

Comparison of *Mycobacterium tuberculosis* DNA Extraction Methods

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Extraction of DNA from sputum samples of patients with tuberculosis is often the most time-consuming step in the entire diagnostic procedure that increases the overall time required to process a clinical specimen. Hence, there is a need to develop a quicker but efficient and cost-effective method. The present study was designed to optimize the time required for DNA extraction without compromising the final yield, purity and therefore the end result for diagnosis. Thirty-five sputum samples were collected from clinically suspected tuberculosis patients and processed using standard decontamination method. DNA from these samples were extracted using three techniques – Qiagen QIAmp DNA mini kit, HiMedia *Mycobacterium tuberculosis* DNA extraction kit and an In-house method (SDS, TE buffer and triton X). All the methods were assessed for yield and purity using Nanodrop followed by Conventional Nested PCR to amplify the IS6110 region. The optimum incubation period in lysis buffer for all three methods was found to be three hours, with no statistically significant change occurring in the yield and purity. The total time required for extraction was maximum for M/s. Qiagen i.e., ~ 6 h and minimum for in-house method ~ 3.45 h. In-house method was also found to be the relatively inexpensive. The in-house method of extraction was found to be the quickest and most cost-effective. However, the time consuming and laborious preparatory procedures may increase the chances of manual error, especially in laboratories with high sample load.

Key words: *Mycobacterium tuberculosis* (MTB), DNA extraction, PCR, yield, purity.

In recent times, more research is being focused on the development of newer diagnostic tools, particularly molecular methods for rapid detection of tuberculosis (TB). The accuracy and reliability of nucleic acid amplification tests (NAT) for TB have been extensively studied and reviewed. However, one of its major limitations is that its use is mostly restricted to research and reference laboratories. With the genome sequence of *Mycobacterium tuberculosis* (MTB) is known, new target molecules are being identified for the

development of new drugs and vaccines.^[1] In addition numerous other diagnostic methods have been developed such as line probe assays, phage-based assay and its variants such as FAST Plaque-TB-MDRi kit, FAST Plaque-TB-Response and molecular beacons for the rapid detection of mutations associated with drug resistance.^[2-8] The ability of these assay to detect MTB in clinical samples is dependent on both the target sequence selected and the efficiency of the DNA extraction procedure.

Several methods for lysis of mycobacterium cell wall and DNA extraction have been evaluated previously including detergents, proteolytic enzymes, mechanical disruption, and temperature changes, alone and in various

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combinations. Extraction of mycobacterial genomic DNA is especially demanding since many mycobacterial species are among the most extreme slow growers, accounting for small amounts of starting material and a robust, waxy cell wall of mycobacteria renders difficulty in lysis of the cell wall. The tightly packed mycolic acid provides the bacillus with an efficient protection and an exceptional impermeability. In addition to the capsule, thick layer of carbohydrate and protein outside the lipid layer impedes the diffusion of large molecules, such as enzymes, and protects the lipid layer itself. Published protocols for mycobacterial DNA preparations and commercially available extraction kits are mainly designed for the isolation of small amounts of genomic material. These include liquid-liquid extraction; adsorption based silica column and enzymatic extraction.

The objective of this study was to compare three different DNA extraction procedures from suspected MTB respiratory specimens (sputum samples): Qiagen QIAmp DNA mini kit, HiMedia *Mycobacterium tuberculosis* DNA extraction kit and the in-house method (SDS, TE buffer and triton X). The efficacy of the most favourable extraction method was evaluated by assessing purity and yield followed by amplification of the extracted DNA by Nested PCR.

MATERIALS AND METHODS

Study Groups:

Thirty-five patients of clinically diagnosed pulmonary tuberculosis were included in the study. Individuals greater than 18 years of age, belonging to either sexes and attending outdoor patient department or admitted to Government Sewree TB Hospital, Mumbai were selected for the study. Individuals less than 18 years of age and pregnant women were excluded from the study. A written informed consent was acquired from all individuals that were included in the study. Ethical approval for the study was taken previously from Institute Ethics Committee.

Sample Collection and Processing:

A total of thirty-five sputum samples were collected from subjects during a period of one year from 2011 to 2012. All samples were transported to the laboratory and stored at 4°C within 4 h of collection and processed immediately for

decontamination of sputum sample by NALC-NaOH Method. MTB screening was done by using Ziehl Neelsen (ZN) staining (Microscopy) and culturing on to L.J medium.^[9] Decontaminated samples were stored at “20°C until further analysis.

Extraction of DNA from MTB:

DNA from the processed sputum sample was extracted by three different methods—

Qiagen QIAmp DNA mini kit (Qiagen, Inc., Valencia, CA), HiMedia *Mycobacterium tuberculosis* DNA extraction kit (HiMedia Laboratories Ltd., Mumbai, India) as per manufacturer's instructions ^[10, 11] and in-house method. Briefly, the in-house protocol involved incubation of sputum samples with TE buffer, 1% triton X at 96°C for 30 min and the centrifugation at 7000 RPM for 8 min. The supernatant was collected and incubated at 65°C for 30 min with 10% SDS. The sample was centrifuged at 5000 RPM for 10 min. Absolute ethanol was added to the supernatant and centrifuged for 12000 RPM for 10 min. The pellet containing DNA was resuspended in TE buffer^[12] (Table 1). The optimum time of lysis in the above methods was assessed using three standard time points (as mentioned in the kit/ reference), three hours and overnight incubation. Standardization and comparison of all three methods was done using MTB standard strain, H37rv. All the thirty five samples were subjected to extraction using all three protocols with a fixed incubation time in lysis buffer of 3 h hereafter.

Quantification of DNA Using Nanodrop Spectrophotometer Technique:

DNA yield from all the three extraction methods was determined on a NanoDrop Spectrophotometer ND-1000 by measuring the absorption at 260 nm, and purity was calculated based on 260/280 ratio.

Statistical Data Analysis:

Yield and purity based on Nanodrop readings for all the techniques viz M/s. Qiagen, M/s. Himedia and in-house method of DNA extraction were statistically analyzed by one-way analysis of variance (ANOVA) test followed by Tukey test. Data using this graph was plotted as mean \pm Standard Error Mean (SEM). All the statistical analysis was carried out using a Graph Pad Prism version 5.0 ^[13-15] (Graphpad Prism Inc, India).

Detection of MTB by Conventional Nested PCR Using Suitable Primers:

Nested Polymerase Chain Reaction was used to amplify the IS6110 sequence of MTB using primer sequences (Primer Design) as mentioned in Table 2. The outer and inner rounds of PCR were carried out using a PCR Master Mix (Fermentas Inc., Maryland, USA) as per manufacturer's instructions with primer concentration being 0.4 μ M and 0.7 μ M respectively. The cycling conditions used were: 94°C/20s, 60°C/20s, 72°C/30s for 35 cycles to give a product size of 556 bp and 285 bp for outer and inner rounds respectively [16]. Amplified products were detected on 1.5% agarose gel infused with SybrSafe® dye for visualization.

RESULTS

The present study was designed to optimize the time required for extraction of DNA without compromising on the final yield, purity and therefore end result for diagnosis. The study included a total of thirty-five tuberculosis suspected sputum samples, all of which were subjected to preliminary screening by simple microscopic detection of AFB using ZN staining technique and culturing on LJ medium. Of these, 24 (68.57%) samples were found to be smear positive while 25 (71.43%) samples were found to be culture positive.

DNA was extracted from standard strain

Table 1. Comparison of the three different DNA extraction procedures

	Primer Name	Position of IS6110	Sequences (5'!3')
Outer	TB1-F	555 - 572	CTCAAGGAGCACATCAGC
	TB2-R	1111 - 1084	TCATAGGAGCTTCCGACC
Inner	TB3-F	590 - 609	CTACGGTGTTTACGGTGCCC
	TB4-R	874 - 855	CTACGGTGTTTACGGTGCCC

Table 2. Amplification primer sequences (primer design) for Nested PCR16

Step	Step Details	Qiagen	Himedia	Manual (In-house)
Step 1	Lysis I	Atl + Sputum *37°C,3hrs	Al + Sputum *90°C, 30 Min	Te Buffer + 1% Triton X + Sputum*96°C,30 Min
Step 2	Protein Degradation	Proteinase K + Al*56°C, 2hrs	Proteinase K*90°C, 30 Min	Na
Step 3	Centrifugation	Na	Above Sol **10,000 Rpm, 1min	Above Sol**7,000 Rpm, 8min
Step 4	Lysis Ii	Na	Lysis Sol C1*70°C, 10min	Sds 10 %*65°C,30 Min
Step 5	Presipitation	Ethanol **10,000 Rpm, 1 Min	Ethanol *10,000 Rpm, 1 Min	Ethanol **12,000 Rpm, 10 Min
Step 6	Washing	Aw1 **10,000 Rpm, 1 Min	Prewash Sol **10,000 Rpm, 1 Min	Na
Step 7	Washing	Aw2 **14,000 Rpm, 10 Min	Wash Sol **13,000 Rpm, 3 Min	Na
Step 8	Elusion	Elusion Buffer Ae**10,000 Rpm, 1 Min	Elusion Buffer Et **10,000 Rpm, 1 Min	Resuspend Pellet Into Te Buffer
Total Time		6 Hrs	4 Hrs	3 Hrs 30 Min

*- Vortex + Incubate; **- Vortex + Centrifuge; ***- Centrifuge;

H37rv using kit based methods—M/s. Qiagen and M/s. Hi-Media and an in-house procedure and the yield and purity of the three different protocols based on incubation time in lysis buffer was compared (Figure 1). Highest yield was obtained on overnight incubation and was considered to be statistically significant ($P < 0.0001$) on comparison with 3 h incubation period. Highest purity was observed in the in-house method with standard time of incubation in lysis buffer and statistically significant in comparison to the others ($P < 0.0001$). Based on the yield and purity, and considering the fact that an overnight incubation shall unnecessarily delay the turn-around time of the test, 3 h incubation time for lysis was considered for further analysis. DNA from all the thirty-five

samples was therefore extracted using all three methods but with a fixed incubation of 3 h in lysis buffer. One-way ANOVA analysis based on yield and purity did not show any statistically significant changes between the three methods of DNA extraction in clinical samples (Figure 2). Therefore, 3 h was considered as the optimum incubation period for DNA extraction from clinical specimens of MTB that did not affect or compromise the end result in any way. Based on cost per reaction, amongst all the three methods, Qiagen was found to be most cost-effective.

Therefore, based on the optimized time, yield, purity and cost-efficacy, the samples were further processed for PCR using DNA extracted by the Qiagen Kit method. Conventional PCR was

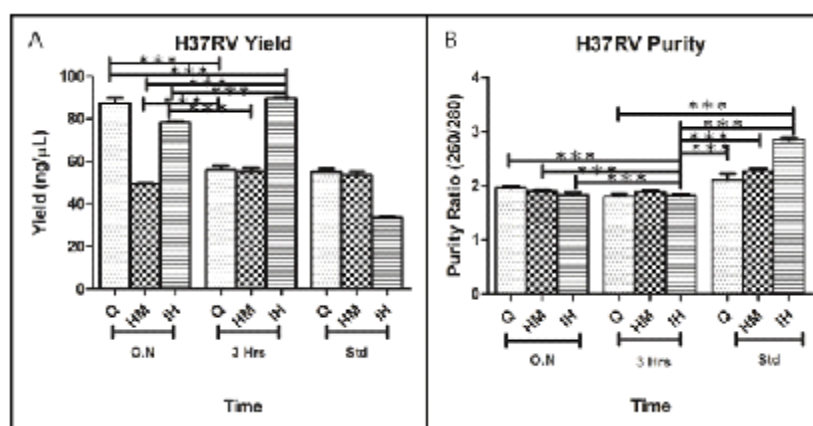


Fig. 1. Comparison of three DNA extraction methods for H37rv in terms of [A] yield and [B] purity. Based on the incubation time in lysis buffer, three time points were considered: O.N- Overnight, 3HR- three hours, Std-according to kit protocol or reference time. Q- M/s. Qiagen, HM- M/s. Hi-Media, IH- in-house. Values are expressed as Mean \pm SEM. **- $P < 0.01$, ***- $P < 0.0001$

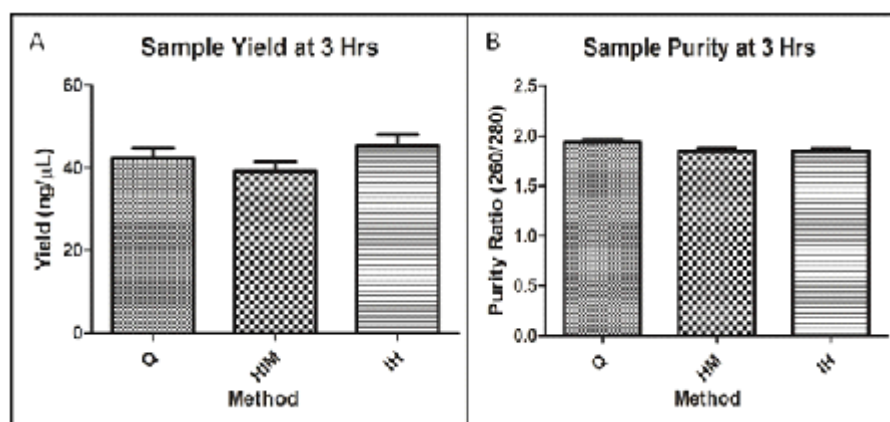


Fig. 2. Comparison of three DNA extraction methods for samples in terms of [A] yield and [B] purity. Q- M/s. Qiagen, HM- M/s. Hi-Media, IH- in-house. Values are expressed as Mean \pm SEM.

carried out for all the thirty-five samples using the primers targeting the IS6110 sequence (Table 2). 31 (88.57%) samples were found to be PCR positive and 4 (11.43%) samples were found to be PCR negative (Figure 3).

DISCUSSION

Globally TB control efforts are based on diagnosis of cases followed by adequate treatment. It is important that correct diagnosis be established early in order to prevent continued transmission and wrong treatment. There is an urgent need for development of more sensitive and rapid techniques. The ever increasing incidence and prevalence rates of tuberculosis among the population in India poses a greater desire to institute efficient diagnostic techniques. PCR is one of those alternative tools that have the potential to improve clinical diagnosis of mycobacterial infections.^[17,18]

Routine diagnosis of MTB includes a rapid and economical method of ZN microscopy. However, recent studies have shown that a significant number of new cases have been reported smear negative by ZN and such individuals can be a potential source of risk to populations in the community, as treatment regimes cannot be initiated timely. In addition, laboratories also employ the culture method that requires long turn around time but offer high sensitivity and specificity. Molecular diagnostic methods like PCR too offer good sensitivity and specificity in a relatively short time, but these methods are

infrastructure intensive and are comparatively more expensive. The ability of this assay to detect MTB is dependent on the efficacy of the DNA extraction procedure.^[19, 20]

The present study was designed to determine the best extraction method for isolation of DNA from sputum samples collected from suspected TB patients and standardization of simple PCR method for detection of MTB. A total of thirty-five tuberculosis suspected sputum samples were included in the study. During the initial screening for MTB it was observed that 24 samples were found to be smear positive while 25 samples were found to be culture positive. This indicates that amongst the routine screening methods, the rapid ZN staining is less sensitive when compared to the conventional culture method. Further, all the samples were subjected to nucleic acid extraction for detection by PCR.

For rapid, sensitive and specific detection by PCR, both the yield and purity of the nucleic acid extracted is of critical importance. Nucleic acid isolation from mycobacteria is difficult due to the presence of thick peptidoglycan layer that makes it resistant to a number of lysis buffers^[21-23]. The time required for cell wall lysis in such cases is important both from yield and purity point of view. The yield of the sample determines the sensitivity of assay while its purity largely affects the specificity. While several methods such as boiling mycobacterial suspension^[24, 25], use of enzymes and silicon beads^[26] or zirconium beads in phenol-chloroform extraction^[27] or simply a combination of two and/or more of the above methods^[28] have

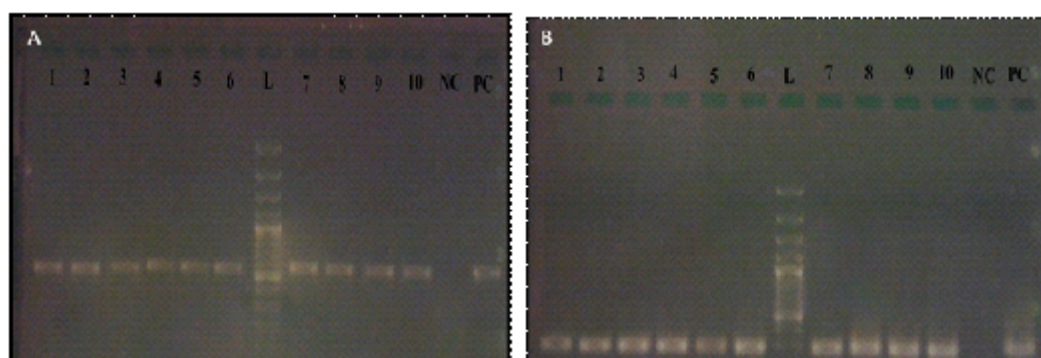


Fig. 3. Detection of tuberculosis IS6110 sequence in DNA extracted from sputum samples by nested PCR. [A]: 556 bp Outer product, [B]: 285 bp Inner product. Lanes- L: Ladder, NC: Negative Control, PC: Positive Control, Lanes 1-10 contains Sputum Samples (HI/TB/1- HI/TB/10).

been used previously in mycobacterial DNA isolation, the present study evaluated three different extraction protocols on the basis of yield and purity.

Two kit based techniques using commercially available kits M/s. Qiagen and M/s. HiMedia were employed and the third was an In-house method designed on the basis of similar chemical methods available in various scientific literatures.^[29,30] For each method, the incubation time for cell lysis: overnight, three hours and standard (according to kit insert procedure/reference) was carefully optimized and DNA was extracted from the standard strain H37Rv for comparative analysis. Yield wise, statistically significant results ($P < 0.0001$) were obtained with overnight incubation suggesting longer the incubation, better the yield. Highest purity however, was observed in the in-house method with standard incubation time in lysis buffer and was statistically significant ($P < 0.0001$) in comparison to the others ($P < 0.0001$). However on comparing yield and purity results of overnight incubation results with that of 3 h, we found that overnight incubation unnecessarily and significantly increases the total turn-around time of the test. Therefore, DNA from the thirty-five clinical samples was extracted using all the three methods but with a fixed incubation time of 3 h in lysis buffer.

At 3 h, however, all the three methods of extraction were found to be efficient as statistically no significant changes were observed in either the purity or yield (Figure 2). A similar comparative study for extracting *M. tuberculosis* DNA from respiratory specimens (spiked sputum) made by Aldous et al. (2005)^[31] showed that column purification is not necessary for the extraction of DNA from sputum samples. In addition, it was observed that even with a fixed time allotted for the lysis step, the total time required for extraction was 6 hours, 4 hours, and 3 hours 45 mins for M/s. Qiagen, M/s. HiMedia and In-house Method respectively.

Apart from yield and purity another factor that plays a pivotal role is the affordability of the diagnostic test, especially in resource deficient setups. The estimated cost per sample was found to be approximately Rs. 200/-, Rs. 400/- and Rs. 100/- for M/s. Qiagen, M/s. HiMedia and In-house

method respectively. Although the time and cost involved was minimum for in-house method, it is both time and labour-intensive procedure considering the preparation of all the chemicals and reagents, making it less suitable in a clinical setup with high sample load. Also, chemicals such as SDS and Triton-X may interfere during PCR. Therefore, among the kit based methods, although the time required for M/s. Himedia is less compared to that of M/s. Qiagen, the latter remains to be a method of choice based on the cost involved.

Hence, DNA extracted via Qiagen kit with 3h of lysis time, all the thirty-five samples were processed for conventional PCR targeting the IS6110 sequence (Table 2) 31 (88.57%) samples were found to be positive by PCR (Figure 3). This indicates that molecular methods are far more sensitive than the conventional methods.

CONCLUSION

The following conclusions can be drawn from the comparative analysis of all the three methods used for nucleic acid extraction i.e., Qiagen, Himedia and In-house method:

- 1 The optimum incubation period in lysis buffer is approximately three hours.
- 2 With 3 hours optimum incubation period for lysis, all the three methods were equally efficient.
- 3 The in-house extraction was found to be most economical ~Rs. 100 per sample and quickest among the three, but may not be preferred due to the labour intensive pre-preparation required and unsuitability for PCR
- 4 With 3 hours lysis time, the quality of nucleic acid remains uncompromised and can be easily detected by PCR

Methods for the detection of mycobacteria are continuously being developed to propose a rapid, accurate and low-cost test. Most of the existing methods can only be used for a limited spectrum of applications, or they are technologically demanding and time consuming. Therefore, majority of workplaces have developed their own isolation procedures. This was only a pilot study and hence, it was not possible to encompass all of them. Nevertheless, the present study shows that while commercially available isolation kits are much more convenient to use, the recommended lysis time may be modified, standardized and optimized so as to reduce the

overall turn around time of the assay without affecting the end result. Thus, optimization of protocols is imperative even when using commercial kits however establishing universal standards for creating global comparisons are equally critical.

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