Novel use of guanidinium isothiocyanate in the isolation of Mycobacterium tuberculosis DNA from clinical material

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Abstract

Nucleic acid amplification technologies offer great promise for the rapid, sensitive and specific diagnosis of tuberculosis. However, the isolation of inhibitor-free DNA from biological specimens is a bottleneck of the PCR assay. Here we describe a simple method for the isolation of PCR-amplifiable DNA of Mycobacterium tuberculosis from all types of samples of pulmonary and extrapulmonary origin tested. Briefly, it involves concentration of the bacilli by high-speed centrifugation, removal of PCR inhibitors by a wash solution containing guanidinium isothiocyanate and the release of bacterial DNA by heating in the presence of detergents and Chelex-100 resin. The entire process is accomplished within \( V \) 3 h. The method has been validated on 780 samples of human, bovine and guinea pig origin including sputum, cerebrospinal fluid, pulmonary fluids, pus, fine needle aspirate, tissue, blood and milk. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

A crucial step in the diagnostic PCR assay for tuberculosis is the isolation of amplifiable template from clinical material. Tissue and cell constituents present in biological material are oftentimes inhibitory to PCR leading to the reporting of false negative results. A natural consequence has been the lowering of test sensitivity, which has led to the quest for improved DNA isolation protocols. Most of the procedures have been developed for sputum since it constitutes the most common and most infectious biological specimen. Sputum in itself presents a challenge as its physical characteristics may vary from containing copious amounts of mucus to being purulent, haemoptytic or tenacious. The same is true for extrapulmonary specimens like pus, tissue biopsy, etc. The commonly employed DNA isolation methods involve the use of enzymes such as lysozyme and proteinase K, cetyltrimethylammonium bromide (CTAB) treatment and extraction with organic solvents [1], detergent-induced lysis in conjunction with proteinase K and lysozyme [2] or lysis using guanidinium isothiocyanate (GITC)-containing solutions [3,4]. Chelex-100 resin has been used in DNA isolation from clinical samples [5,6]. Though many methods have been published, no single method is available that is universally applicable to all types of clinical specimen. Commercial houses market kits for pathogen DNA isolation optimized for sputum. However, they are out of place in high disease incidence countries like India owing to their exorbitant cost and the increasing prevalence of extrapulmonary TB. Therefore there is a need for a reproducible, simple and inexpensive protocol for mycobacterial DNA isolation from all types of biological samples. Here, we report a method for the isolation of Mycobacterium tuberculosis DNA that fulfills these requirements. We have employed GITC to efficiently wash away PCR inhibitors from clinical samples. Mycobacterial DNA is subsequently obtained upon bacterial lysis by heating with Chelex-100 and detergents.
2. Materials and methods

2.1. Clinical specimens

Seven hundred and eighty samples were included in the development and validation aspects of this study. These comprised 300 sputa and 216 other pulmonary and extrapulmonary samples of human origin, namely, CSF (n = 130), pus (n = 14), fine needle aspirate (n = 14), pleural effusion (n = 10), bronchoalveolar lavage (n = 11), tissue biopsy (n = 15), blood (n = 17) and paraffin-embedded tissue (n = 5). The samples were provided by hospital consultants, namely, Drs. M. Dudeja, Sunderlal Jain Charitable Trust Hospital; S.K. Sharma and S. Singh, AIIMS; New Delhi; V.M. Katoch, JALMA, Agra and consultants at Sri Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. Guinea pig spleens (n = 4) were also successfully processed by this method (Dr. H.K. Prasad, personal communication) including milk (n = 65), rectal pinch (n = 65) and prescapular lymph gland biopsy (n = 65).

2.2. DNA isolation method

2.2.1. Preprocessing

Sputum: Add 0.2 vol. of 2.5% N-acetyl l-cysteine (NALC, in 68 mM phosphate buffer pH 7.5). Vortex and stand for 5–10 min. Centrifuge at 25,000 × g (high speed) at room temperature for 15 min. Discard supernatant. Tissue: Mince and homogenize tissue in 5 M GITC, 50 mM Tris–Cl, pH 7.5, 25 mM EDTA, 0.5% Sarcosyl, 0.2 M β-mercaptoethanol (inhibitor removal solution, IRS) in a mini bead beater (Biospec, USA) using 1-mm glass beads for 30–60 s. Centrifuge at 600 × g for 3 min. Centrifuge the supernatant at high speed and discard supernatant. Paraffin-embedded tissue: Deparaffinize tissue by xylene treatment. Proceed as above. Blood: Centrifuge at high speed. Wash pellet with 1 M NaCl. Milk: Centrifuge at high speed. Discard supernatant. Remove milk fat by wiping tube wall with a cotton swab. Pus: To purulent and thick samples, add an equal volume of phosphate-buffered saline and vortex. Centrifuge at high speed; discard supernatant. CSF, BAL, pleural effusion: Centrifuge at high speed, discard supernatant.

2.2.2. Inhibitor removal

Resuspend pellet from step 1 in an equal volume of IRS. Note: For highly mucoid, tenacious or purulent samples, complete resuspension in IRS may require incubation at 37°C for up to 60 min or an additional wash with IRS. An additional wash with the IRS may also be necessary for tissue samples. Centrifuge at high speed and discard supernatant. Rinse pellet with water. Dry pellet.

2.2.3. DNA extraction

To pellet, add approximately 5 vols. of Chelex-100 resin [5] (Bio-Rad, USA), Tween 20 and Triton X-100 at a final concentration of 10%, 0.3% and 0.03% respectively. Heat the tubes at 90°C for 40 min. Use supernatant for PCR.

2.3. Stability and handling of IRS

The IRS was stable for at least 2 months at room temperature if stored in a dark and airtight container. The IRS was prepared in a fume hood and GITC-containing waste was disposed off in 10 M NaOH ensuring that the final concentration did not fall below 0.3 M [3].

2.4. PCR

DNA amplification was assessed by the devR assay that is specific for organisms of the M. tuberculosis complex [7,8]. Briefly, a 40-µl reaction was set up containing 0.5 µM of primers devRf (5'-GGTGAGGCAGATGGTCGGTCGC-3') and devRr (5'-CGCGGGCTTGGTCGACGGTGTC-3'), 1.5 mM of MgCl₂, 0.2 mM dNTPs, 1 U of Taq DNA polymerase and 4 or 10 µl of specimen DNA (neat and 1/10 dilution). In parallel, inhibitor check reactions were set up containing 10–20 ng of M. tuberculosis DNA and sample DNA. The thermal cycling parameters were 10 min at 94°C, 40/45 cycles each of 1 min at 94°C, 1 min and 30 s at 65°C/70°C, and a final extension of 10 min at 72°C. 23S rDNA PCR assay was performed on paraffin-embedded tissue as described [7]. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

![Image](image-url)
3. Results

3.1. Standardization of DNA isolation procedure

*M. tuberculosis* DNA isolation was attempted from several acid-fast bacillus (AFB) positive sputa as described [5]. A substantial degree of PCR inhibition was noted with more than half the samples (particularly purulent and/large volume samples, Fig. 1A). Very faint amplification was observed when the sputum samples were diluted 50–100-fold with 10 mM Tris–1 mM EDTA, pH 8.0, likely due to dilution of inhibitors (Fig. 1B). In order to improve DNA recovery and to effectively remove inhibitors, two approaches were attempted. First, the efficacy of various detergents including sodium dodecyl sulfate (SDS), Triton X-100, Tween 20 and Nonidet P-40 (NP-40), in lysis of *tubercle bacilli*, cultured in 7H9 medium containing albumin dextrose and Tween 80, was evaluated. The detergents were used in conjunction with 10% Chelex-100 [5]. The inclusion of SDS at the recommended concentration of 0.1% [5] inhibited DNA amplification, suggesting PCR inhibition by SDS. The inclusion of 0.03% Triton X-100 and 0.3% Tween 20 either alone (not shown) or together, consistently resulted in the best bacterial lysis in comparison to a mixture of NP-40, SDS and Tween 20 [5] (Fig. 1C). Second, an inhibitor removal step was introduced. Ten +4 grade AFB positive sputa of various physical characteristics containing ~10^7 bacilli/ml were used during standardization. The samples were divided into two equal aliquots and one set was treated with IRS prior to DNA isolation. Good amplification was obtained from all the IRS-treated samples and no inhibition was noticeable. However, PCR inhibition was noticeable with seven out of 10 samples not treated with IRS. The IRS was efficiently removed caseous material, mucin or tissue debris as indicated by a substantial decrease in post-wash pellet size. A representative gel picture is shown (Fig. 1D). The IRS treatment effectively removed inhibitory material and also did not compromise with DNA yield from sputum of varying volume (5–0.5 ml, Fig. 2A) and of varying bacterial load (AFB +2 grade to smear negative, Fig. 2B). The process was validated on >300 sputa ranging from +4 grade to smear-negative specimens.

3.2. Sensitivity of the IRS method

Sensitivity was evaluated on four +3 grade sputa. Briefly, NALC-treated sputa were divided into aliquots ranging from 1 ml to 3.5 µl, the volumes were made up to 1 ml by the addition of Tris–EDTA. DNA was isolated as described and PCR analysis was performed. DNA amplification was detectable with sputum volumes as low as 7–15 µl which was equivalent to DNA from ~50 to 100 bacilli. A typical amplification profile is shown in Fig. 3A. The applicability of the IRS method to tissues was also examined. Guinea pigs infected subcutaneously with *M. tuberculosis* H37Rv (four animals) were sacrificed 47 days post infection. Spleens were homogenized, plated as neat and serial dilutions on Lowenstein Jensen slants, DNA isolated as described and subjected to *M. tuberculosis* PCR analysis in parallel. The *devR* gene was amplified from all dilutions of spleen homogenate tested. The highest dilution of spleen homogenate contained ~30 genome equivalents of DNA (based on recovered cfu). A representative result is shown in Fig. 3B.

3.3. Comparison of IRS method with proteinase K-SDS-CTAB method

Duplicate aliquots of five +3 grade sputa (125–15 µl volume) were subjected to DNA extraction by the IRS
method and by another method used in our laboratory employing proteinase K-SDS-CTAB [1]. A typical result is shown in Fig. 4. The lower limit of detection for the IRS method was 15 $\mu$l compared to 31 $\mu$l for the proteinase K-SDS-CTAB method. The improved performance of the IRS method over the latter method with particular reference to inhibitor removal was verified on 80 sputa of varying bacterial load. Sixteen out of 40 DNA samples isolated by proteinase K-SDS-CTAB method showed PCR inhibition and required further purification. In contrast, not a single sample out of the 40 DNA samples isolated by the IRS method showed PCR inhibition (not shown).

3.4. Application to extrapulmonary specimens

The IRS method was successfully applied to the isolation of inhibitor-free DNA from all types of extrapulmonary samples tested, including fluids and tissue samples of human and bovine origin (see Section 2). Representative PCR assays with $M. tuberculosis$ DNA isolated from CSF, pleural effusion, bronchoalveolar lavage, pus, FNAC, blood, bovine milk, tissue biopsy and paraffin-embedded tissue are shown in Fig. 5.

4. Discussion

We have developed a rapid and simple method for the extraction of PCR-amplifiable $M. tuberculosis$ DNA from clinical samples. For sample volumes less than 1.3 ml, the DNA isolation process can be performed in a 1.5 ml microcentrifuge tube. Our method exploits the tough cell wall characteristics of mycobacteria that confer resistance to 5 M GITC. In a departure from the conventional use of this chaotropic agent in effecting lysis of cells for nucleic acid isolation [3,4,9,10], our method is based on a wash with a GITC-containing buffer. The introduction of a 'cleaning-before-lysis' step results in effective removal of biological constituents (and potential PCR inhibitors) present in the specimen and creates a better environment for efficient lysis of mycobacteria rendering further purification of DNA unnecessary. Residual inhibitors, if present at all, are adsorbed by Chelex-100 resin during the DNA isolation step.

The methods currently employed for the isolation of mycobacterial DNA from clinical samples suffer from one or many drawbacks that include long processing times, use of organic solvents and enzymes and multiple steps resulting in either DNA loss or in the inefficient removal of PCR inhibitors. Multistep methods increase false positive results due to the risk of cross-contamination and also augment false negative reporting due to losses in DNA recovery. These drawbacks have been overcome in the IRS method. The processing time is reduced to $\sim 3$ h, resulting in the reporting of test findings on the same day as the receipt of the sample. The process involves the use of reagents that are readily available and inexpensive. Most importantly, the process can efficiently remove all kinds of inhibitors present in clinical samples. The method has been validated on 780 pulmonary and extrapulmonary samples. Efficient removal of the PCR inhibitors permitted the addition of more DNA equivalents to the PCR reaction, thereby increasing the sensitivity of the assay procedure. Enhanced sensitivity is invaluable when handling low volume and/or paucibacillary specimens.

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References


