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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,

resuspended in 100 μL of TE, and dissolved by incubating at 37 °C for 1 h in a final volume of 100 μL of TE buffer (10 mM Tris, and 1 mM EDTA, pH 8) (Scharlau Chemie, Spain) [9].

Commercial kits (Wizard Genomic DNA Purification Kit and QIAamp DNA Mini Kit)

Extraction with both commercial kits was used following the manufacturer's recommended protocol for the isolation of DNA from small volumes of corporal fluids. The samples were eluted in 100 μL of TE buffer supplied with the kit.

Assessment of quality and quantity from DNA

The quality (OD_{260}/OD_{280}) and quantity (OD_{260}) of the DNA obtained were measured using a BioPhotometer Plus (Eppendorf, Hamburg, Germany). Fifty microliters of 1/10 DNA dilution in bi-distillated water (sterilized DNase/RNase free) ($_{bd}$water) was employed, and five measurements were performed for each sample. The OD_{260}/OD_{280} ratio was calculated for an indication of nucleic-acid purity.

Polymerase chain reaction for the detection of M. leprae

The efficiency of the DNA extraction methods was evaluated by amplifying the RLEP sequence that is specific and sensitive for *M. leprae* using the nested primers: Lp1: TGCATGTCATG GCCTTGAGG and Lp2: CACCGATACCAGCGGCAGAA (129 bp), and Lp3: TGATGGGTCGGCGTGGTC and Lp4: CAGAAATGG TGCAAGGGA (99 bp) in the second PCR reaction [10].

All PCRs were performed containing 5× Green GoTaq Buffer (Promega Corporation, Madison, WI, USA), 4 mM of $MgCl_2$, 0.3 μM of each primer (Sigma–Aldrich, Germany), 0.2 mM dNTPs (10 mM dNTP mix; Amersham Biosciences, Buckinghamshire, UK), 0.625 U of Taq DNA polymerase (5 U/μL; Promega Corporation), and $_{bd}$water to a final volume of 25 μL. In the first reaction, 5 μL of DNA was used as template. In the second reaction, 2 μL from the first reaction was used as the DNA template.

A tube with water instead of template was always included as a negative control in both reactions, and a positive control of *M. leprae* DNA was obtained from the growth of an inoculum in an armadillo, kindly donated by the Sanatorio de Fontilles, Alicante, Spain (2 μg of *M. leprae* genomic DNA isolate: NHDP-63 in 500 μL of TE buffer).

In both rounds, the amplification was performed by heating at 94 °C for 1 min, followed by 30 cycles at 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final extension at 72 °C for 5 min using a thermal cycler (Peltier gradient thermal cycler; Nahita, Hyogo, Japan). The amplified product (10 μL) with 5× Green GoTaq Flexi Buffer (Promega Corporation) was analyzed by electrophoresis on 2% agarose gel (Promega Corporation) with 0.4 μg/mL of ethidium bromide (Sigma–Aldrich, USA) in Gel Electrophoresis Apparatus GNA-100 (Pharmacia, Stockholm, Sweden) with 70v (Techware [Sigma–Aldrich, Germany]) for 45 min.

Test of an inhibitor role

A control PCR assay was used to confirm the potential inhibition, using specific primers designed for β-actin human sequence (accession number NM_007393.3) (forward: CCTTCCTGGGCATGGAGTCCTG, reverse: GGAGCAATGATCTT GATCTTC), which amplify a 202 bp region. The PCR reaction was performed in 25 μL total volume containing 5× Green GoTaq Buffer (Promega Corporation), 1.5 mM of $MgCl_2$, 0.025 U of Taq DNA polymerase (1.25 units; Promega Corporation), 200 nM of dNTP mix (10 mM), 0.5 μM of each oligonucleotide primer, 5 μL of the extracted DNA, and $_{bd}$water. The amplification was performed as follows: strand separation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1.5 min. Finally, there was a 7 min at 72 °C for further strand extension. Ten microliters of the amplified PCR product was analyzed by electrophoresis on 2% agarose gel (Promega Corporation) with 0.4 μg/mL of ethidium bromide (Sigma–Aldrich, USA) in Gel Electrophoresis Apparatus GNA-100 (Pharmacia) with 70v (Techware [Sigma–Aldrich, Germany]) for 45 min.

Statistical analysis

The results recorded were introduced in an Excel data sheet (Microsoft Office 2010), and Table 1 was designed with this software. The data were analyzed using the GraphPad Prism

Table 1 – Quantity and quality of DNA extracted from the four different DNA extraction methods.

	Chelex 100		Phenol/chloroform		Wizard Genomic DNA Purification Kit (Promega Corporation)		QIAamp DNA Mini Kit (QIAGEN)	
	OD 260 nm	OD_{260}/OD_{280} ratio	OD 260 nm	OD_{260}/OD_{280} ratio	OD 260 nm	OD_{260}/OD_{280} ratio	OD 260 nm	OD_{260}/OD_{280} ratio
HCS	66.76 ± 7.19	1.08 ± .03	4.70 ± 1.09*	1.59 ± .16*	1.00 ± .18*	1.43 ± .09*	3.54 ± .38*	1.67 ± .10*
LCS	48.68 ± 7.40	1.05 ± .08	.96 ± .22*,**	1.24 ± .09	.14 ± .08*,**	1.04 ± .60	1.65 ± .17*	1.44 ± .09
NS	14.87 ± 2.06	.99 ± .03	.30 ± .18*,**	.99 ± .02	.10 ± .09*,**	1.02 ± .08	1.08 ± .11*	1.13 ± .07
NCS	39.83 ± 7.85	1.01 ± .08	.77 ± .36*,**	1.16 ± .14*,**	.43 ± .29*,**	.98 ± .43**	1.33 ± .34*	1.65 ± .13*

HCS = high-codification slides; LCS = low-codification slides; NCS = negative control slides; NS = negative slides; OD = optical density.

* Significant difference with the Chelex-100-resin method.

** Significant difference with the QIAGEN method.

version 5.01 for Windows (GraphPad, San Diego, CA, USA). The Kruskal–Wallis test was used to compare the differences observed among the DNA extraction methods. Significant differences were considered for $p < .05$.

Results

The DNA concentration obtained from the HCS group (66.67 ± 7.19 ng/μL) with the Chelex-100-resin extraction method was the highest among all the extraction methods ($p = .021$). Additionally, the DNA concentrations from the HCS group obtained with the QIAGEN kit and phenol–chloroform were not significantly different, with averages of 3.54 ± 0.38 ng/μL and 4.70 ± 1.09 ng/μL, respectively. The DNA concentration from the Promega kit was the lowest (1.00 ± 0.18 ng/μL; $p = .032$) among all the methods used (Table 1). The Chelex 100 resin presented an OD_{260}/OD_{280} ratio of 1.08 ± 0.03, and showed significant differences with the rest of the extraction methods ($p = .025$).

In the LCS group, the highest DNA concentration was obtained from the Chelex 100 resin (48.68 ± 7.41 ng/μL). The DNA concentration from QIAGEN (1.65 ± 0.17 ng/μL) was statistically significantly different from the phenol–chloroform and Promega methods ($p = .033$). The OD_{260}/OD_{280} ratio (1.44 ± 70.09 ng/μL) from QIAGEN was the highest, and the differences were statistically significant ($p = .016$) when compared with the Chelex 100 resin and Promega kit (Table 1).

In the NS group, the DNA concentration obtained from Chelex 100 resin was the highest (14.87 ± 2.06 ng/μL) and statistically significant compared with the rest ($p = .030$). The differences in the DNA concentration (1.08 ± 0.03 ng/μL) with the QIAGEN method were statistically significant ($p = .023$) compared with the phenol–chloroform (0.30 ± 0.18 ng/μL) and Promega methods (0.10 ± 0.09 ng/μL), respectively (Table 1). At the same time, differences were observed in the extraction methods, and the QIAGEN method's OD_{260}/OD_{280} ratio (1.13 ± 0.07 ng/μL) was the highest among all the extraction methods (Table 1).

In the NCS group, the DNA concentration obtained from the Chelex 100 resin was the highest (39.83 ± 7.85 ng/μL) and statistically significant compared with the rest ($p < .001$). The differences in the DNA concentration (1.33 ± 0.34 ng/μL) with the QIAGEN method were statistically significant ($p = .001$) compared with the phenol–chloroform (0.77 ± 0.36 ng/μL) and Promega methods (0.43 ± 0.29 ng/μL), respectively (Table 1). At the same time, differences were observed in the extraction methods, and the QIAGEN method's OD_{260}/OD_{280} ratio (1.65 ± 0.13 ng/μL) was the highest among all the extraction methods, and the differences with the other extractions methods were significant ($p < .001$) (see Table 1). Statically, differences were observed between Chelex 100 resin (1.01 ± 0.08) compared with the rest of the methods ($p < .001$).

Additionally, the efficiency of the four extraction methods was compared and defined as their ability to produce high-quality DNA for positive amplification in the PCR technique. Amplification was observed in 59 out of 152 (38.8%) samples with the RLEP target. In the HCS samples, 100% positive amplification of *M. leprae* was obtained with the four methods; in the LCS samples, only the QIAGEN and phenol–chloroform methods showed 100% amplification of the RLEP target, but there was no RLEP amplification with the Promega method. In the NS group, QIAGEN showed positive amplification of RLEP in all the samples, but no amplification was detected with the rest of the methods (see Fig. 1). No amplification was observed in the NCS group by any method.

Considering the results obtained by each method, the following amplifications of *M. leprae* DNA were obtained: 23.7% (9 out of 38) Chelex 100 resin, 26.3% (10 out of 38) Promega kit, 44.7% (17 out of 38) phenol–chloroform method, and 60.5% (23 out of 38) QIAGEN method.

Discussion

Molecular techniques have emerged as a support for the conventional laboratory diagnosis for the confirmation of infection with *M. leprae* [11]. The first and very important step in these techniques is DNA extraction [6].

DNA extraction from ZNS slides can be a problem due to the need to obtain good-quality DNA to be amplified by PCR. All methods underwent a common pretreatment step with proteinase K and 10% sodium dodecyl sulfate, because some authors recommend this combination for improving the yield of the DNA extraction methods [12]. They claim that the pretreatment step yields better results than direct extraction [12],

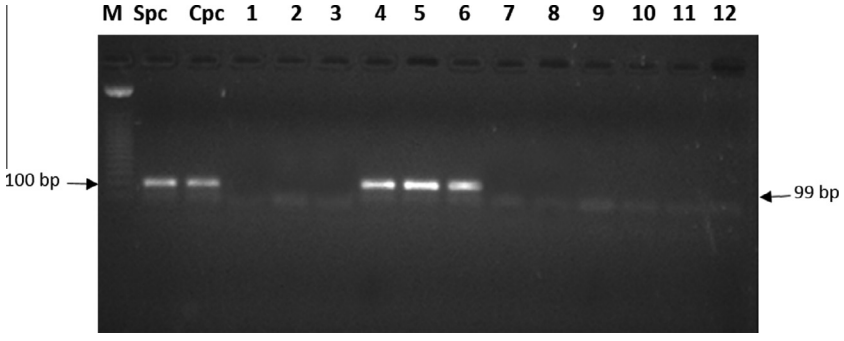

Fig. 1 – Chelex 100 resin method. Amplification of the RLEP region from genomic DNA from negative-slide (NS) samples. DNA extracted from a nodule of a Cuban multibacillary patient using the QIAGEN extraction method; 1, 2, 3: NS samples extracted with Chelex 100 resin; 4, 5, 6: NS samples extracted with the QIAGEN kit; 7, 8, 9: NS samples extracted with the phenol/chloroform method; 10, 11, 12: NS samples extracted with Promega kit. Cpc = Cuban positive control; M = 100 bp DNA marker (Promega); Spc = Spain positive control; 4 fg/μL of purified genomic *Mycobacterium leprae* DNA from *Dasypus novemcinctus*.

and this buffer solution has been used as a direct DNA extraction method in ZNS slides [1,13]. The yields in those studies were low, but we consider that, in our case, this combination obtains high concentrations and eliminates pollutants from the stained slides.

The successful amplification of a target sequence by PCR depends largely on the amount and quality of the extracted nucleic acids from the biological samples [14].

The mycobacterial cell-wall complex has a waterproof structure, which hinders their lysis and subsequent release of the genetic material [15,16]. The efficiency of cell lysis, the ratio of DNA/RNA extracted, and the residual extraction reagents will influence this process [16].

The quick and simple Chelex 100 chelating-resin procedure has been widely used for DNA extraction [17]. Our results for the HCS group are consistent with those obtained by Suárez et al. [4] who used this method to obtain good-quality DNA from positive slides, although Fontes et al. [13] concluded that it is not good enough for this purpose. Additionally, the Chelex-100-resin method is not capable of removing all the cellular components and other contaminants that may be present in the sample, and this can be the cause behind no amplification of the RLEP region, although no inhibition in our samples was observed [18]. In our study, the samples from LCS, NS, and NCS groups showed no positive amplification of the RLEP region, which may be due in part, in LCS and NS, to the presence of a very low bacterial load.

The phenol–chloroform mixture has been used to obtain DNA from mycobacterial culture [19], and obtains a good yield of DNA and high efficiency for HCS and LCS, while for NS and NCS, there was no amplification of *M. leprae* DNA. Probably due to the several steps of performance. To our knowledge, there is no previous report of the use of this extraction method for ZNS slides. However, our results indicate that it can be used to obtain good-quality DNA from positive slides in different molecular techniques, although it has several steps of sample handling that can reduce performance and increase cross contamination [20].

The Wizard Genomic DNA Purification Kit from Promega presented very low results with all the extraction methods considering quality, quantity, and efficacy. In the LCS, NS, and NCS groups, there was no DNA *M. leprae* amplification, and the analysis of the β-actin PCR control showed that there is no inhibition of the reactions. To our knowledge, there are no previous reports of the use of this extraction method for ZNS slides. Our results suggest that the Promega kit is not adequate for *M. leprae* DNA extraction from ZNS slides.

The relation between lower yield of DNA with lower yield of bacteria is difficult to explain. We consider a possible explanation that the samples from multibacillary patients have a result of the infiltration of the ear lobes and elbows normally present in this type of disease, and much more evident than in the paucibacillary patients, as a result will be more concentrated on the slides and present more total DNA, but we do not think it is related to the BI.

The OD_{260}/OD_{280} ratio is an indication of nucleic-acid purity. Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8. Low ratios could be caused by protein or phenol contamination. The QIAGEN method obtained high concentrations of DNA recovered with the highest purity value. This method is very efficient even for samples with highly degraded DNA [5]. Several studies comparing different extraction methods, including commercial kits, have validated the QIAGEN efficacy for DNA extraction [19].

The amplification of the target sequence not only depends on the extraction method used. There are other factors related, such as the number of copies of the gene in the microorganism, the size of the region to be amplified, and the PCR conditions [6]. In our case, the size of the sequence to be amplified is small and is present in up to 32 copies in the genome *M. leprae*, besides using a nested PCR increases the sensitivity of the test [21]. These factors should be taken into account because they can increase the sensitivity of the test, especially in clinical samples with low concentration of bacilli and/or degraded genomic material [10].

The two commercial methods showed significant differences between them, which may be due to the basic principle of these methods. The Promega kit is not an affinity method and requires several steps, which may result in low concentrations. The QIAGEN method is an affinity method that increases the purity and efficiency of the DNA obtained. Poma et al. [22] compared three commercial kits for DNA extraction for *Trypanosoma cruzi* artificially infected murine blood, and showed that the QIAGEN method had a high efficiency in comparison with other commercial kits.

The ZNS slides from paucibacillary patients are negative by the Ziehl–Neelsen staining method, but this does not necessarily indicate that the patient is not infected. This is one of the limitations for *M. leprae* detection by conventional methods, due to their low sensitivity for detecting the presence of bacilli. A negative ZNS slide only indicates that the concentration of bacilli is below 10,000 bacilli/mL [2]. When the QIAGEN kit was used for DNA extraction of the NS group, amplification of the RLEP sequence was detected, and in the NCS group, no amplification was detected. The positive amplification from NS reveals that the QIAGEN kit is a very efficient method for extracting DNA from samples with low levels of genomic material. Our results agree with Kamble et al. [1] who used a PCR to detect the presence of *M. leprae* in negative ZNS slides, and concluded that the use of negative slides may be useful for obtaining high-quality DNA. Molecular techniques have demonstrated high sensitivity that can increase the number of cases diagnosed compared with the conventional techniques [1].

The importance of identifying, diagnosing, and treating infected individuals increases the possibilities of interrupting the chain of transmission of the infection, and at the same time preventing the emergence of new cases and the aggravation of injury (disabilities) of diagnosed cases. Although the diagnosis with PCR is not applicable in most areas with a high burden of leprosy, the ZNS slides can be easily sent to reference laboratories for later molecular analysis.

Conclusion

This study reveals that ZNS slides can be a source of good-quality DNA useful for PCR. The results of this study

demonstrate the ability of QIAamp DNA Mini Kit (QIAGEN) for obtaining high-quality DNA to be used in molecular techniques even from negative ZNS slides, and the usefulness of the phenol–chloroform method for extracting DNA from positive ZNS slides. The DNA extraction and PCR from the ZNS slides can be a useful tool to confirm the diagnosis of paucibacillary patients, which usually is very difficult by conventional methods.

A good DNA extraction from ZNS slides can be useful not only for diagnosis, but also for the application of other molecular techniques, such as the genetic characterization of the pathogen or resistance to antibiotics, and also retrospective studies, which, given the impossibility to cultivate *M. leprae*, will provide new elements for the understanding of the epidemiology of the disease.

Conflicts of interest

There was no financial support.

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REFERENCES

[1] R.R. Kamble, V.S. Shinde, S.P. Madhale, et al, 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 (2010) 57–59.
[2] C.C. Shepard, D.H. McRae, A method for counting acid-fast bacteria, Int. J. Lepr. Other Mycobact. Dis. 36 (1968) 78–82.
[3] R. Dayal, M. Agarwal, M. Natrajan, et al, PCR and in-situ hybridization for diagnosis of leprosy, Indian J. Pediatr. 74 (2007) 645–648.
[4] O. Suárez, P. Suffys, A. Entenza, et al, Extracción de ADN de *Mycobacterium leprae* de láminas positivas de pacientes multibacilares, Rev. Leprol. 28 (2012) 455–458.
[5] K. Phillips, N. McCallum, L. Welch, A comparison of methods for forensic DNA extraction: chelex-100® and the QIAGEN DNA Investigator Kit (manual and automated), Forensic Sci. Int. Genet. 6 (2012) 282–285.
[6] Y. de Armas, V. Capó, L. López, et al, Comparación de tres métodos de extracción de ADN de tejidos embebidos en parafina, Biotecnol. Apl. 28 (2011) 40–43.
[7] Ministerio de Salud Pública, Programa de Control de Lepra, second ed., Ciencias Médicas (ed.), Havana, Cuba, 1999.
[8] Ministerio de Salud Pública, Lepra. Normas Técnicas para el Control y Tratamiento, Ciencias Medicas (ed.), Havana, Cuba, 2008.
[9] A. Ani, S. Okpe, M. Akambi, Comparison of a DNA based PCR method with conventional methods for the detection of *M. tuberculosis* in Jos, Nigeria, J. Infect. Dev. Ctries. 3 (2009) 470–475.
[10] H.D. Donoghue, J. Holton, M. Spigelman, PCR primers that can detect low levels of *Mycobacterium leprae* DNA, J. Med. Microbiol. 50 (2001) 177–182.
[11] A.N. Martinez, R. Lahiri, T.L. Pittman, et al, Molecular determination of *Mycobacterium leprae* viability by use of real-time PCR, J. Clin. Microbiol. 47 (2009) 2124–2130.
[12] C.J. Huijsmans, J. Damen, J.C. van der Linden, et al, Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: effects on downstream molecular applications, BMC Res. Notes 3 (2010) 239.
[13] A. Fontes, H. Gomes, M. de Araujo, et al, 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 (2012) 143–149.
[14] L. Bachmann, B. Däubl, C. Lindqvist, et al, PCR diagnostics of *Mycobacterium tuberculosis* in historic human long bone remains from 18th century burials in Kaiserebersdorf, Austria, BMC Res. Notes 1 (2008) 83.
[15] U. Reischl, Application of molecular biology-based methods to the diagnosis of infectious diseases, Front. Biosci. 1 (1996) e72–e77.
[16] R. Kotlowski, A. Martin, A. Ablordey, et al, One-tube cell lysis and DNA extraction procedure for PCR-based detection of *Mycobacterium ulcerans* in aquatic insects, molluscs and fish, J. Med. Microbiol. 53 (2004) 927–933.
[17] K. Nagdev, R. Kashyap, P. Deshpande, et al, Determination of polymerase chain reaction efficiency for diagnosis of tuberculous meningitis in Chelex-100 extracted DNA samples, Int. J. Tuberc. Lung Dis. 14 (2010) 1032–1038.
[18] J. Chang, S. Zhang, L. Li, Comparing and evaluating six methods of extracting human genomic DNA from whole blood, Fa Yi Xue Za Zhi 25 (2009) 109–111.
[19] O. Okwumabua, E. Shull, M. O'Connor, et al, Comparison of three methods for extraction of *Mycobacterium avium* subspecies paratuberculosis DNA for polymerase chain reaction from broth-based culture systems, J. Vet. Diagn. Invest. 22 (2010) 67–69.
[20] J. Sidorova, B. Biderman, E. Nikulina, et al, A simple and efficient method for DNA extraction from skin and paraffin-embedded tissues applicable to T-cell clonality assays, Exp. Dermatol. 21 (2012) 57–60.
[21] S. Jamil, S.M. Wilson, M. Hacket, A colorimetric PCR method for the detection of M. leprae in skin biopsies from leprosy patients, Int. J. Lepr. Other Mycobact. Dis. 62 (1994) 512–520.
[22] H. Poma, C. Davies, D. Gutierrez, et al, Comparación de la eficiencia de extracción de ácidos nucleicos utilizando distintos kits comerciales y empleando la qPCR. Efecto de las sustancias inhibidoras, Rev. Argent. Microbiol. 44 (2012) 144–149.