Comparison of DNA Extraction Protocols for *Mycobacterium Tuberculosis* in Diagnosis of Tuberculous Meningitis by Real-time Polymerase Chain Reaction

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ABSTRACT

Background: Several nucleic acid amplification techniques are available for detection of *Mycobacterium tuberculosis* (MTB) in pulmonary and extrapulmonary samples, but insufficient data are available on the diagnostic utility of these techniques in tubercular meningitis where bacilli load is less. The success of final amplification and detection of nucleic acid depends on successful extraction of DNA from the organism. **Aims:** We performed this study to compare four methods of extraction of MTB DNA from cerebrospinal fluid (CSF) samples so as to select one method of DNA extraction for amplification of nucleic acid from clinical samples. **Materials and Methods:** Four methods of extracting MTB DNA from CSF samples for testing by real-time polymerase chain reaction (PCR) were compared: QIAGEN^R protocol for DNA purification using QIAamp spin procedure (manual), AMPLICOR^R respiratory specimen preparation kit, MagNA Pure^R kit extraction, combined manual DNA extraction with automated extraction by MagNA Pure^R. Real-time PCR was performed on COBAS TaqMan 48 Analyzer^R with known positive and negative controls. **Results:** The detection limit for the combined manual and MagNA Pure^R extraction protocol was found to be 100 copies of MTB DNA per reaction as against 1,000 copies of MTB DNA per reaction by the QIAGEN^R, AMPLICOR^R, and the MagNA Pure^R extraction protocol. **Conclusion:** The real-time PCR assay employing the combination of manual extraction steps with MagNA Pure^R extraction protocol for extraction of MTB DNA proved to be better than other extraction methods in analytical sensitivity, but could not detect less than 10^2 bacilli /ml.

Key words: Extraction, Real-time PCR, Tubercular meningitis

INTRODUCTION

Infection of the central nervous system (CNS) is one of the most devastating clinical manifestations of tuberculosis. Early diagnosis and prompt institution of antitubercular treatment are deciding factors for the final outcome of the patient. Diagnosis of tubercular meningitis (TBM) is still a complex issue because of inconsistent clinical presentations and lack of a rapid, sensitive, and specific test.[1,2]

Since the beginning of this decade, polymerase chain reaction (PCR) and other amplification techniques have been introduced for the diagnosis of infections with *Mycobacterium tuberculosis* (MTB).[3-6] Although no amplification system known today provides sufficient sensitivity to replace culture as a reliable screening tool, but can be used as supplementary tests as they are specific and offer rapid turnaround time as compared to cultures.[6,7] Moreover, nucleic acid amplification (NAA) tests can be useful in patients on antitubercular therapy and for monitoring treatment response.[2] Real-time PCR offers a distinct advantage of simultaneous amplification and detection in one run without the need for additional steps for detection of amplicons. The same reaction tube is used for amplification in real time, and there are no sample transfers, reagent additions, or gel separation steps, thereby overcoming the risk of contamination.[8] The ability of these assays to detect MTB in clinical samples is largely dependent on the efficiency of DNA extraction procedure used, as MTB has a complex cell wall structure that is impermeable and difficult to lyse. Effective extraction of mycobacterial DNA from CSF samples require the following steps:[9,10]

- Shock treatment (heating and freezing) to weaken the mycobacterial cell wall along with the use of lysozyme

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to dissolve proteinaceous debri
- Chemical treatment to lyse the mycobacterial cell wall
- DNA purification
- Elution of DNA

Several methods for mycobacterial cell wall lysis and DNA extraction have been evaluated for samples such as sputum and extrapulmonary samples but limited number of studies has been done solely on CSF.[4,5,11,12] Diagnosis of TBM remains a challenge as the number of bacilli in CSF samples are quite low as compared to that in pulmonary samples; moreover, CSF is a precious sample with limited amounts available for diagnostic purpose. The objective of this study was to compare four protocols for extracting MTB DNA from CSF samples. The effectiveness of each extraction protocol was assessed by subjecting each sample thrice to real-time PCR assay.

MATERIALS AND METHODS

Sample preparation

A first-day positive Mycobacterium Growth Indicator Tube (MGIT) of H37 Rv in BACTEC 960 system, which contains approximately 10^6 CFU/ml of MTB, was taken as the standard for preparing dilutions of 10^3, 10^2, $10^{1.5}$, and 10 CFU/ml in normal CSF samples (with no cytological, biochemical, and microbiological abnormalities, and culture negative for mycobacteria). All normal CSF samples were stored at -20°C and were thawed immediately before spiking with known concentration of MTB. Four sets of the above-mentioned dilutions were prepared, and each set was subjected to a different DNA extraction protocol. In order to avoid contamination, samples were processed in a separate biosafety cabinet and all plasticware used for DNA extraction were DNAase free, disposable, and different sets of micro-pipettes were used at each step (ie, for sample processing, DNA extraction, and master mix preparation) with unidirectional workflow for all procedures. All samples in each set of dilutions were centrifuged at 24,000 *g* for 1 hr in a refrigerated microcentrifuge and 200 µl of the deposit were subjected to each of the four different extraction protocols. To check the reproducibility all the experiments (ie, four extraction protocols with the different sets of dilutions) were run in triplicate.

Methods of DNA extraction

DNA extraction protocols evaluated are as follows [Table 1].

Protocol 1

QIAGEN® protocol for DNA purification from blood and body fluids using QIAamp spin procedure (manual): The QIAamp DNA purification procedure comprises four steps and was carried out using QIAamp mini spin columns in a standard microcentrifuge strictly following the manufacturers' instructions.

1. The samples (200 µl of the deposit) were lysed by incubation with proteinase K and a special lysis buffer containing guanidine hydrochloride, and later treated with ethanol and centrifuged.
2. The sample was applied to the QIAamp mini spin column. DNA was adsorbed onto the QIAamp silica membrane during a brief centrifugation step.
3. DNA bound to the QIAamp membrane was washed in two centrifugation steps to remove any residual contaminants without affecting DNA binding.
4. Purified DNA was eluted from the mini spin column in a concentrated form in the elution buffer containing tris chloride and ethylenediaminetetraacetic acid (EDTA).

Protocol 2

AMPLICOR® respiratory specimen preparation kit for manual DNA extraction as described by the manufacturer with certain modifications for CSF Sample: Amplicor protocol basically involves three steps:

1. CSF sample (200 µl of the deposit) was given shock treatment (freezing and thawing) and treated with lysozyme and incubated. The sample was later washed with the wash solution (Tris-HCl and EDTA) provided in the kit and centrifuged.
2. Supernatant was discarded and bacteria in the deposit were lysed by incubation in the lysis reagent (NaOH, EDTA, and sodium azide).
3. Specimen was made amplification ready by the addition of neutralization reagent (Tris-HCl buffer, magnesium chloride, and sodium azide).

Protocol 3

MagNA Pure® kit extraction protocol for MTB to be

Table 1: Summary of various DNA extraction protocols

Protocols	Shock treatment	Treatment with lysozyme	Chemical lysis	DNA purification	DNA precipitation
Protocol 1	+	–	Proteinase K and guanidine HCl	Silica membrane	Tris HCl and EDTA
Protocol 2	–	+	NaOH, EDTA and sodium azide	Tris HCl buffer, MgCl and Sodium azide	
Protocol 3	–	–	Proteinase K and chaotropic salts	Magnetic glass particles	Low salt elution buffer
Protocol 4	+	+	Proteinase K and chaotropic salts	Magnetic glass particles	Low salt elution buffer

processed in the MagNA Pure Compact Instrument (Roche) as described by the manufacturer: The nucleic acid isolation procedure was based on the proven MagNA Pure Magnetic glass particle technology. The principal steps of a MagNA Pure Compact nucleic acid isolation procedure are as follows:

1. The samples were lysed by incubation with Proteinase K and a special lysis buffer containing a chaotropic salt.
2. Magnetic Glass Particles (MGPs) were added and nucleic acids were immobilized on the MGPs surfaces.
3. Unbound substances (eg, proteins, cell debris, PCR inhibitors, etc) were removed by several washing steps.
4. Purified nucleic acids were eluted from the MGPs

Protocol 4

Combination of manual DNA extraction steps with automated extraction protocol of MagNA Pure^{R} for MTB: This extraction protocol involves two additional steps before following the MagNA Pure compact nucleic acid extraction protocol:

1. The deposit was subjected to heat (95°C for 5 min), freezing in ice (-80°C for 5 min), and thawing.
2. Sample was treated with 50 µl of lysozyme and incubated at 37°C for 30 min.

The samples were thereafter treated as described in the MagNA Pure extraction protocol. The DNA samples extracted from all samples were subjected to amplification by real-time PCR in the Cobas Taqman 48 instrument (Roche).

Amplification of mycobacterial DNA by real-time PCR

The DNA extracts from all extraction protocols were later used along with the COBAS TaqManR MTB Master Mix containing Mycobacterium genus-specific primers, oligonucleotide probes, and Mycobacterium Internal Control; these were then processed in COBAS TaqMan 48 AnalyzerR for automated amplification and detection. Known positive and negative controls were put up in each run to test the validity of amplification and detection. The COBAS Taqman MTB Test uses Mycobacterium genus-specific primers to define a sequence within the gene coding for 16S rRNA. The Mycobacterium Internal control is a non-infectious, recombinant linearized plasmid DNA with primer-binding regions identical to those of the MTB target sequence and a randomized internal sequence of length and base composition similar to that of the MTB target sequence. It also has a unique probe-binding region that differentiates the Mycobacterium Internal control amplicon from target amplicon.

RESULTS

The detection limit was found to be 1,000 copies of MTB DNA per reaction by the QIAGEN^{R}, AMPLICOR^{R}, and the MagNA Pure^{R} extraction protocol in the real-time PCR assay. Less than 100 copies were not detected by any of the above extraction methods. The detection limit for the combined manual and MagNA Pure^{R} extraction protocol was found to be 100 copies of MTB DNA per reaction for the real-time PCR assay [Table 2]. The detection limit was same in all separate runs of real-time PCR, showing good reproducibility.

DISCUSSION

The advent of NAA holds great promise in the diagnosis of TBM. This technique has good specificity (98%), but suffers with a low sensitivity of 56%, making it not an ideal test for the diagnosis of TBM.[6,7] The success of final amplification and detection of DNA depends on the extraction of good quality DNA.[9] The present study was done to standardize an extraction protocol, which should be at least comparable to automated culture (Bactec MGIT 960 system detect 100 bacilli/ml) in terms of sensitivity but highly specific for MTB complex, with a low turnaround time. We evaluated four different DNA extraction protocols for MTB in dummy CSF samples containing low concentrations of MTB bacilli ranging from approximately 10 to 10^3 bacilli/ml of CSF.

Successful detection of DNA by amplification methods depends on the purity and quality of the extracted DNA. It was found that QIAGEN^{R} method (protocol 1), AMPLICOR^{R} respiratory specimen preparation kit (protocol 2), and MagNA Pure^{R} kit extraction (protocol 3) were almost comparable, with a sensitivity of 10^3 bacilli/

Table 2: Real-time PCR positivity with different extraction protocols

Extraction Protocols	Samples											
	Run 1				Run 2				Run 3			
	10^3	10^2	$10^{1.5}$	10	10^3	10^2	$10^{1.5}$	10	10^3	10^2	$10^{1.5}$	10
Protocol 1	+	–	–	–	+	–	–	–	+	–	–	–
Protocol 2	+	–	–	–	+	–	–	–	+	–	–	–
Protocol 3	+	–	–	–	+	–	–	–	+	–	–	–
Protocol 4	+	+	–	–	+	+	–	–	+	+	–	–

ml, but a combination of manual DNA extraction steps with automated extraction protocol of MagNA Pure^{R} (protocol 4) could detect MTB in samples with 10^2 bacilli/ml. The detection limit was 100% comparable in all three separate runs. No DNA extraction protocol could detect less than 10^2 bacilli/ml, emphasizing that PCR cannot replace any of the conventional diagnostic tools, especially culture for MTB. But it can be used as an important adjunct for confirmation of diagnosis and to diagnose TBM in patients on antituberculous therapy and monitor response to treatment.

All protocols in our study were kit based and much modification could not be done except for the inclusion of some additional steps, but an attempt was made to compare some of the steps with commonly used extraction protocols. Shock treatments like freezing at -20°C and boiling of bacterial suspensions for 10 min in a suitable buffer has been found to be helpful in the extraction of mycobacterial DNA in a number of studies.[10,13] Shock treatment was included in protocols 1 and 4 but omitted in protocols 2 and 3 prior to lysis. Lysis of bacterial cell walls was achieved by using detergents by workers in the past.[14] Effective lysis was also achieved in a study with the use of Sodium Dodecyl Sulfate (SDS), Triton X-100, and lysozyme.[14] In this study, lysozyme was used in protocols 2 and 4. Chemical lysis was achieved in protocol 1 with the use of Proteinase K and Guanidine HCl. Proteinase K was also used in protocols 3 and 4. The use of Proteinase K was found to be helpful in removing DNA-bound proteins resulting in improvement in quality of template DNA [Table 2].

CONCLUSION

The real-time PCR assay employing the combination of manual extraction steps with MagNA Pure^{R} extraction protocol for extraction of MTB DNA proved to be better than other extraction methods in analytical sensitivity. This study highlights that though physical methods of lysis (shock treatment), use of lysozyme and Proteinase K along with MagNA Pure^{R} can definitely improve the quality of the extracted DNA but it is still difficult to diagnose TBM in patients having less than 10^2 bacilli/ml. This method of DNA extraction with real-time PCR is now being evaluated for the diagnosis of TBM in our setup.

Further research is needed to evaluate new techniques of DNA extraction and PCR to increase the sensitivity of diagnosis and search for alternative methods of diagnosis such as detection of specific immune responses in CSF or detection of specific mycobacterium proteins.

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