Furthermore, adequate care and transmission control depend on early and reliable pathogen detection. Here, we describe a qPCR test for routine diagnosis of leprosy-suspected patients. The reaction simultaneously amplifies two specific Mycobacterium leprae targets (16S rRNA and RLEP), and the human 18S rRNA gene as internal control. The limit of detection was estimated to be 2.29 copies of the M. leprae genome. Analytical specificity was evaluated using a panel of 20 other skin pathogenic microorganisms and Mycobacteria, showing no cross-reactivity. Intra- and inter-operator Cq variation was evaluated using dilution curves of M. leprae DNA or a synthetic gene, and no significant difference was observed between three operators in two different laboratories. The multiplex assay was evaluated using 97 patient samples with clinical and histopathological leprosy confirmation, displaying high diagnostic sensitivity (91%) and specificity (100%). Validation tests in an independent panel of 50 samples confirmed sensitivity and specificity of 97% and 98%, respectively. Importantly, assay performance remained stable for at least five months. Our results show that the newly developed multiplex qPCR effectively and specifically detects M. leprae DNA in skin samples, contributing to an efficient diagnosis that expedites the appropriate treatment.
Author summary

Leprosy is a chronic dermato-neurological disease caused by *Mycobacterium leprae*, an obligate intracellular bacterium. Diagnosis of leprosy often relies on skin examinations for clinical signs, bacilli staining from skin smears and invasive skin biopsies. However, the spectrum of clinical manifestations and, often, low bacilli numbers can hinder accurate diagnosis. Timely detection is a challenge in leprosy diagnosis, relying on clinical examination and requiring trained health professionals. Proper intervention for adequate care and transmission control depends on early and reliable pathogen detection. Quantitative PCR methods for detecting bacterial DNA are more sensitive and could aid in differentially diagnosing leprosy from other dermatological conditions. In this work, we present a new multiplex PCR that was assessed for quality control standards, and the data indicate that the assay is stable and reproducible. The results presented here are the basis of a novel and robust tool with potential to increase the accuracy of leprosy diagnosis in routine or reference laboratories.

Introduction

Leprosy is a neglected infectious disease that still represents a public health issue [1] with more than 200,000 cases every year worldwide. Diagnosis is generally late and, although a specific and effective treatment is available, it is likely that transmission occurs before the patient is diagnosed and adequately treated, thus contributing to sustained transmission. The high number of young patients (under 15 years old) and patients with disabilities due to the advanced stage of the disease, confirms this hypothesis [1]. Furthermore, clinical forms vary to a great extent, from localized (tuberculoid) to disseminated (lepromatous) forms, making diagnosis difficult. Evidence suggests that early diagnosis could prevent transmission and help epidemiological control [2].

Methods such as bacterial index detection by microscopy and histopathological examination have been the main complementary tools for the diagnosis of leprosy [2–4]. Classical bacteriological methods cannot confirm leprosy since *M. leprae* does not grow *in vitro*. In addition, there is no reliable marker to estimate the risk of disease progression [5,6]. In this regard, the sequencing of *M. leprae* genome [7] was a milestone towards the improvement of direct *M. leprae* detection, leading not only to better characterization of genomic targets unique to *M. leprae* strains but also to an extensive comparison of different mycobacteria.

At the time when the first sequences became available, the polymerase chain reaction (PCR) technique was laborious and very expensive, averting its universal application. However, as PCR was further developed, it became more affordable, versatile and reliable, with fully automated systems becoming commercially available from different companies [8–10]. For tuberculosis, routine tests using PCR reduced the turnaround time, allowing same day treatment initialization, which might impact resistance prevalence [11–13]. Cost-effective nucleic acid detection assays are relatively widespread, but assays for some neglected diseases are still missing. In leprosy, the situation is even more difficult due to reduced and late investments directed to diagnostic tests [14].

In the last few years, many studies have been carried out using the PCR technique to detect *M. leprae* DNA in clinical specimens. PCR has been used especially under challenging diagnoses such as equivocal paucibacillary [4,15–18] or monitoring household contacts [19,20]. In this context, several different targets have been described in an attempt to establish the most sensitive and specific assay [16,20–28]. However, most of the PCR protocols were developed,
evaluated, and validated using reagents or tests produced without good manufacturing practices (GMP). Also, most of the studies enroll only leprosy patients and do not recruit patients with other common dermatological diseases that are differential diagnosis to leprosy. Thus, the development and validation of an assay over different laboratories has become a necessity.

Here, we present the development and validation of a multiplex real-time PCR assay aiming to standardize the leprosy molecular diagnostic assay. The protocol was designed to simultaneously detect two *M. leprae* targets (16S rRNA and RLEP genes), previously used in several studies [4,16,19,26,29], and one mammalian target (18S rRNA gene), that serves as reaction control [30]. Cross-reactivity was evaluated using DNA from 20 related mycobacterial and other skin pathogenic species, and no match was found. The new assay was validated using 97 skin biopsies and an independent panel enrolling 50 samples retrieved from patients previously characterized by clinical examination and histopathology, showed high sensitivity and specificity. The new multiplex PCR was also assessed for quality control standards and the data indicate that the assay is stable and reproducible. The results presented here are the basis of a novel and robust tool with potential to increase the accuracy of leprosy diagnosis in routine or reference laboratories.

**Material and methods**

**Ethics statement**

The Ethics Committee of the Oswaldo Cruz Foundation approved this study (CAAE: 38053314.2.0000.5248, number: 976.330-10/03/2015). Written informed consent was obtained from all patients. If participant was a minor, written formal consent was obtained from the parent/guardian.

**Clinical samples**

Leprosy patients were enrolled at the Leprosy clinic from the Oswaldo Cruz Foundation in the city of Rio de Janeiro, Brazil. Skin biopsies were collected using a 6 mm punch and stored in 70% ethanol at -20˚C until processing. The samples were included according to patient enrollment consecutively and are representative of the period between 2010 and 2018.

Ninety-seven samples (53 skin biopsies from leprosy patients and 44 skin biopsies from patients with other skin diseases) were used for qPCR tests. Clinical and demographic characteristics of all patients are shown in Table 1.

Leprosy patients were defined according to the clinical, bacteriological, and histopathological Ridley-Jopling (R&J) classification and the operational classification in multibacillary (MB) or paucibacillary (PB) forms according to the WHO [31]. Leprosy or other dermatological diseases (ODD) patients were treated according to their respective condition. Leprosy paucibacillary (PB) or multibacillary (MB) patients were treated according to the Ministry of Health recommendations, while ODD patients were treated accordingly for each specific disease.

**Replication Study**

To validate the conditions and analysis parameters established with the clinical samples from Oswaldo Cruz’s Leprosy Clinic, we tested a distinct collection of 50 skin biopsy samples that were also obtained at the Leprosy Clinic. The second set of samples was sent to the Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, Switzerland, where DNA samples were extracted and characterized by conventional PCR according to a previously published protocol [32]. Then, purified DNA was sent back to the Leprosy Clinic at Oswaldo Cruz Foundation,
where it was blindly analyzed with the qPCR developed in the present study. After analysis, blinding was removed and the results were compared. Of these 50 samples, fifteen were from patients with other skin diseases, 27 patients had MB leprosy and eight from PB leprosy. The group presented a 1.27:1 ratio of males to females. The mean age was 44.8 ($\pm$ 17.72 SD), and the range was 8–77. Details on the clinical characteristics are shown in S1 Table.

**Mycobacterial isolates samples**

*M. leprae* Thai-53 purified from athymic BALB/c (*nu/nu*) mouse footpads was kindly provided by Dr. Patricia Rosa at the Lauro de Souza Lima Institute, Bauru, São Paulo, Brazil. Purified DNA from *M. leprae* was used as positive control and in analytical sensitivity studies. DNA from 21 mycobacterial samples were used for the analytical specificity study. *L. amazonensis* and *L. braziliensis* was kindly provided by Dr Elisa Cupolillo by the Laboratório de Pesquisa em Leishmaniose (IOC-FIOCRUZ), and *M. avium, M. gordonae, M. manteni, M. africanum* subtype I, *M. africanum* subtype II, *M. bovis, M. bovis* (BCG), *M. canetti, M. fortuitum, M. gordonae, M. intracellulare, M. kansasii, M. microti, M. pinnipedia, M. simiae, and M. tuberculosis* DNA was extracted at the Laboratório de Biologia Molecular Aplicada a Micobactérias (IOC--FIOCRUZ) as published elsewhere [33].

**Synthetic DNA**

The synthetic DNA (gBlock) was purchased from Integrated DNA Technologies (IDT) and consists of a double-stranded DNA containing the sequences of the three genomic targets.

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**Table 1. Clinical and demographic characteristics of the leprosy and other dermatological disease cases.**

| Characteristics | Types | 1st panel | 2nd panel |
|-----------------|-------|-----------|-----------|
|                 |       | Leprosy group (n = 53) | ODD group (n = 44) | Leprosy group (n = 35) | ODD group (n = 15) |
| Gender          | Male  | 32        | 13        | 25        | 3         |
|                 | Female| 21        | 31        | 10        | 12        |
| Age             | 1–15  | 2         | 3         | 3         | 1         |
|                 | 16–30 | 8         | 7         | 5         | 2         |
|                 | 31–45 | 14        | 6         | 11        | 2         |
|                 | 46–60 | 20        | 19        | 9         | 9         |
|                 | >60   | 9         | 9         | 7         | 1         |
| WHO classification | PB    | 18        | NA        | 8         | NA        |
|                 | MB    | 35        | NA        | 27        | NA        |
| Clinical form   | I     | 6         | NA        | 0         | NA        |
|                 | TT    | 1         | NA        | 3         | NA        |
|                 | BT    | 11        | NA        | 5         | NA        |
|                 | BB    | 5         | NA        | 6         | NA        |
|                 | BL    | 3         | NA        | 7         | NA        |
|                 | LL    | 27        | NA        | 14        | NA        |
| Bacterial index | 0     | 23        | 38        | 8         | 15        |
|                 | 0–2   | 6         | 0         | 2         | 0         |
|                 | 2–4   | 11        | 0         | 13        | 0         |
|                 | 4–6   | 13        | 0         | 12        | 0         |

Other Dermatological Disease (ODD). Operational classifications [paucibacillary (PB) or multibacillary (MB)]. PB individuals were classified as Tuberculoid (TT), Borderline tuberculoid (BT), and Indeterminate (I). MB individuals were classified as Borderline-borderline (BB), Borderline lepromatous (BL) or Lepromatous (LL). NA: Not Applicable.

https://doi.org/10.1371/journal.pntd.0009850.t001

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(RLEP, 16S rRNA, and 18S rRNA) (S1 Text). The lyophilized DNA was reconstituted to 10 ng/μL (corresponding to 1.83 × 10^9 copies per reaction) in TE pH 8.0, following the supplier’s protocol.

DNA extraction

DNA extraction from the biopsies was carried out using the DNeasy Blood and Tissue extraction kit (Qiagen, Germany). The total extracted DNA was quantified with NanoDrop (Thermo-Fisher Scientific, Waltham, MA, USA) and stored at -20˚C. *M. leprae* DNA from nude mice footpad was purified using TRizol reagent (Life Technologies, Carlsbad, California) following the manufacturers’ instructions, as previously described [3]. DNA used in the replication study were extracted using QIAmp UCP Pathogen Mini kit (Qiagen GmbH, Hilden, Germany).

Standard curve and 95% limit of detection (LoD_{95%}) assessment

The standard curve was used for determination of the limit of detection and assay stability. A series of 10-fold dilutions was prepared from either *M. leprae* or synthetic DNA, using DNA purified from human blood obtained from healthy donors as a sample matrix. The dilution series used for the standard curve and the LoD_{95%} determination spans concentrations from 0.5 fg/reaction to 5 ng/reaction of purified *M. leprae* DNA (approximately 1.4 × 10^{-1} to 1.4 × 10^6 genome-equivalents/reaction, considering the *M. leprae* genome size to be 3.3 Mbp), and 1.83 × 10^0 to 1.83 × 10^7 copies/reaction (equivalent to 0.5 ag/reaction and 5 pg/reaction, respectively) of synthetic DNA.

Quantitative PCR (real-time PCR assays)

A multiplex real-time qPCR assay targeting simultaneously two *M. leprae* regions and an internal reference human sequence was developed. The primers and hydrolysis probes were designed to detect regions from RLEP and 16S rRNA genes [29] from *M. leprae*, and the human 18S rRNA [30] (Table 2). According to BLAST and *in silico* PCRs, the RLEP primers are specific to *M. leprae* without any complete (whole oligonucleotide) identical matching to *M. lepromatosis* or any other mycobacteria. However, 16S rRNA primers completely match *M. lepromatosis* genome sequences, except for a single base mismatch in the probe sequence. Reactions were performed on an ABI7500 Standard instrument (Thermo-Fisher Scientific, Waltham, MA, USA), using Multiplex PCR Mastermix (IBMP/FIOCRUZ PR, Curitiba, Brazil). For each reaction, 5 μL of DNA solution was added for a 25 μL final volume. Reaction mixtures were prepared in triplicates and amplified at 95˚C for 10 min, and 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions included a positive control (mouse foot-pad *M.

| Target | Forward | Reverse | Probe | Final concentration | Fluorophore |
|--------|---------|---------|-------|---------------------|-------------|
| 16S rRNA | 5´-GCATGTTCTTGGTGTTGGAAGC-3´ | 5´-CACCCCACACAAAGTGTAT-3´ | 5´-CATCCTGCAGCGCAGCA-3´ | 0.5 μM | FAM |
| RLEP | 5´-GACGAGCACTCAGTGTTGAA-3´ | 5´-CGGTAGAAGGTGGGCCTAT-3´ | 5´-CGGCAGGCGCGATCAGA-3´ | 0.2 μM | VIC |
| 18s rRNA | 5´-GAAACTGCGAATGGCTCATAAATCA-3´ | 5´-CCGCAGGACGCGACGACGCA-3´ | 5´-GGAGGAGCAGGACGACAG-3´ | 0.06 μM | CY5 |

https://doi.org/10.1371/journal.pntd.0009850.t002
leprae DNA and/or high-bacterial load lepromatous leprosy patient purified DNA), and water as a non-template control (NTC; PCR reaction without any template DNA).

**Stability**

The stability of the new multiplex qPCR was evaluated the synthetic DNA template diluted in TE to the concentrations of approximately $2 \times 10^8$, $2 \times 10^7$, $2 \times 10^6$, $2 \times 10^5$, $2 \times 10^4$, $2 \times 10^3$, $2 \times 10^2$, $2 \times 10^1$, 10, 5, and 2.5 copies per reaction.

All reagents (oligomix 25X and qPCR mix) were maintained in independent aliquots at -20°C at the Leprosy Laboratory (FIOCRUZ-RJ). Tests with the dilution series described above were repeated weekly for the first month, and then once a month for five months.

**Data analyses and statistics**

Qualitative (diagnostic sensitivity and specificity, accuracy) and quantitative (intra- and inter-laboratory repeatability and reproducibility, analytical sensitivity and specificity) validation tests were performed. The 95% limit of detection (LoD$_{95\%}$) was calculated by fitting a Probit model to the estimated detection probabilities. Samples that fall under the “equivocal” category were considered negative when calculating the diagnostic sensitivity, specificity and the predictive values. These parameters were calculated according to Altman and Bland [34]. Data were processed and analyzed using customized scripts for R version 3.5.1 (downloaded from http://www.Rproject.org/).

**Results**

**Analytical performance**

Primers and hydrolysis probes designed to target 16S rRNA and RLEP sequences of *M. leprae* were tested in multiplexed reactions to concomitantly detect the human 18S rRNA sequence. Optimal fluorescence thresholds were chosen based on the common practice that it should be positioned on the lower half of the fluorescence accumulation curves plot from the 10-fold dilutions, crossing most if not all fluorescence signals on the exponential segment of the curve on a logarithmic scale (Fig 1). Therefore, after setting the baseline to the automatic function, fluorescence threshold values chosen for determining $C_p$ (Crossing point) values for each target were set to intercept the positive controls and avoid the negative ones, being established as follows: 0.2 for RLEP, 0.15 for 16S rRNA, and 0.16 for 18S rRNA.

The analytical 95% limit of detection (LoD$_{95\%}$) was determined from a series of tests in which DNA extracted from *M. leprae* was diluted from 5 ng to 100 ag/reaction. Fig 2 shows the fitted Probit models and the obtained LoD$_{95\%}$ for 16S rRNA and RLEP, which were experimentally determined as approximately 450 fg of DNA (ca. 126 *M. leprae* genomes) for the 16S rRNA gene and about 4.60 fg of DNA (ca. 1.3 *M. leprae* genomes) for the RLEP gene.

The developed multiplex reaction was evaluated against a collection of microorganisms to assess the specificity of the primers and probes under these conditions. The selection included several mycobacteria, as well as a few other pathogens associated with skin diseases such as Leishmaniasis (Fig 3). We only considered any species as cross-reactive if all the technical replicates displayed amplification for at least one of the targets, which was not the case for any of the species tested. Most positive amplifications observed correspond to RLEP, which was detected in two out of three replicates in *M. fortuitum* and *M. kyroniense*. Even though some reactions presented 16S rRNA signals above the threshold, these amplifications are very uncharacteristic and are easily distinguishable from a proper amplification when compared with the positive control with 500 fg/reaction of *M. leprae*. 
Repeatability and reproducibility

Three independent operators performed three replicate runs each, in consecutive days, and evaluated the repeatability and reproducibility of the multiplex reactions. For each replicate, a new dilution series for the synthetic gene was prepared from a concentrated aliquot to be used as a template. The data (S2 Table) shows that all intra-operator replicates were remarkably reproducible, with only one point (Op. 1, 16S rRNA $1.83 \times 10^{2}$) displaying a relative standard deviation (rRSD%) above 5%, but still well below 10%. The inter-operator variability was also very low, and the largest variation was observed for the 16S rRNA target. Nonetheless, the rRSD% was between 1.38 and 11.57 across the dilution range, which shows an excellent reproducibility for a quantitative test (see also S3 Table).

The accuracy of the determinations performed by the multiplex real-time qPCR assay was also estimated using the synthetic DNA. To evaluate the intra- and inter-repeatability (or intermediate precision) for operators, we calculated the arithmetic mean, standard deviation, and relative standard deviation percentage of three independent experiments. It is noteworthy that the detection of the human target 18S rRNA does follow the same dilution trend for the other targets because the synthetic template was not diluted in human DNA.

In summary, for both *M. leprae* targets we observed that all points showed excellent reproducibility and repeatability. As expected, detection of the human target 18S rRNA loses
Fig 2. Analytical 95% limit of detection (LoD\textsubscript{95\%}) for 16S rRNA and RLEP in multiplexed qPCR. *Mycobacterium leprae* DNA was diluted in DNA extracted from whole blood from healthy donors and tested from 5 ng to 0.5 fg/reaction. Probability of detection was calculated for 16S rRNA and RLEP (top and bottom panels, respectively) from nine independent experiments, and a Probit model was fit to the data (black lines). The gray ribbon around the model fit indicates the 95% CI on the predicted probability. Dotted lines indicate the interpolation to determine the concentration at a 95% probability. The calculated LoD\textsubscript{95\%} is displayed on each plot in femtograms of DNA/reaction.

https://doi.org/10.1371/journal.pntd.0009850.g002
reproducibility as it becomes scarce in the reaction due to the dilution factor. It is noteworthy that there is no variation in the detection of the human target 18S rRNA when M. leprae DNA was present in the synthetic control molecule, i.e., in a 1:1 ratio, supporting the notion that the multiplexed reactions do not interfere with each other.

Stability

Storage stability was assessed by performing monthly evaluations of reactions with different concentrations of the synthetic DNA molecule for 5 months. Most of the data points tested varied below the established limit of three standard deviations above the average of all time points. Fig 4 shows the C_p obtained for the three evaluated targets (16S rRNA, RLEP, and 18S rRNA) in representative concentrations for brevity, over a 5-month period. The test remained reliable for the entire range of concentrations tested.

Diagnostic performance

The implemented setup involved the interrogation of two target sequences from M. leprae to classify clinical samples correctly while mitigating possible false positives. To evaluate the diagnostic performance, we first established optimal parameters for the analysis, considering possible cross-reactions that may occur in the laboratory routine. The C_p cutoff for both targets were determined iteratively by analyzing the receiver operating characteristic (ROC) curves for each combination of cutoff cycles. S1 Fig shows the ROC curves for a subset of the best-performing combinations of cutoff values for 16S rRNA and RLEP. Data for the full range of C_p cutoff combinations are listed in the S4 Table.

Based on these results the best combination of cutoff values (35.5 for 16S rRNA and 34.5 for RLEP) showed a sensitivity of 91% and specificity of 100%, positive and negative predictive values (PPV and NPV) were 100% and 90%, respectively. These values were similar between
MB and PB samples, as shown in the S5 Table. These parameters for analysis and summarized them in the decision algorithm presented in Table 3.

Next, the molecular diagnosis obtained using the new multiplex PCR, was compared to the clinical diagnosis of each sample (Fig 5 and S6 Table). Results show that the qPCR reaction and classification algorithm correctly characterized 48 of the 53 samples previously described as "Leprosy" by the clinical outcome. Of the 5 misclassified samples, one was classified as negative for *M. leprae* and four were in the "equivocal" quadrant. All the misclassified samples have a Bacterial Index of 0.

None of the 44 samples characterized as "Other skin diseases" were classified as *M. leprae*-positive by our reaction and decision algorithm. Thirty-eight of these samples were classified as "Negative" and 6 as "equivocal".

**Assay validation**

Conditions established with the training cohort were tested on an independent set of samples, which were previously characterized using a distinct qPCR method described in Girma et al. [32]. The comparison between the original classification and the new results is shown in Fig 6 and S1 Table. We tested 50 samples, of which 34 were previously characterized as positive and 16 as negative.

| Table 3. Decision algorithm for classification of samples based on the data obtained with the new multiplex qPCR. |
|---|---|
| **Results** | **Classification** |
| 18S rRNA negative | Extraction failure (repeat extraction) |
| 18S rRNA C<sub>p</sub> between 13 and 32 | Valid reaction (proceed with classification) |
| RLEP < 34.5 and 16S rRNA < 35.5 | *M. leprae* detected |
| RLEP < 34.5 and 16S rRNA ≥ 35.5 | Equivocal (mark patient for new sample collection and testing) |
| RLEP ≥ 34.5 | *M. leprae* undetected |

https://doi.org/10.1371/journal.pntd.0009850.t003
The 50 samples were classified according to our algorithm, resulting in 33 correctly classified as positive and 11 correctly classified as negative. Of the four samples classified as equivocal, two were negative for the reference method and one was positive. The sensitivity, specificity and accuracy calculated for this sample set were 97.1%, 100% and 98%, respectively.

Taken together, these results demonstrate that the multiplexed reaction for *M. leprae* RLEP and 16S rRNA is able to classify samples precisely, by combining the strengths of each molecular target and improving on their use in isolation. This setup is also able to flag samples with low bacilli burden for further monitoring, avoiding unnecessary treatment of uninfected patients and proper follow up of *M. leprae* carriers.

**Discussion**

Leprosy is a chronic infectious disease with a wide range of clinical forms, each distinguished by immunological and histopathological features. Leprosy can be tuberculoid, which is a localized form exhibiting few or no bacteria, or lepromatous, which is a systemic form with high loads of mycobacteria. Among the tuberculoid patients, there is a range of skin granulomatous diseases phenotypically comparable to leprosy [5].

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Fig 5. Distribution of $C_p$ values obtained for the training panel. Each point represents a different sample (mean $C_p$s of a technical duplicate). Filled circles represent leprosy samples and open dots represent negative samples, as defined by the clinical assessment. Points aligned to the top and right margins indicate samples in which 16S rRNA or RLEP, respectively, were not detected within 45 cycles. Bacterial index is shown as a color gradient (samples for which bacterial index information was not available are filled in gray). Dotted red lines indicate the cutoff values from Table 3. Equivocal or misclassified samples are annotated with the operational classification (false negatives) or with the diagnosis for clinic-negative samples.

https://doi.org/10.1371/journal.pntd.0009850.g005
The use of PCR for leprosy diagnosis has been extensively tested [4,16,35–46]. However, limitations towards the experimental designs for some published studies were identified. We observed that most studies: (i) test only samples from leprosy patients, creating difficulties in determining some diagnostic parameters such as specificity; (ii) were performed on small sample sizes; and (iii) do not have independent validation on the same assay or an evaluation of the same protocol in different centers. Furthermore, no studies have used reagents produced under good manufacturing practices (GMP), a set of guidelines that allow for traceability and batch-to-batch reproducibility of characteristics such as physical parameters and performance of the reagents [47].

In this study, we solved some of these issues by (i) developing and validating an assay based on the two most tested targets in the literature with better accuracy so far [7,8,43,48] (ii) following guidelines for validation of diagnostic tests [47,49,50], and (iii) using GMP grade reagents. We were also able to include a reaction for the detection of human 18S rRNA gene in the sample, to assess the quality of DNA extraction and reagent performance in the same reaction as the M. leprae determination occurs.

RLEP and 16S rRNA are the most frequent markers used in leprosy studies, displaying PCR sensitivity values up to 80% for each target. However, it is important to note that the sensitivity

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**Fig 6. Validation of parameters with an independent sample panel.** Samples previously characterized by Girma et al [32] were subjected to the new qPCR described in the present study. Each point represents a different sample. Filled circles represent leprosy samples and open dots represent negative samples. Points aligned to the top and right margins indicate samples in which 16S rRNA or RLEP, respectively, were not detected within 45 cycles. Bacterial index is shown as a color gradient (samples for which bacterial index information was not available are filled in gray). Dotted red lines indicate the cutoff values from Table 3.

https://doi.org/10.1371/journal.pntd.0009850.g006
of targets varied between sample types, clinical settings, and also between studies of the same authors [8,9]. Tatipally et al. [9] showed that using more than one marker in a multiplex format of conventional endpoint PCR yields significantly higher PCR positivity.

In the current study, a multiplex qPCR assay simultaneously amplifies two specific *M. leprae* targets (16S rRNA and RLEP), and the mammalian 18S rRNA gene as internal reaction control. The assay validation comprised analytical performance, diagnostic sensitivity and specificity, as well as reproducibility and repeatability. Development of multiplex qPCR assays provides a greater challenge than designing singleplex assays because it often requires extensive optimization as primer-dimers and non-specific interactions may interfere with amplification of the desired targets. Additionally, it is important that the amplification of two or more targets does not preferentially amplify one of the targets [51,52]. Combining multiple primers and probes did not affect the efficiency of the triplex qPCR in comparison to the corresponding singleplex reactions used in Martinez et al. [16]). They evaluated the independent detection of 16S rRNA and RLEP using the same primers and probes and obtained 0.51 and 0.91 for sensitivity and 0.73 and 1 for specificity, respectively. Barbieri et al. [4] also used the same 16S rRNA target to evaluate paucibacillary leprosy samples and obtained 0.57 for sensitivity and 0.91 for specificity. Here, we evaluated a panel with 53 leprosy and 44 non-leprosy patient samples, and later a different sample panel (50 patient samples) and achieved high sensitivity (> 90%) and specificity (100%) for both panels tested.

However, we understand that the small number of paucibacillary (PB) individuals in our study is a limitation. In fact, the greatest importance of using qPCR as a complementary diagnosis is precisely for PB samples. Generally, PB patients exhibit low (or zero) bacterial index and the histopathology examination does not distinguish them from other dermatoses. Therefore, these are the cases where clinical evaluation alone might not be able to determine the diagnosis, and where a qPCR confirmation becomes more important. However, due to the scarcity of bacterial DNA in these samples, it is known that the detection of *M. leprae* in PB patients by real-time PCR is difficult [4].

The reactions we developed in this study predict the equivocal classification of early-stage infections based on the finding from Martinez et al. [16], who showed that RLEP displays higher sensitivity than 16S rRNA whereas the ribosomal gene displays higher specificity. Thus, samples lacking 16S rRNA amplification but with RLEP amplification with a *Cp* lower than the threshold are suggested to be re-analyzed.

In general, our data (Fig 5) show a correlation between BI and *Cp* values. Biopsies from patients with higher BI values were deemed positive for bacteria earlier in the amplification cycle, as seen by the lower *Cp* values and high copy numbers of bacilli.

The “analytical sensitivity” or “limit of detection” of an assay is defined as the ability of the assay to detect very low concentrations of a given substance in a biological specimen [47]. The result of the limit of detection (LoD95%) determination when tested on a purified *M. leprae* sample indicated a higher sensitivity for RLEP (4.6 fg of DNA/reaction, equivalent to approximately 1.3 *M. leprae* genomes) versus 16S rRNA (450 fg of DNA/reaction, approximately 126 *M. leprae* genomes). This difference in sensitivity was expected since the 16S rRNA is a single copy gene [29] and the RLEP presents an average of 36 copies per genome [26].

Applicability in a reference laboratory setting was also considered during the development of these reactions. Intra and inter-operator variability were low, ensuring consistent results in routine testing (S3 Table). Moreover, reagents remained stable for at least five months, allowing for adequate stock maintenance (Fig 4).

Leprosy is a silent disease with a very long incubation time. Currently, transmission can only be halted if patients obtain early diagnosis. High-risk individuals, which are the patients’ close contacts, should be traced and treated whenever leprosy is detected. Recently, it has been
suggested that novel policies towards this group of contacts such as immuno- and chemoprophylaxis are effective to help control the disease burden [15,53,54]. These approaches provide a screening of the high-risk population that, coupled with a pharmacological or immunological intervention, has been suggested to decrease disease incidence.

In some situations, clinical diagnosis needs the accuracy of a laboratory analysis, and qPCR is a reliable technique to enable diagnostic confirmation [10]. Indeed, we confirmed that the availability of molecular tests can be very helpful in diagnosing patients during contact monitoring [55]. When contacts present a leprosy-like lesion, a positive PCR has resulted in a leprosy diagnosis with 50% sensitivity and 94% specificity [55]. Other indirect methods based on simultaneous detection of host humoral as well as cellular immune responses directed against the bacteria are also promising new diagnostic tools. Recently, lateral flow assays (LFA), combining detection of mycobacterial components and host proteins, proved to be specific and sensitive [56–62]. The signature detected by this platform identified 86% of the leprosy patients, with a specificity of 90% (AUC: 0.93, p < 0.0001) [60]. Thus, a multicentric study comparing different available methods such as qPCR and LFA is still necessary. It is noteworthy that our data showed accuracy, sensitivity, and specificity values quite similar to LFA.

We believe that the diagnosis of tropical and neglected diseases needs molecular-based methods such as PCR, especially due to the robustness and capillarity of the technique in clinical analysis laboratories worldwide. Towards that future, we present a real-time quantitative PCR produced with GMP reagents that adheres to all quality control specifications, allowing batch-to-batch performance reproducibility and repeatability, and that can be used in research and clinical laboratories with reasonable infrastructure in endemic countries. Finally, we envision the multiplex qPCR assay developed adapted to more affordable, rapid, point-of-care tests to be used in low-resourced settings, enabling on-site early and specific diagnosis of leprosy, hopefully helping disease control.

Supporting information

S1 Text. Sequences of the synthetic DNA template control.

S1 Fig. Diagnostic performance of the new multiplex qPCR. Different combinations of cutoff values for 16S rRNA (panels) and RLEP (color scale) were tested on a patient panel (n = 97). For each combination of cutoff values, the sensitivity and specificity were calculated and plotted as ROC curves. Here, only C_p cutoff values for 16S rRNA between 35 and 36.5 are shown. The combinations resulting in a specificity of 1 and the highest sensitivity for each condition are annotated.

S1 Table. Validation multiplex real-time qPCR assay study results.

S2 Table. Reproducibility and Repeatability results from synthetic DNA.

S3 Table. Precision measurement for repeatability and reproducibility.

S4 Table. List of C_p cutoff value combinations with associated sensitivity and specificity scores.
S5 Table. Sensitivity, Specificity, PPV, NPV and accuracy of the multiplex qPCR reaction calculated for Multibacillary and paucibacillary samples.
(XLSX)

S6 Table. Individual C_p values for targets included in the multiplex real-time qPCR assay (16S rRNA/RLEP/18S rRNA), sociodemographic, and laboratory variables for patient samples included in this study.
(XLSX)

Acknowledgments
The authors are grateful to the entire team of dermatologists, nurses, and technicians that collaborate at the Souza Araújo Clinic from the Leprosy Laboratory at the Oswaldo Cruz Institute. The authors are also grateful for the excellent technical assistance by Aline Burda Farias, Nilton José Fidêncio and Sylvia Mara Bohn at IBMP. We thank the Laboratório de Pesquisa em Leishmaniose (IOC-FIOCRUZ) and Laboratório de Biologia Molecular Aplicada a Micobacterias (IOC-FIOCRUZ) for donating the DNA from mycobacterial samples used in the analytical specificity study.

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References

1. WHO SEARO. A guide for surveillance of antimicrobial resistance in leprosy: 2017 update. https://www.who.int/lep/resources/9789290226192/en/

2. Sarno EN, Duppre NC, Sales AM, Hacker MA, Nery JA, Matos HJ de. Leprosy exposure, infection and disease: a 25-year surveillance study of leprosy patient contacts. Mem Inst Oswaldo Cruz. 2012 Dec; 107(8):1054–9. https://doi.org/10.1590/s0074-02762012000800015 PMID: 23295758

3. Shepard CC, McRae DH. A method for counting acid-fast bacteria. Int J Lepr Other Mycobact Dis. 2019; 16; 36(1):78–82.

4. Barbieri RR, Manta FSN, Moreira SJM, Sales AM, Nery JAC, Nascimento LPR, et al. Quantitative polymerase chain reaction in paucibacillary leprosy diagnosis: A follow-up study. PLoS Negl Trop Dis. 2019; 13(3):e0007147. https://doi.org/10.1371/journal.pntd.0007147 PMID: 30935722

5. Reibel F, Cambau E, Aubry A. Update on the epidemiology, diagnosis, and treatment of leprosy. Med Mal Infect. 2015; 45(9):383–93. https://doi.org/10.1016/j.medmal.2015.09.002 PMID: 26428602

6. White C, Franco-Paredes C. Leprosy in the 21st century. Clinical Microbiology Reviews. American Society for Microbiology; 2015; 28(1):80–94. https://doi.org/10.1128/CMR.00079-13 PMID: 23295758

7. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, et al. Massive gene decay in the leprosy bacillus. Nature. 2001; 409(6823) :1007–11. https://doi.org/10.1038/35059006 PMID: 11234002

8. Goulart IMB, Goulart LR. Leprosy: Diagnostic and control challenges for a worldwide disease. Arch Dermatol Res. 2008; 300(6):269–90. https://doi.org/10.1007/s00403-008-0857-y PMID: 18461340

9. Ratnapala S, Srikantam A, Kasety S. Polymerase Chain Reaction (PCR) as a Potential Point of Care Laboratory Test for Leprosy Diagnosis—A real-life experience. Trop Med Infect Dis. 2018; 3(4):107. https://doi.org/10.3390/tropicalmed3040107 PMID: 30725432

10. Krakl P, Ricchi M. A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. Front Microbiol. 2017; 8(FEB):1–9.

11. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid Molecular Detection of Tuberculosis and Rifampicin Resistance. N Engl J Med. 2010; 363(11):1005–15. https://doi.org/10.1056/NEJMoa0907847 PMID: 20825313

12. Scott L, David A, Govender L, Furrer J, Rakgokong M, Waja Z, et al. Performance of the Roche Cobas MTB Assay for the Molecular Diagnosis of Pulmonary Tuberculosis in a High HIV Burden Setting. J Mol Diagnostics. 2020; 22(10):1225–1237.

13. Silva Feliciano C, José Bazzo Menon L, Maria Pala Anselmo L, Dippenaar A, Mark Warren R, Araújo Silva W Jr, et al. Xpert MTB/RIF performance to diagnose tuberculosis and rifampicin resistance in a reference centre in southern Brazil. 2019 Aug 5; 5(3):00043–2019.

14. Fonseca B de P, Albuquerque PC, Zicker F. Neglected tropical diseases in Brazil: lack of correlation between disease burden, research funding and output. Trop Med Int Heal. 2020; 25(11):1373–1384

15. Steinmann P, Reed SG, Mirza F, Hollingsworth TD, Richardus JH. Innovative tools and approaches to end the transmission of Mycobacterium leprae. Lancet Infect Dis [Internet]. 2017; 17(9):e298–305. https://doi.org/10.1016/S1473-3099(17)30314-6 PMID: 28693856

16. Martínez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, et al. Evaluation of real-time qPCR-Based assays for leprosy diagnosis directly in clinical specimens. J Clin Microbiol. 2011 Jul; 50(1):1–8. https://doi.org/10.1128/JCM.001354-2011 PMID: 22022631

17. Nobre ML, Amorim FM, de Souza MCF, de Neves-Manta FS, Esquenazi D, Moraes MO, et al. Multibacillary leprosy and the elderly: A field for further research. Lepr Rev. 2017; 88(4):510–9.

18. da Silva Martinez T, Nahas AA, Figueira MMR, Costa AV., Gonçalves MA, Goulart LR, et al. Oral lesion in leprosy: Borderline tuberculoid diagnosis based on detection of mycobacterium leprae DNA by qPCR. Acta Derm Venereol. 2011; 91(6):704–7. https://doi.org/10.2340/00015555-1175 PMID: 21879249

19. Gama RS, Gomides TAR, Gama CFM, Moreira SJM, de Neves-Manta FS, de Oliveira LBP, et al. High frequency of M. leprae DNA detection in asymptomatic household contacts. BMC Infect Dis. 2018 Dec 2; 18(1):153. https://doi.org/10.1186/s12879-018-3056-2 PMID: 29609530

20. Reis EM, Araujo S, Lobato J, Neves AF, Costa AV, Gonçalves MA, et al. Mycobacterium leprae DNA in peripheral blood may indicate a bacilli migration route and high-risk for leprosy onset. Clin Microbiol Infect. 2014; 20(5):447–52. https://doi.org/10.1111/1469-0691.12349 PMID: 24033793

21. Goulart IMB, Cardoso AM, Santos MS, Gonçalves MA, Pereira JE, Goulart LR. Detection of Mycobacterium leprae DNA in skin lesions of leprosy patients by PCR may be affected by amplicon size. Arch Dermatol Res. 2007; 299(5–6):267–71. https://doi.org/10.1007/s00403-007-0758-5 PMID: 17530267
22. Caleffi KR, Hirata RDC, Hirata MH, Caleffi ER, Siqueira VLD, Cardoso RF. Use of the polymerase chain reaction to detect Mycobacterium leprae in urine. Brazilian J Med Biol Res. 2012; 45(2):153–7. https://doi.org/10.1590/s0100-879x2012007500011 PMID: 22886535

23. Maltemp FG, Baldin VP, Lopes MA, Siqueira VLD, Scordo RBDL, Cardoso RF, et al. Critical analysis: Use of polymerase chain reaction to diagnose leprosy. Brazilian J Pharm Sci. 2016; 52(1):163–9.

24. Azevedo M de CS, Ramuno NM, Fachin LRV, Tassa M, Rosa PS, Belone A de FF, et al. qPCR detection of Mycobacterium leprae in biopsies and slit skin smear of different leprosy clinical forms. Brazilian J Infect Dis. 2017; 21(1):71–8.

25. Arunagiri K, Sangeetha G, Sugashini PK, Balaraman S, Showkath Ali MK. Nasal PCR assay for the detection of Mycobacterium leprae pr gene to study subclinical infection in a community. Microb Pathog. 2017; 104:336–9. https://doi.org/10.1016/j.micpath.2017.01.046 PMID: 28137508

26. Truman RW, Andrews PK, Robbins NY, Adams LB, Krahenbuhl JL, Gillis TP. Enumeration of Mycobacterium leprae Using Real-Time PCR. Small PLC, editor. PLoS Negl Trop Dis. 2008 Nov 4; 2(11):e328. https://doi.org/10.1371/journal.pntd.0000328 PMID: 18982056

27. Banerjee S, Sarkar K, Gupta S, Mahapatra PS, Gupta S, Guha S, et al. Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts—a pilot study from India. BMJ Infect Dis. 2010; 10:1–8. https://doi.org/10.1126/bmj.3951.10415506 PMID: 20044936

28. Donohoe HD, Holton J, Spigelman M. PCR primers that can detect low levels of Mycobacterium leprae DNA. J Med Microbiol. 2001; 50(2):177–82. https://doi.org/10.1099/0022-1317-50-2-177 PMID: 11211226

29. Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, et al. Molecular Determination of Mycobacterium leprae Viability by Use of Real-Time PCR. J Clin Microbiol. 2009; 47(7):2124–30. https://doi.org/10.1128/JCM.00512-09 PMID: 19439537

30. Manta FS de N, Leal-Calvo T, Moreira SJM, Ribeiro-Alves M, Rosa PS, et al. Ultra-sensitive detection of Mycobacterium leprae: DNA extraction and PCR assays. Poonawala H, editor. PLoS Negl Trop Dis. 2020; 14(5):e0008325. https://doi.org/10.1371/journal.pntd.0008325 PMID: 32453754

31. Ridley D, Jolping W. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis. 1966; 34(3):255–73. PMID: 5950307

32. Girma S, Avanzi C, Boboasha K, Desta K, Idriess MH, Busso P, et al. Evaluation of Auramine O staining and conventional PCR for leprosy diagnosis: A comparative cross-sectional study from Ethiopia. PLoS Negl Trop Dis. 2018; 12(9):1–14. https://doi.org/10.1371/journal.pntd.0007533 PMID: 30180159

33. Van Soolingen D, Hermans PWM, De Haas PEW, Soll DR, Van Embden JDA. Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: Evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol. 1991; 29(11):2578–86. https://doi.org/10.1128/jcm.29.11.2578-2586.1991 PMID: 1685494

34. Altman DG, Bland JM. Statistics Notes: Diagnostic tests 1: sensitivity and specificity. BMJ. 1994; 308(6943):1552. https://doi.org/10.1136/bmj.308.6943.1552 PMID: 8019315

35. Kramme S, Bretzel G, Panning M, Kawuma J, Drosten C. Detection and quantification of Mycobacterium leprae Viability by Use of Real-Time PCR. J Clin Microbiol. 2009; 47(7):2124–30. https://doi.org/10.1128/JCM.00512-09 PMID: 19439537

36. Scollard DM, Gillis TP, Williams DL. Polymerase chain reaction assay for the detection and identification of Mycobacterium leprae DNA in different types of clinical samples for the diagnosis of leprosy. J Med Microbiol. 2005 Jun 1; 44(3):311–6. https://doi.org/10.1002/mcm.20415 PMID: 1574011

37. Phetsuksiri B, Rudeekasin J, Supakulp S, Wachapong S, Mahotarn K, Brennan PJ. A simplified reverse transcriptase PCR for rapid detection of Mycobacterium leprae in skin specimens. FEMS Immunol Med Microbiol. 2006; 48(3):319–28. https://doi.org/10.1111/j.1574-695X.2006.00152.x PMID: 17052269

38. Da Cunha FMB, Werneck MCM, Scolla RH, Werneck LC. Pure neural leprosy: Diagnostic value of the polymerase chain reaction. Muscle and Nerve. 2006; 33(3):409–14. https://doi.org/10.1002/mus.20465 PMID: 16315323

39. Santos AR, De Miranda AB, Sarno EN, Suffys PN, Degrange WM. Use of PCR-mediated amplification of Mycobacterium leprae DNA in different types of clinical samples for the diagnosis of leprosy. J Med Microbiol. 1993; 38(4):298–304. https://doi.org/10.1099/00222665-39-4-298 PMID: 8411091

40. Scollard DM, Gillis TP, Williams DL. Polymerase chain reaction assay for the detection and identification of Mycobacterium leprae in patients in the United States. Am J Clin Pathol. 1998; 109(5):642–6. https://doi.org/10.1093/ajcp/109.5.642 PMID: 9576586

41. Martinez AN, Britto CFPC, Nery JAC, Sampaio EP, Jardim MR, Sarno EN, et al. 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. 2006; 44(9):3154–9. https://doi.org/10.1128/JCM.02250-05 PMID: 16954241

42. Rudeaneksin J, Srisungngam S, Sawanpanyalert P, Sittiwakin T, Likanonsakul S, Pasadom S, et al. LightCycler™ real-time PCR for rapid detection and quantitation of Mycobacterium leprae in skin specimens. FEMS Immunol Med Microbiol. 2008; 54(2):263–70. https://doi.org/10.1111/j.1574-695X.2008.00472.x PMID: 18783434

43. Martínez AN, Talhari C, Moraes MO, Talhari S. PCR-Based Techniques for Leprosy Diagnosis: From the Laboratory to the Clinic. Franco-Paredes C, editor. PLoS Negl Trop Dis. 2014; 8(4):e2655. https://doi.org/10.1371/journal.pntd.0002655 PMID: 24722358

44. Tiwari V, Malhotra K, Khan K, Maurya PK, Singh AK, Thacker AK, et al. Evaluation of polymerase chain reaction in nerve biopsy specimens of patients with Hansen’s disease. J Neurol Sci. 2017; 380:187–90. https://doi.org/10.1016/j.jns.2017.07.038 PMID: 28870564

45. Carvalho RS, Foschiani IM, Renata M, Nogueira S, Marta SN. Early detection of M. leprae by qPCR in untreated patients and their contacts: results for nasal swab and palate mucosa scraping Early detection of M. leprae by qPCR in untreated patients and their contacts: results for nasal swab and palate mucosa sc. Eur J Clin Microbiol Infect Dis. 2018 Oct; 37(10):1863–1867. https://doi.org/10.1007/s10096-018-3320-9 PMID: 30008126

46. Woods SA, Cole ST. A rapid method for the detection of potentially viable Mycobacterium leprae in human biopsies: a novel application of PCR. FEMS Microbiol Lett. 1989; 65(3):305–9. https://doi.org/10.1016/0378-1097(89)90235-8 PMID: 2693204

47. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev. 2010; 23(3):550–76. https://doi.org/10.1128/CMR.00074-09 PMID: 20610823

48. Turankar RP, Pandey S, Lavania M, Singh I, Nigam A, Darlong J, et al. 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 Mycobacteriology. 2015; 4(1):54–9. https://doi.org/10.1016/j.ijmyco.2014.11.062 PMID: 26655199

49. Mattocks CJ, Morris MA, Matthijis G, Swinnen E, Corveleyn A, Dequeker E, et al. A standardized framework for the validation and verification of clinical molecular genetic tests. Eur J Hum Genet. 2010; 18(12):1276–88. https://doi.org/10.1038/ejhg.2010.101 PMID: 20664632

50. National Association of Testing Authorities A (NATA). Guidelines for the validation and verification of chemical methods. 2013;(December 2006):1–6.

51. Markoutlos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: A practical approach. J Clin Lab Anal. 2002; 16(1):47–51. https://doi.org/10.1002/jcla.2058 PMID: 11835531

52. Lorenz TC. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. J Vis Exp. 2012; 63:3998. https://doi.org/10.3791/3998 PMID: 22664923

53. Gillini L, Cooreman E, Wood T, Pemmaraju VR, Saunderson P. Global practices in regard to implementation of preventive measures for leprosy. Phillips RO, editor. PLoS Negl Trop Dis. 2017; 11(5):e0005399. https://doi.org/10.1371/journal.pntd.0005399 PMID: 28472183

54. Richardus JH, Tiwari A, Barth-jaeggi T, Arif MA, Banstola NL, Baskota R, et al. Leprosy post-exposure prophylaxis with single-dose rifampicin (LPEP): an international feasibility programme. 2020;(20):10–4. https://doi.org/10.1016/S2214-109X(20)30396-X PMID: 33129378

55. Manta FSN, Barbieri RR, Moreira SJM, Santos PTS, Nery JAC, Duppre NC, et al. Quantitative PCR for leprosy diagnosis and monitoring in household contacts: A follow-up study, 2011–2018. Sci Rep. 2019 Dec 1; 9(1):1–8. https://doi.org/10.1038/s41598-018-37186-2 PMID: 30626917

56. Bobosha K, Tjon Kon Fat EM, van den Eeden SJF, Bekele Y, van der Ploeg-van Schip JJ, de Dood CJ, et al. Field-Evaluation of a New Lateral Flow Assay for Detection of Cellular and Humoral Immunity against Mycobacterium leprae. PLoS Negl Trop Dis. 2014; 8(5). https://doi.org/10.1371/journal.pntd.0002845 PMID: 24810599

57. Van Hooij A, Fat EMTK, Van Den Eeden SJF, Wilson L, Da Silva MB, Salgado CG, et al. Field-friendly serological tests for determination of M. Leprae-specific antibodies. Sci Rep. 2017; 7(1):1–8. https://doi.org/10.1038/s41598-016-0028-x PMID: 28127051

58. Yang S, Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 2004; 4(6):337–48. https://doi.org/10.1016/S1473-3099(04)01044-X PMID: 15172342

59. Corstjens PLAM, van Hooij A, Tjon Kon Fat EM, Alam K, Vrolijk LB, Diamini S, et al. Fingerstick test quantifying humoral and cellular biomarkers indicative for M. leprae infection. Clin Biochem. 2019; 66:76–82. https://doi.org/10.1016/j.clinbiochem.2019.01.007 PMID: 30895682
60. Van Hooij A, Van Den Eeden S, Richardus R, Tjon E, Fat K, Wilson L, et al. Application of new host biomarker profiles in quantitative point-of-care tests facilitates leprosy diagnosis in the field. EBioMedicine. 2019; 47:301–8. https://doi.org/10.1016/j.ebiom.2019.08.009 PMID: 31422044

61. van Hooij A, Tió-Coma M, Verhard EM, Khatun M, Alam K, Tjon Kon Fat E, et al. Household Contacts of Leprosy Patients in Endemic Areas Display a Specific Innate Immunity Profile. Front Immunol. 2020; 11:1–12. https://doi.org/10.3389/fimmu.2020.00001 PMID: 32038653

62. Ti O-Coma M, Kiebasa SM, Van Den Eeden SJF, Mei H, Roy JC, Wallinga J, et al. Blood RNA signature RISK4LEP predicts leprosy years before clinical onset-NC-ND license EBioMedicine. 2021; 68:103379. https://doi.org/10.1016/j.ebiom.2021.103379 PMID: 34090257