British Journal of Medicine & Medical Research
8(2): 147-156, 2015, Article no.BJMMR.2015.435
ISSN: 2231-0614

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Evaluation of Simple and Cost-Effective DNA Preparation and Subsequent PCR Amplification for Clinically Relevant Mycobacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Author FR designed the study, author VJG wrote the protocol, authors FR and VJG wrote the first draft of the manuscript and author VJG managed the experimental process. Authors FR, JAR and VJG managed the literature searches. Author DHC managed the statistical analyses of the study. Authors GIM and AMG performed the growth and reading of the culture media and identified the mycobacterial species. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2015/16945
Editor(s):
(1) Rui Yu, Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, USA.
Reviewers:
(1) Anonymous, India.
(2) Travería Gabriel Eduardo, Universidad Nacional de La Plata, Facultad de Ciencias Veterinarias, Argentina.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1114&id=12&aid=8894

Original Research Article

Received 19th February 2015
Accepted 3rd April 2015
Published 20th April 2015

ABSTRACT

Aim: Slow growth rate in culture renders the traditional isolation, identification, and drug susceptibility testing of clinically important mycobacteria inadequate when there is an urgent need for a precise diagnosis in order to initiate patient treatment. Molecular methods all rely on mycobacterial DNA isolation which in turn has become an essential step of the process. Our study aimed to evaluate DNA isolation protocols from mycobacteria of clinical interest.
Methods: Therefore, in order to determine an optimal method we evaluated 8 inexpensive, rapid and easy DNA isolation methods from 30 mycobacterial cultures (10 *Mycobacterium tuberculosis*

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and 20 Non-tuberculous Mycobacteria) for subsequent direct detection by PCR.
Results: Six of those 8 methods reliably allow the isolation of good DNA yields and quality, the optimal protocol being the one that includes a 1% Triton X-100 lysis solution. Protocols using SDS 1% as a lysis solution did not yield DNA suitable for PCR amplification.
Conclusion: Six of the methods we evaluated can easily be implemented in resource limited settings for routine use, potentially contributing to a better management of mycobacterial infections.

Keywords: Mycobacteria; polymerase chain reaction; DNA isolation; diagnostics; resource limited settings.

1. INTRODUCTION

Mycobacteria have gained a particular place in the bacterial world for the clinical importance of some important human pathogens, such as *Mycobacterium tuberculosis* (MTB), *Mycobacterium leprae* and *Mycobacterium ulcerans*, the etiological agents of tuberculosis, leprosy, and Buruli ulcer, respectively [1,2]. MTB infections alone are a public health problem worldwide claiming around 1.5 million lives annually [3].

Non-tuberculous Mycobacteria (NTM) are for the most part ubiquitous environmental organisms, some of which can cause diseases in humans [4]. NTM consist of more than 100 species with more than 30 described within the last 10 years. These microorganisms are currently an important and increasing cause of infections in patients with HIV, immunological defects, aging, cancer, and those who undergo immunosuppressive therapies [5]. In the recent years, NTM such as *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium abscessus* and *Mycobacterium peregrinum* have been characterized in clinical isolates from patients presenting lesions after esthetic surgeries or mesotherapia which confirms the surge of these infections [6-8].

Traditionally, mycobacteria are identified by phenotypic traits, such as morphological features, optimal growth temperature, pigmentation and biochemical profiles [9,10]. However, conventional biochemical tests have been associated with long turnaround times, leading to significant delays in diagnosis [11]. Other methods based on lipid analysis, such as high-performance liquid chromatography thin-layer chromatography, and gas-liquid chromatography, are cumbersome and expensive and are used only in select clinical laboratories [12].

Molecular identification by nucleic acid probes or polymerase chain reaction (PCR) derived methods such as Restriction Fragment Length Polymorphism (RFLP) have significantly decreased the time of procedure [13-17]. However, identification of mycobacteria by any of those methods is impaired by difficulties in extracting genomic DNA due to the complex structure of the lipid rich mycobacterial cell wall that is resistant to simple lysis processes with strong alkali or acids [18-20]. As a result, most of the simple and commonly used nucleic isolation procedures result in poor quality and low yield of nucleic acids [19-21].

Several methods of mycobacterial cell wall lysis and DNA extraction have been evaluated, including detergents, proteolytic enzymes, mechanical disruption, and temperature changes alone and in various combinations [21-28].However these can be complex and some are expensive. In addition, standardized analytic procedures are not completely safe for mycobacteriology laboratory staff [29]. Therefore, before DNA extraction, pathogenic mycobacteria must be inactivated to render it safe for manipulation outside of a containment level 3 facility [30-34].

Technical complexity as well as the equipment and consumables required make the use of enzymatic lysis, detergents, and other commercial extraction methods time consuming and expensive. In order to find the optimal procedures to minimize processing time, costs and bio safety risks, we evaluated8 protocols for extracting DNA from cultures of MTB and NTM for the direct PCR detection of mycobacteria.

2. MATERIALS AND METHODS

2.1 Mycobacterial Strains and Culture Conditions

2.1.1 *Mycobacterium tuberculosis*

A total of 1 H37Rv laboratory reference strain and 10 MTB isolates obtained from clinical pecimens processed at the Bacteriology Unit of

the Corporation for Investigations in Biology (CIB) and randomly selected were grown in the automated BACTEC™ MGIT™ 960 medium (Becton-Dickinson, Franklin Lakes, NJ, USA). Two hundred microliters of the MGIT 960 cultures were then inoculated onto Löwenstein-Jensen (LJ) slants and incubated for 4 weeks at 37ºC until growth became apparent. Cell suspensions were then prepared in Middle brook 7H9 broth and adjusted to the concentration of a 1.0 McFarland Standard, which is equivalent to $3x10^8$ mycobacteria/mL.

2.1.2 Non-tuberculous mycobacteria

Twenty isolates of NTM (*M. abscessus* (5), *M. fortuitum* (4), *M. avium* (4), *M. marinum* (3), *M, chelonae* (2), *M. peregrinum* (1) *and M. kansasii* (1))were obtained from clinical specimens processed at the CIB Bacteriology Unit. NTM were initially grown on LJ medium for 10 to 15 days at 37ºC until growth was observed. Bacterial suspensions of a 1.0 McFarland Standard were then prepared in Middle brook 7H9 medium.

2.2 Heat Inactivation

One mL of the Middle brook 7H9 broth suspension of H37Rv containing $3x10^8$ mycobacteria was transferred to 3 sterile 1.5 ml screw-capped tubes that were subsequently heated in a dry-heat block (Multi-Block heater, Thermo Scientific, USA) at 95°C. Ten sets of 3 sterile 1.5 mL tubes were prepared and heated for 10 different times, increasing at 5minute increments from 0 to 45 minutes.

After heat treatment, the efficacy of the inactivation procedure was assessed by culture on Thin Layer Agar Middle brook 7H11 (TLA 7H11) for 6 weeks at 35°C and 6% CO2. Growth control was assessed using the H37Rv strain not subjected to heat treatment, which was cultured in parallel under the same conditions in triplicate. The shortest heating time inducing a total inactivation was subsequently used for inactivating the MTB clinical isolates before growth control assessment as described for the reference strain. The same minimal heat activation time was applied to the 20 NTM however, no subsequent growth control was performed considering that their transmission and pathogenic characteristics were not constituting a hazard.

2.3 DNA Extraction and Quantification

A total of 10 heat-treated MTB and 20NTM isolates were evaluated for 8 DNA extraction protocols that differed by the lysis solution used (Table 1). Each isolate was resuspended in Middle brook 7H9 broth and adjusted to a 1.0 Mac Farland as described above. For each DNA extraction protocol, an aliquot of 200 µL corresponding to $6x10^7$ bacteria of each suspension was transferred into a sterile 1.5 ml screw-capped tube to which an equal volume of lysis solution was added prior to thermal treatment. After cooling at room temperature, tubes were briefly centrifuged to collect droplets. The 8 extraction protocols performed are listed in Tables 1 and 4 of them include a 1 min sonication at room temperature. All work was performed in a BSL3 environment, using class II type A2 bio safety cabinets and personal protective equipment including N95 particle respirators, gloves and protective gowns.DNA concentrations were determined using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) by measuring the absorbance at 260 nm.

Table 1. DNA extraction protocols

Protocol	A	B	C	D	E	F	G	H
Lysis solution	**Water**	**NaCl0.85%**	**1% SDS**	**1% Triton-X 100**	**Water**	**NaCl0.85%**	**1% SDS**	**1% Triton-X 100**
Inactivation at 95C for 5 min	v	v	v	v	v	v	v	v
Centrifugation at 10000 x g for 5 min	v	v	v	v	v	v	v	v
Sonication for 1 min	x	x	x	x	v	v	v	v

Description of the 8 (from A to H) DNA extraction protocols applied to the MTB and NTM isolates. Four different lysis solutions were used. Protocols A to D differ from protocols E to H by the application (or not) of a 1 min sonication step. (v): procedure was applied. (X): procedure was not applied

2.4 PCR Amplification of Mycobacterial DNA

Amplification of MTB DNA was performed by using T4(5´ CCTGCGAGCGTAGGCGTCGG 3´) and T5 (5´ CTCGTCCAGCGCCGCTTCGG 3´) primers specific for the IS*6110*MTB complex-specific insertion element [35]. To detect NTM DNA the amplification of a 439 bp fragment of the gene coding for the 65 KDa heat shock protein (*hsp*65) which is conserved among mycobacteria, was performed using Tb-11 (5´ ACCAACGATGGTGTGTCCAT 3´) and Tb-12 (5´ CTTGTCGAACCGCATACCCT 3´)primers [36]. PCR reactions were performed in 50 µL reaction mixtures using 10 ng of template DNA and 1.25U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania) following the manufacturer´s recommendations on a BioRadDNA Engine ® (BioRad, Hercules, CA, USA).Cycling parameters for MTB amplification were an initial denaturation at 95°C for 5 min, followed by 39 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min. A final extension at 72°C for 7 min was added. Amplification of the NTM*hsp*65 fragment was performed as described for MTB except that the annealing temperature was 62°C. The amplified DNA products were visualized by ethidium bromide staining on a 2% agarose gel.

2.5 Data Analysis

The data were analyzed and plotted using the Prism 5.0 software (Graph Pad, La Jolla, CA, USA). The average of the DNA quantities obtained after each of the 8 extraction protocols for the mycobacterial isolates was calculated, followed by an ANOVA test to identify the differences between averages. Statistically significant differences were identified using the post hoc Bonferroni test, with a 95% (IC 95%) confidence interval. A value of $p \leq 0.01$was considered significant.

3. RESULTS

3.1 Heat Inactivation

Heat kill experiments were performed at 95°C during various times from 0 to 45 min with a 5 min increment using the control H37Rv strain. For all the exposure times, no growth was observed after 6 weeks incubation at 35°C in TLA 7H11 medium, indicating a complete inactivation after at least a 5 min thermal treatment. According to these first results and to maximize safety and prevent the risk of a biohazard contamination and infection, the 10 MTB clinical isolates were heated at 95°C for 5 min before culture for 6 weeks in TLA 7H11, also resulting in 0% organism viability.

3.2 Nucleic Acids Extraction and Quantification

The 8 DNA isolation procedures were applied independently to the 30 mycobacterial isolates (10 MTB and 20 NTM) and the total DNA yield was quantified. Results are presented as mean±SD in Table 2. Among the 4 methods tested that do not require sonication, the highest DNA yield resulted from protocol D using 1% Triton-X 100. The DNA yield differences between protocol D and all the other protocols are significant at $p<0.01$ as indicated in Table 3. Among the 4 methods that include a 1 min sonication, the highest DNA yield resulted from protocol H, which corresponds to D plus sonication. Again, DNA yield differences between protocol H and all the other protocols are significant at $P<0.01$ (Table 3). Therefore in both groups of protocols (with and without sonication), 1% Triton-X 100 appeared to be the most efficient lysis solution. Also, the implementation of the 1 min sonication step improved the total DNA yield only when switching from protocols D to H ($p<0.001$). However, there was no significant difference between protocols A and E, B and F, and C and G.

3.3 DNA Amplification by PCR

In order to verify that the quality of the isolated DNA was suitable for further use, PCR amplification was carried out on the DNA isolated by each of the 8 protocols. For each protocol, PCR was performed with the MTB *IS*6110 specific T4 and T5 primers on 10 ng of isolated MTB DNA from the H37Rv reference strain as well as the 10 MTB clinical isolates. All the MTB isolates resulted in a similar amplification profile with an amplicon of 123 bp. Fig. 1A shows the typical amplification profile of a randomly chosen MTB isolate from which DNA was isolated applying protocols A to H. No amplification at all was observed with protocols C and G, both using 1% SDS as the lysis solution. Tb-11 and Tb-12 primers, used for amplification of NTM amplified a fragment of 439 bp (Fig. 1B). All NTM DNAs amplified similarly with the various isolation protocols except for protocols. DNA isolated using protocols C and G was not used for NTM amplification since no product had been obtained with MTB.

Table 2. Total DNA yield averages obtained from mycobacterial isolates applying protocols A to H

Protocol	DNA yield (mean (SD))							
	A	B	C	D	E	F	G	H
MTB (n=10)	13.0 (7.8)	18.5 (7.0)	21.6 (3.7)	301.6 (64.7)	20.6 (14.3)	27.04 (13.0)	21.59 (2.2)	396.5 (99.6)
NTM (n=20)	14.1 (16.9)	21.9 (31.2)	13.9 (14.3)	401.3 (121.5)	20.3 (28.3)	24.4 (32.8)	15.5 (15.4)	496.5 (167.3)
MTB + NTM (n=30)	13.7 (14.4)	20.8 (25.6)	16.4 (12.3)	365.7 (114.3)	20.4 (24.2)	25.3 (27.5)	17.53 (12.8)	462.0 (153.4)

Standard deviation (SD), MTB (Mycobacterium tuberculosis), NTM (Non-tuberculous Mycobacteria);Average DNA yield averages (in μg) obtained after isolation process applying protocols A to H. Standard Deviation is indicated in hyphens

Table 3. Significance of the differences in total DNA yields obtained applying protocols D and H compared to the other protocols

Comparison of protocol (1)	Compared with protocol (2)	Difference (1-2)	*p*	95% CI for difference	
D	A	351.9	<0,001	294.8	409.1
	B	344.8	<0,001	287.7	402.0
	C	349.2	<0,001	292.0	406.3
	E	345.2	<0,001	288.0	402.3
	F	340.3	<0,001	283.2	397.5
	G	348.1	<0,001	291.0	405.3
	H	-96.3	<0,001	-153.9	-38.6
H	A	448.2	<0,001	391.6	504.9
	B	441.2	<0,001	384.5	497.8
	C	445.5	<0,001	388.8	502.1
	D	93.3	<0,001	38.6	153.9
	E	441.5	<0,001	384.9	498.1
	F	436.6	<0,001	380.0	493.3
	G	444.4	<0,001	387.8	501.1

Comparison of the significance and confidence intervals of the differences between DNA yield averages obtained after isolating mycobacterial DNA with protocols A to H. The 2 protocols that yielded the greatest amount of DNA, D and H respectively (1) are compared to the other methods (2) in terms of total DNA yield difference (1-2), significance (p) and 95% confidence interval.

3.4 Comparison of DNA Isolation Methods for Processing Time and Cost

It was measured that protocols A to D (without sonication) require 11 min to complete, and protocols E to H (with sonication) can be achieved in 12 min. The cost of each DNA isolation method was calculated based on the current prices of consumables, equipment and reagents. All protocols required the same consumables. Differences in reagents are reflected in the lysis solution composition. The least expensive protocols were A and E (0.11 USD), followed by B and F (0.15 USD) and D and H (0.20 USD). Finally, protocols C and G were the most expensive (0.58 USD).

4. DISCUSSION

Heating of the culture is widely used for the inactivation of MTB, however, some current procedures for heat inactivation may not be perfectly adequate as heat killing at 80ºC for 20 min for the inactivation of MTB is not safe nor effective [25]. These findings were later supported by several studies that showed that heating at 95ºC in a heat block was not adequate to kill suspensions of mycobacteria. However, heating at 100ºC in a boiling-water bath or a forced-air oven for at least 5 min kills mycobacteria, suggesting that temperatures below 100ºC do not kill mycobacteria [24,37-38].

Our results show that inactivation at 95ºC for at least 5 min using a dry-heat block is sufficient for

inactivation of MTB, therefore setting-up a basis to work safely in the subsequent DNA processing steps and avoid the risk of acquiring a laboratory infection. TLA 7H11 was selected to check the viability of MTB after heat inactivation because previous studies had shown that this method is more sensitive and faster than growth on the traditional LJ medium [39,40]. Given the high pathogenicity of MTB, we recommend that each laboratory willing to implement this heat-killing method should test it first with all the growth and viability controls before its routine use. Previous maintenance and temperature check of the heater should be performed carefully since an inadequate temperature indication could have serious consequences.

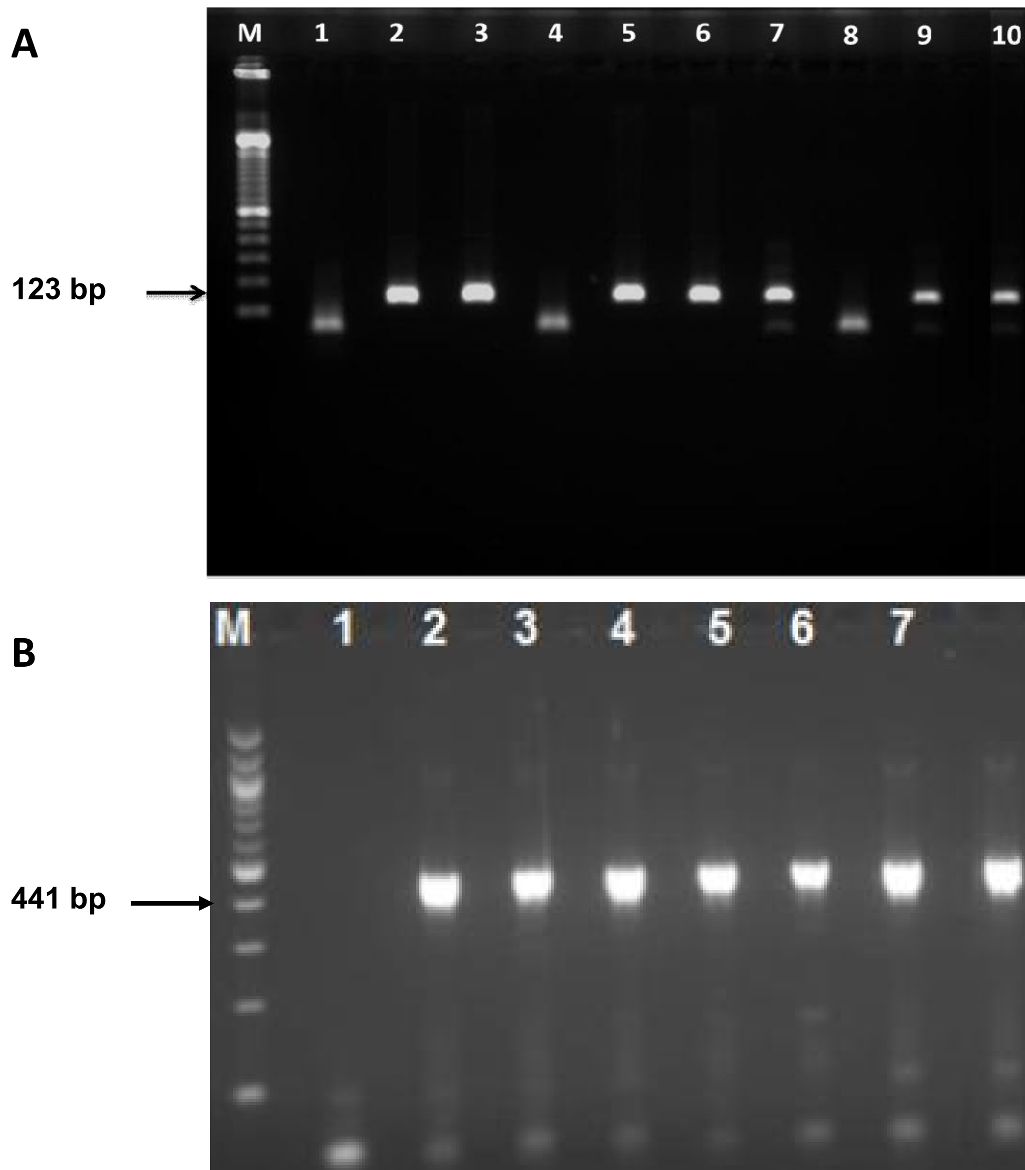

Fig. 1. Electrophoresis profile of PCR amplifications

Ethidium bromide stained 2% agarose gel electrophoresis profile of a typical PCR amplification of a randomly chosen MTB (1A) and NTM (1B) isolate using the 8 protocols. Lanes are as follows: 1A: M: molecular weight marker (100 bp DNA ladder), 1: negative control; 2: protocol A; 3: protocol B; 4: protocol C; 5: protocol D; 6: protocol E; 7: protocol F; 8: protocol G; 9: protocol H; 10: H37Rv positive control; 1B: M: molecular weight marker (100 bp DNA ladder), 1: negative control; 2: protocol A; 3: protocol E; 4: protocol B; 5: protocol lF; 6: protocol D; 7: protocol H; 8: H37Rv positive control

In order to determine an optimal extraction method (quick, cheap, safe method and with enough quantity and excellent quality of DNA), our study evaluated different mycobacterial DNA extraction protocols.

Comparison of our different extraction methods showed that 1% Triton-X 100 lysis with or without sonication provided the greatest yield of DNA (Table 2) with statistically significant differences with respect to other extraction methods (Table 3). It is known that Triton-X 100 prevents the aggregation of mycobacteria in suspension [21,41]. Additionally, heating of the mycobacterial isolates facilitates the detergent action on the mycobacterial cell wall, thus enhancing the effect of cell lysis. Our results corroborate those published by Awua and col. in which 1% Triton-X 100 was shown to be the lysis solution providing the highest DNA yield [21]. However in that study, the solvent of the Triton-X 100solution was Tris/EDTA whilst in our case, it is simply water.

The implementation of a 1 min sonication step improved the total DNA yield only for the Triton X-100. Indeed, the availability of a sonicator is not common in many laboratories, and even though its use in protocols D and H is beneficial, the already satisfactory DNA yield obtained with protocol D makes it optional.

The absence of amplification observed in extracts obtained using 1% SDS is likely to be due to residual SDS. It has been suggested that substances such as phenol, heparin, haemoglobin and SDS may be potent inhibitors of PCR [19,20,42,43]. Their use could require additional DNA purification in order to remove inhibitors. This extra step makes the use of 1% SDS impractical.

In addition to time, easiness, safety and DNA quality and quantity, another important point to be considered when selecting a DNA isolation method for routine use is cost [44]. For the protocols evaluated in this study, the cost per extraction ranged from USD 0.11 to USD 0.58. The cost of the protocols that included sonication did not increase with respect to protocols that did not include that step because the 1 min use of the sonicator was too short to have a measurable impact on the cost. However, one cannot elude that the initial purchase of a sonicator is out of reach in most resource limited settings. The most expensive and least reliable protocols were the ones using SDS (C and G). At this point in addition to cost, we believe that they should not be recommended for the inconsistency in amplification already mentioned above. The remaining 3 sets of methods, A and E (USD 0.11), B and F (USD 0.15) and finally D and H (USD 0.20) have a cost varying very little from one to another. These differences in cost are not very important for a laboratory that processes only a few mycobacterial cultures per week, but it will become critical if the number of DNA isolations is on a much larger scale.

The election of a suitable mycobacterial DNA isolation protocol will also depend on the DNA quality needed for subsequent analysis. No visible difference in amplification was observed between protocols A, B, D, E, F and H, indicating that they all provide a DNA with a similar quality for both MTB and NTM, at least for PCR analysis.

For this study, cost considerations were a factor that determined our choice of sample size (10 MTB + 20 NTM), and we opted for an approach that allowed us to obtain significant *p* and 95% CI values (Table 3). As it has been several times advocated, setting a sample size goal that must be reached regardless of cost, or simply ignoring costs is often impractical for investigators when a more realistic approach can produce valuable results [45-47].

5. CONCLUSION

Six of the methods we evaluated can easily be implemented in resource limited settings for routine use, potentially contributing to a better management of mycobacterial infections.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. This study is classified in the category "research without risk". For this study apply only experimental laboratory techniques involving MTB isolates and at no time any biological, physiological, psychological or social interventions on humans were performed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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