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Comparison of four DNA extraction methods for the detection of *Mycobacterium leprae* from Ziehl–Neelsen-stained microscopic slides

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ABSTRACT

Objective/background: The diagnosis of leprosy has been a challenge due to the low sensibility of the conventional methods and the impossibility of culturing the causative organism. In this study, four methods for *Mycobacterium leprae* nucleic-acid extraction from Ziehl–Neelsen-stained slides (ZNS slides) were compared: Phenol/chloroform, Chelex 100 resin, and two commercial kits (Wizard Genomic DNA Purification Kit and QIAamp DNA Mini Kit).

Methods: DNA was extracted from four groups of slides: a high-codification-slide group (bacteriological index [BI] $\geqslant 4$), a low-codification-slide group (BI = 1), a negative-slide group (BI = 0), and a negative-control-slide group (BI = 0). Quality DNA was evidenced by the amplification of specific repetitive element present in *M. leprae* genomic DNA (RLEP) using a nested polymerase chain reaction.

Results: This is the first report comparing four different extraction methods for obtaining *M. leprae* DNA from ZNS slides in Cuban patients, and applied in molecular diagnosis. Good-quality DNA and positive amplification were detected in the high-codification-slide group with the four methods, while from the low-codification-slide group only the QIAGEN and phenol–chloroform methods obtained amplification of *M. leprae*. In the negative-slide group, only the QIAGEN method was able to obtain DNA with sufficient quality for positive amplification of the RLEP region. No amplification was observed in the negative-control-slide group by any method. Patients with ZNS negative slides can still transmit the infection, and molecular methods can help identify and treat them, interrupting the chain of transmission and preventing the onset of disabilities.

Conclusion: The ZNS slides can be sent easily to reference laboratories for later molecular analysis that can be useful not only to improve the diagnosis, but also for the application of other molecular techniques.

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Introduction

Leprosy is a chronic granulomatous disease caused by an infection with *Mycobacterium leprae* that affects the skin and the peripheral nervous system. The conventional diagnosis is based on the observation of clinical symptoms, and supported by bacteriological analysis (Ziehl–Neelsen-stained slides [ZNS slides] and histopathology). The observation of acid-fast bacilli in the ZNS slides confirms the diagnosis, but if the slide is negative (acid-fast bacilli are not visualized), it does not necessarily mean that the person is not infected [1]. The reproducibility of ZNS slide results depends on the technician and laboratory expertise, because at least 10^4 -bacilli/g of tissue are required for a reliable microscopic detection in stained slides [2]. However, the molecular detection of the bacilli by polymerase chain reaction (PCR) is more sensitive and specific compared with the conventional methods, and has been used for rapid detection of microorganisms in clinical samples [3]. The detection of *M. leprae* in ZNS slides by PCR has been reported and shown to have advantage over conventional microscopic and serological methods [1,4]. DNA extraction from ZNS slides can be a difficult task, when there are low levels of genomic DNA and/or it is degraded. Other problems that may occur are contaminants and inhibitors of PCR, the partial degradation of the DNA molecules, and the duration of the protocols [5,6]. To achieve good results, good quality DNA is essential.

In this paper, we evaluate the quantity and quality of the genomic DNA from four extraction methods, and the success of the subsequent PCR amplification of the *M. leprae* RLEP region specific repetitive element (RLEP).

Materials and methods

Samples

One hundred and fifty-two archived skin-smear ZNS slides were obtained from the collection of the National Reference Laboratory for Leprosy at "Pedro Kourí" Tropical Medicine Institute. All the slides following the classical protocol of The National Leprosy Program guidelines were obtained from both ear lobes and elbows of the patients [7,8].

The slides were recoded and classified into four groups by their bacteriological index (BI) according to the Ridley–Jopling classification: 40 slides with a BI $\geqslant$ 4 were denominated high-codification slides (HCS), 40 slides with a BI = 1 were denominated as low-codification slides (LCS), 12 slides with a BI = 0 were denominated negative slides (NS), and 60 slides with a BI = 0 were denominated negative-control slides (NCS). The HCS, LCS, and NS used for DNA extraction were obtained from patients with leprosy definitive diagnosis in 2010, and the NCS were obtained from patients in which another diagnosis was confirmed. All samples were stored at room temperature pending the extraction procedure.

All the slides from the groups were randomized and allocated into one of the following four different *M. leprae* DNA extraction methods: Chelex 100 resin (Sigma–Aldrich, Hamburg, Germany), phenol–chloroform–isoamyl alcohol, and two commercial kits (Wizard Genomic DNA Purification Kit and QIAamp DNA Mini Kit). When the allocation was completed, each extraction method included 10 HCS, 10 LCS, 3 NS, and 15 NCS.

Ethic statement

This research protocol was reviewed and approved by the Committee of Ethics of the "Pedro Kourí" Tropical Medicine Institute, CEI-IPK code: 03-10.

No written informed consent was obtained from patients because all slides were archived in the National Reference Laboratory. No patient data were used in this paper.

Processing of slides and DNA extraction

Pre-treatment

Xylene treatment was used to remove the immersion oil from the slides. The slides were embedded for 15 min in xylene (Merck, Darmstadt, Germany), and then dried. Once dried, 200 μL of NET-10 buffer (pH 8.0) (10 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl) (VWR International, Leuven, Belgium) was added on each ZNS slide, and the smear was scraped using a pipette filter tip and collected in a 1.5 mL microfuge tube with 20 μL of proteinase K (20 mg/mL) (Merck) and 40 μL of 10% sodium dodecyl sulfate (Amersham Biosciences, Uppsala, Sweden). The tubes were incubated with an agitation system at 65 °C (Memmert B40 incubator; Memmert, Schwabach, Germany) overnight, and then at 97 °C for 10 min in a heater (Labnet, Belgic). The mixture was centrifuged at 19,664.6*g* (Digicen 21R; Orto Alresa, Madrid, Spain) for 6 min, and the supernatant was carefully transferred to a new 1.5 mL clean microfuge tube.

Chelex-100-resin method

Samples were mixed with an equal volume of 5% Chelex 100 (Sigma–Aldrich, St. Louis, MO, USA) in Tris–EDTA (TE) buffer, incubated for 10 min at 100 °C, and centrifuged at 19,664.6*g* for 5 min. The supernatant was transferred to a new 1.5 mL clean microfuge tube and stored at 4 °C [6].

Phenol–chloroform–isoamyl alcohol method

For this method, 250 μL of phenol–chloroform–isoamyl alcohol (25:24:1) (Merck) was added to the tubes containing the samples, and mixed gently for 5 min. The mixture was centrifuged at 18,152*g* for 10 min. The top aqueous DNA layer, while avoiding the interface, was transferred to a new 1.5 mL clean tube. Additionally, 250 μL of chloroform–isoamyl alcohol (24:1) (Merck) was added and mixed gently for 5 min, and centrifuged at 18,152*g* for 10 min. The top aqueous DNA layer was transferred again to a 1.5 mL clean tube while avoiding the interface, and 400 μL absolute ethanol (Merck) and 15 μL 3 M sodium acetate, pH 6.0 (Sigma–Aldrich, USA), were added and mixed by inversion. The DNA was stored at −80 °C for 20 min for precipitation. The samples were centrifuged at 18,152*g* for 10 min, the supernatant was discarded, and the pellet was washed with 500 μL of 70% ethanol, and centrifuged at 18,152*g* for 10 min. The pellet was air dried,

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