Original Article

COMPARISON OF THREE MYCOBACTERIAL DNA EXTRACTION METHODS FROM EXTRAPULMONARY SAMPLES FOR PCR ASSAY

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Abstract

Sensitivity of the molecular diagnostic tests of extrapulmonary tuberculosis largely depends upon the efficiency of DNA extraction methods. The objective of our study was to compare three methods of extracting DNA of *Mycobacterium tuberculosis* for testing by polymerase chain reaction. All three methods; heating, heating with sonication and addition of lysis buffer with heating and sonication were implicated on 20 extrapulmonary samples. PCR positivity was 2 (10%), 4 (20%) and 7 (35%) in the samples extracted by heating, heat+sonication and heat+sonication+lysis buffer method respectively. Of the extraction methods evaluated, maximum PCR positive results were achieved by combined heat, sonication and lysis buffer method which can be applied in routine clinical practice.

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Introduction

Isolation of nucleic acid (DNA) from mycobacteria is difficult due to its complex cell wall structure.[1] Therefore, most of the simple and commonly used DNA extraction methods result in poor quality and low yield of DNA, which is also affected by type of sample used.[2] However, the sensitivity of molecular diagnosis is largely dependent on the efficiency of cell lysis and extraction of DNA.[3] Several methods for mycobacterial cell wall lysis and DNA extraction have been used like simple boiling in distilled water, disruption by glass bead or sonication, enzymatic lysis, chemical lysis or combination of these techniques.[4] The objective of this study was to compare three methods of extracting *M. tuberculosis* DNA from various types of extrapulmonary specimens.

Materials and Methods

The study included 20 samples, of which 8 samples were positive for mycobacteria in ABF smear and/or culture and 12 were negative. Samples were collected from the patients strongly suspected for having extrapulmonary tuberculosis on the basis of cyto-chemical and histopathological evidences. Samples were centrifuged at 3000 rpm for 15 minutes and sediment was reconstituted with 2 ml distilled water. Reconstituted deposit was used for direct examination by Ziehl-Neelsen staining[5] and cultured by inoculation on Lowenstein- Jensen media.[6]

During DNA extraction in heat method aliquots of the sediments were washed with distilled water and boiled at 95°C for 30 minutes in a water bath. After centrifugation at 10,000 rpm for 5 minutes supernatant was collected in a 1.5 ml tube.[7,8] In combined heat and sonication method, after removal from water bath lysate was sonicated in an ultrasonication bath (Branson 1200 E4, Branson Co, Danbury, CT) for 15 min at 30 W.[9,10] Then supernatant was recovered in the same way. In the third method, at first deposit was suspended in 135 µl of lysis buffer [Prepared by mixing 20 µM Tris/HCl (pH 8.3), 1mg proteinase K/ml, 0.5% Tween 20 and 10ml sterile distilled water] instead of distilled

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water and incubated at 56°C for 3 hours. Then the lysate cooled to room temperature and centrifuged at 12000 rpm for 15 min. Resultant pellet was resuspended with 100 µl distilled water and rest of the method repeated as method-2. Briefly, heating, sonication, centrifugation and finally collection of the supernatant in 1.5 ml tube.[11,12]

Amplification and detection procedures

A 123 bp sequence of mycobacterial genome was amplified with IS6110 primer. The amplification was performed in 25 µl reaction mixtures with the cycling program of initial denaturation at 94°C for 5 min; followed by 31 cycles of denaturation at 94°C for 45 sec, annealing 68°C for 45 sec and an extension at 72°C for 2 min; a final extension step at 72°C for 7 min.[13] After amplification, 5 µl of the reaction mixtures was electrophoresed on ethidium bromide-containing 1.5% agarose gels and visualized by UV transillumination.

Results

Three DNA extraction methods were applied in 8 AFB smear and/or culture positive and 12 negative samples. Maximum positivity was seen in the method using heat, sonication and lysis buffer in combination. Among 8 AFB smear and/or culture positive cases 6 (75%) were positive by this method whereas 4 (50%) were positive by heat-sonication and 2 (25%) by only heating method. Among 12 AFB smear and/or culture negative samples one (8.3%) sample was PCR positive by heat+ sonication+lysis buffer method. None of the AFB negative sample was positive by only heating or heat + sonication method.

Table 1: *Comparison of PCR results with the DNA extracted by three different methods (n=20).*

Method of extraction	PCR positivity in		
	Smear and/or culture +ve samples n=8	Smear and/or culture –ve samples n=12	Total n=20
Heat only	2 (25%)	0 (0%)	2(10%)
Heat+sonication	4 (50%)	0 (0%)	4(20%)
Heat+sonication +lysis buffer	6 (75%)	1 (8.3%)	7(35%)

Discussion

Several DNA extraction methods can be employed for the isolation of mycobacterial nucleic acid from clinical samples. But in case of samples of extrapulmonary TB more precise method is required due to the paucibacillary nature of these samples. Moreover in highly TB prevalent country like Bangladesh the method should be simpler and reasonably cost effective. In this context, kit based extraction offers quality-controlled reagents with optimized compositions for all steps, but they are relatively costly and have variable sensitivity.[14] Heating is simplest and widely used method but its sensitivity in smear negative pulmonary as well as extrapulmonary samples is not satisfactory.[7,8,15] In fact, association of physical disintegration by bead beating or sonication in a specific enzyme and detergent containing buffer is appropriate for mycobacterial cell wall lysis.[14]

In the present study two available modifications of the heat method was carried out by adding lysis buffer (prepared by mixing Tris/HCl 20 µM (pH 8.3), proteinase K 1mg/ml, and Tween20 0.5%) and sonication. Modifications were approached based on DNA extraction methods used in other studies.[9,10, 11] In this study only 2 (10%) samples were positive initially by heat method. Additional sonication step for 15 minutes increased PCR positivity to 4 (20%) and after adding lysis buffer it was further increased to 7 (35%).

Though the main disadvantage of the sonication method was the need of a sonicator for cell lysis and the third method needs extra 3 hours incubation but in consideration of proper diagnosis it can be acceptable. This incubation time can be lowered by further experiment as different authors mentioned different ranges of incubation times.[8,11,12] Lastly, from our experiment it is apparent that combination of lysis buffer and sonication with heating is considerably bring better DNA yield in detecting *M. tuberculosis* by PCR, especially in samples with extrapulmonary samples that have low number of mycobacteria. Obviously the limitation of our study is small sample size and lack of observation of quality and quantity of recovered DNA. Further study with large number of pulmonary and extrapulmonary samples following necessary modification may strengthen our findings.

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