Brazilian Journal of Microbiology (2011) 42: 774-777
ISSN 1517-8382

ASSESSMENT OF THE QUALITY OF DNA EXTRACTED BY TWO TECHNIQUES FROM *MYCOBACTERIUM TUBERCULOSIS* FOR FAST MOLECULAR IDENTIFICATION AND GENOTYPING

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Submitted: March 31, 2010; Returned to authors for corrections: May 30, 2010; Approved: November 04, 2010.

ABSTRACT

We report a comparative study of two DNA extraction techniques, thermolysis and chemical lysis (CTAB), for molecular identification and genotyping of *M. tuberculosis*. Forty DNA samples were subjected to PCR and the results demonstrated that with thermolysis it is possible to obtain useful data that enables fast identification and genotyping.

Key words: DNA extraction - *Mycobacterium tuberculosis* - thermolysis - CTAB

Tuberculosis (TB) is responsible for the death of approximately 3 million people per year and it is estimated that one third of the human population is infected latently with *Mycobacterium tuberculosis*, Brazil occuping 16th place in the world ranking (13). The development of fast molecular biology techniques to identify and genotype *M. tuberculosis* isolates is crucial for the prevention and control of tuberculosis. Although techniques based on amplification of nucleic acids are highly sensitive and reduce the time required for identification or epidemiological study of *M. tuberculosis*, there are few studies assessing the quality of *M. tuberculosis* DNA extracted from cultures. Several techniques for DNA extraction have been described (6, 9), for application in distinct diagnostic methods.

Here we aimed to find a fast and easy method to extract DNA of sufficiently high quality to be used in the molecular identification and genotyping of *M. tuberculosis* isolates. Two methods for DNA extraction from *M. tuberculosis* cultures were compared and the extracted DNA was utilized for identification of the isolates by PCR and their genotyping by the spoligotyping technique.

A total of 40 *M. tuberculosis* clinical isolates were analyzed. The first method of extraction was a modified thermolysis technique (10) and the second was chemical lysis with cetyltrimethylammonium bromide (CTAB) (12). In the thermolysis method, a loopful of mycobacteria grown on Lowenstein-Jensen medium was suspended in 300 mL of TE buffer (10 mM Tris, 1 mM EDTA pH 8.5) and subjected to 3 cycles of boiling and freezing for 20 minutes at -20ºC. In the CTAB method, the samples were inactivated by heating for 18 - 24 hours at 80ºC in a dry bath. After that, proteinase K and sodium dodecyl sulfate (SDS) were added and the samples incubated for 10 minutes at 65ºC. 5M NaCl and CTAB solution were then added and incubated for another 10 minutes at 65ºC. Chloroform: isoamyl alcohol (24:1) mixture was added and

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mixed until a milky mixture was obtained, which was centrifuged to 11,750 rcf for 5 minutes. The upper phase was transferred to a new tube containing isopropanol and the samples were vortexed and frozen for 18 hours. After thawing, the samples were centrifuged for 30 minutes at 4ºC and the supernatant was discarded. After drying, the samples were washed with 70% ethanol and centrifuged for 20 minutes at 11,750 rcf at 4°C. The supernatant was discarded and the pellets were dried at room temperature. The samples were resuspended in TE buffer and incubated for one hour at 65ºC and, finally, frozen for 2 hours at -20ºC.

For the molecular identification by PCR, a pair of universally accepted primers were used to amplify a fragment of the insertion sequence IS*6110* (PCR-IS*6110*), a specific sequence for the *M. tuberculosis* complex (11). For the PCR reaction, 21.5 µL of 1x PCR-Master Mix (Fermentas™, USA), 0.5 µL of each primer (INS-1 5'-CGTGAGGGCATCGAGGT GGC-3' and INS-2 5'GCGTAGGCGTCGGTGACAAA-3') and 2.5 µL of genomic DNA were mixed. After this, the reaction was performed in the PTC-100 Thermo cycler (MJ Research) under the following conditions: initial cycle of 95ºC for 10 minutes, followed for 30 cycles of 94°C for 1 minute, 56°C for 2 minutes and 72°C for 1 minute and a final cycle of 72°C for 7 minutes. Subsequently, 10 µL of amplified product was loaded on 1% agarose gel, resolved by electrophoresis and stained with ethidium bromide.

Strains were genotyped by the spoligotyping technique, which is a standard method for *M. tuberculosis*. Spoligotyping is a PCR-based technique that simultaneously detects and types *M. tuberculosis* through patterns of multiple, well-conserved 36-bp direct repeats (DRs) with nonrepetitive spacer sequences, 34 to 41 bp long (5).

In the spoligotyping technique, two amplifications of the extracted DNA were performed. In the first amplification reaction, 20 µL of 1x PCR-Master Mix (Fermentas™, USA), 1 µL of each primer at 5 µM (DRa 5'biotinylated-GGTTTTGGG TCTGACGAC-3', and DRb 5'-CCGAGAGGGGACGGAAA C-3') (5) and 1 µL of extracted DNA. In the second amplification reaction, PCR-amplified products were used as template. The reactions were performed in a PTC–100 Thermo cycler (MJ Research), with an initial cycle of 15 minutes at 95ºC, followed for twenty cycles of 60 seconds at 95ºC, 60 seconds at 55ºC and 30 seconds at 72ºC and a final cycle of 5 minutes at 72ºC. Following DNA amplification, 20 µL of amplicons were added to 150 µL of 2x SSPE/0.1% SDS solution and heated at 100ºC for 10 minutes. Next, 150 µL of this mixture were applied to a membrane and incubated for 60 minutes at 60ºC for hybridization.

The membrane was washed twice with 2x SSPE/0.5% SDS solution and incubated in 1:4,000-diluted streptavidin-peroxidase conjugate (Zymed Laboratories, USA) for 60 minutes at 42ºC. Afterwards, the membrane was washed twice with 2x SSPE and incubated for 1 minute with ECL™ (GE Healthcare, UK), for chemoluminescence. As the DRa primer is labeled with biotin, the amplified DNA could be used for hybridization to spacer nucleotides, which were covalently bound to a membrane (5). After 30 minutes contact with the membrane, X-ray film was labeled and the spoligopatterns were visualized. DNA from *M. bovis* BCG and *M. tuberculosis* $H_{37}Rv$ were used as positive controls and 2x SSPE/0.1% SDS solution as negative control. The results of spoligotyping of DNA extracted by both lysis techniques were analyzed comparatively, using the Fourth International Spoligotyping Database (SpolDB4) (2).

Out of these two extraction techniques, thermolysis proved to be easier and faster. In thermolysis, the DNA was extracted after 2 hours, while in chemical lysis with CTAB, the extracted DNA was obtained after 30 hours. In laboratories that work with pathogenic bacteria such as *M. tuberculosis*, the thermolysis technique is of special interest because the cyclic procedures of heating and freezing cause the death of the bacteria, preventing contamination. Several studies have demonstrated the usefulness of thermolysis for inactivation and DNA extraction (4, 8).

In the molecular identification tests, the PCR results indicated that both techniques (thermolysis and CTAB) can be used for DNA extraction, as illustrated in Figures 1a and Figure 1b, respectively. Regarding genotyping by spoligotyping, it was possible to obtain the spoligotypes with the DNA extracted by both techniques (thermolysis and CTAB), as illustrated in Figures 2a and 2b, respectively.

The spoligotypes found belong to 5 families and 12 sub-families: LAM2, LAM4, LAM6, LAM9, LAM3 S Convergent, H1, H3, S, T1, T4-CEU1, U Likely T3 and U. These results showed that the spoligotyping technique can be successfully performed with DNA extracted by thermolysis, because in the molecular epidemiology, the quality and the efficiency of amplification depend on the efficiency of DNA extraction methods (1) and the quality of *Taq* polymerase (3).

In conclusion, thermolysis is a simple, fast method that can be employed in the extraction of *M. tuberculosis* DNA. The quality of the DNA obtained by thermolysis was sufficient to allow molecular identification by PCR and genotyping by spoligotyping, contributing to the rapid diagnosis and control of tuberculosis for molecular epidemiology.

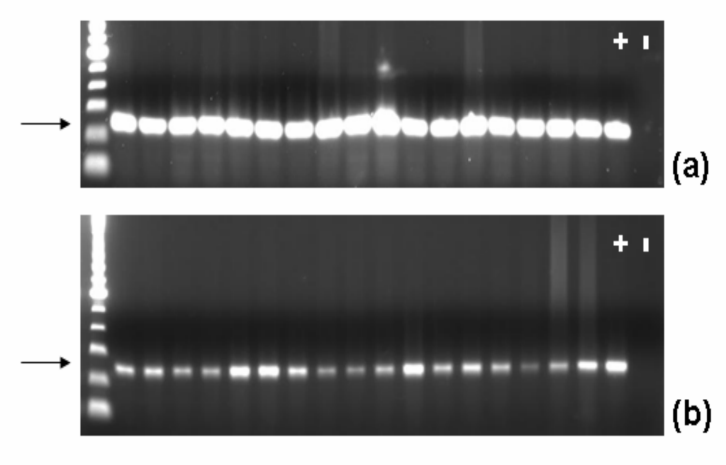

Figure 1. Multiplex PCR amplification products from various *Mycobacterium tuberculosis* DNAs extracted by (a) thermolysis and (b) CTAB methods after electrophoresis on a 1% agarose gel and staining with ethidium bromide. Arrows indicate the fragments with 245 bp.
Positive (+) ($H_{37}Rv$) and negative (-) controls and a 100 bp molecular ladder were used.

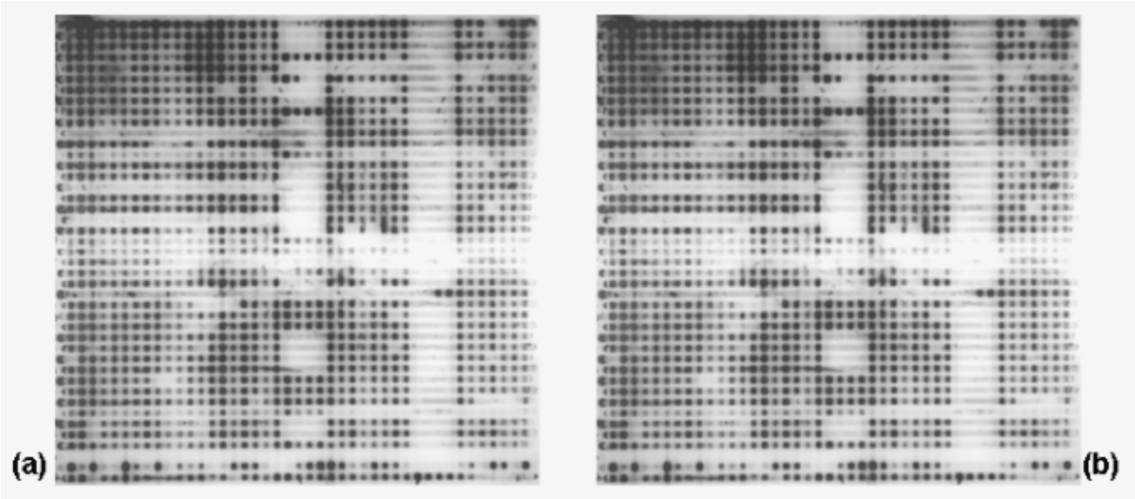

Figure 2. Spoligotype patterns obtained from various *Mycobacterium tuberculosis* DNAs extracted by (a) thermolysis and (b) CTAB methods. The samples on the 3 lowest rows are the negative control and the two positive controls ($H_{37}Rv$ and *M. bovis*).

ACKNOWLEDGEMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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