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COMPARISON OF THREE EXTRACTION METHODS FOR ISOLATION OF *M.TUBERCULOSIS* DNA FOR GENOTYPIC STUDIES AND EFFICACY OF HEAT KILLING AFTER BOILING.

Microbiology

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ABSTRACT

Molecular methods involving nucleic acids have become an indispensable part of any scientific study in the modern era. Towards this, the isolation of DNA/RNA from the organisms is a crucial step where the purity and quantity need to be high to arrive at a meaningful result. In this study, three DNA extraction procedures have been tried with the primary aim to find its suitability in genotypic analysis of *Mycobacterium tuberculosis*. A total of 104 isolates of *M.tuberculosis* from solid culture were subjected to Boiling method, CTAB method, and a commercial kit based system and the extracted DNA was analyzed for the amount and the purity. The highest DNA concentration resulted from the commercial extraction method (value 89.09 µg/ml ± 14.69), while the lowest one resulted from the CTAB method (value 35.62 µg/ml ± 8.4); while the yield from boiling method was higher compared to the CTAB method (51.67 µg/ml ± 9.21). Spectrophotometric analysis for A260/280 ratio showed that purity of DNA was high in CTAB method compared to remaining two extraction methods. The extracted DNA by the three methods all showed distinct bands when amplified with MIRU20 primer. Culture of material after heat extraction was cultured on solid medium to check the viability, and out of 104 samples, a total of 4.3% remained viable. Thus the boiling method is a fast and reliable means of extracting DNA for genetic analysis of *M.tuberculosis*, but a small number of isolates which escape the killing remains a matter of concern.

KEYWORDS

M.tuberculosis, DNA extraction, Boiling method, Genotypic study.

INTRODUCTION

In the present era, we are witnessing a rapid transition from conventional culture techniques to molecular methods in microbiology, and it is particularly evident for *Mycobacterium tuberculosis*, where speed of detection is crucial. To achieve this, the quality and quantity of DNA which can be extracted from an organism is of critical importance.

Isolation of genomic DNA from *Mycobacterium* species has always been a time consuming cumbersome procedure primarily due to the thick and waxy cell wall of mycobacteria species which hampers lysis of the bacterial cell (1). A variety of methods can be used for DNA isolation from different biological materials, ranging from boiling the sample in distilled water, autoclaving, disruption by glass beads or sonication, and in recent years, by the use of different enzymes and surfactants seen in most commercial kits. All these methods of mycobacterial cell wall lysis and DNA extraction have been evaluated alone, or in various combinations to arrive at the most suitable procedure which can be universally adopted. (2, 3, 4)

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* (5). Successful detection of DNA by amplification methods depends on the purity and quality of the extracted DNA.

By looking at the multitude of methods which can be used, it can be deduced that no single method is perfect if the yield, purity, and ease of performance are considered together. Many of the commonly employed methods of isolating DNA yield either low quantity (due to incomplete lysis of bacterial cell wall) or poor quality of mycobacterial DNA, resulting in low sensitivity of the test; while those which yield high quality product are frequently time consuming and complex.

Another important factor which need to be considered in any extraction method is to determine at which point *M. tuberculosis* preparations can be considered inactivated and thus be safely removed from containment. Heating of the culture is widely used, and at least one report has deemed heating at 80°C as sufficient for inactivation (6). However, other reports have raised concerns as to the efficacy of heating and even of further treatment with a combination of lysozyme and proteinase K (7, 8).

Against this backdrop, in this study, three different DNA extraction methods used for mycobacteria were done, namely: (i) Boiling extraction method, (ii) Chemical lysis with Cetyltrimethylammonium bromide (CTAB), and (iii) Commercial Lyte star TB extraction kit method. The extracted DNA was quantitatively and qualitatively evaluated so that it can be used in genotyping studies; together with the efficacy of boiling in inactivating the organism.

MATERIAL AND METHODS

Inoculum preparation:

A total of 104 mycobacterial isolates were included in this study. Two loop full of organism was removed from a solid culture slope (Lowenstein Jensen medium) and added to 400 µl of TE (10 mMTris-HCl [pH 8.0], 1 mM EDTA) buffer in 3 aliquots in Eppendorf tubes. The following procedures were done with the individual aliquots:

(i)TE boiling extraction: The method proposed by Kocagoz et al (9) with minor modifications was adopted. The content of aliquoted tube-1 was briefly mixed on a vortex mixer. This tube was submerged in a water bath preheated and maintained at 90ºC for 20min and then centrifuged at 16,000 rpm for 5 min. A 100-µl aliquot of the supernatant was transferred to a sterile tube and stored at-20°C until PCR testing.

(ii) CTAB Extraction method: For this we used the method described earlier (10). Aliquoted TE buffer tube-2 was briefly mixed on a vortex mixer. The tube was kept in a boiling water bath for 8 to 10 minutes and chilled in melting ice for 8 minutes (heat shock). 40 µl of lysozyme (20mg/ml) was added to the tube and incubated at 37ºC for 2 hours in shaking water bath. After 2 hours incubation 5 µl of proteinase k (10mg/ml) and 56 µl of 10% SDS (sodium dodecyl sulfate) were added to the tubes and kept in water bath at 65ºC for 30 minutes. After 30 minutes 80µl of 5M NaCl and 64µl of CTAB (10% CTAB- 4% NaCl) were added and incubated in shaking water bath at 65ºC for 30 minutes. To this final total volume of 645 µl, equal volume of

chloroform:isoamyl alcohol (24:1 ratio) was added and kept at room temperature for 5 minutes. The tubes were centrifuged at 11000 rpm for 5minutes and upper layer of the aqueous phase was transferred to another tube without disturbing the lower protein debris. 600µl of isopropanol was added to the new tubes containing supernatant followed by precipitation at -80ºC for 30 minutes. Again the tubes were centrifuged at 11000 rpm for 15 minutes. Supernatant was discarded and 150µl of chilled ethanol was added slowly for washing the DNA pellet. Tubes were centrifuged at 11000 rpm for 5 minutes, supernatant was discarded and tubes were kept in concentrator for drying. DNA pellet was dissolved in 30µl of low TE aliquoted tubes and stored at -20°C until PCR testing.

(iii) **LYTE STAR TB/NTM PCR EXTRACTION 2.0 KIT (ADT Biotech, Malaysia):** The instructions of the manufacturer were used for this method. Briefly, pretreatment solution 1 (1x concentration) and pretreatment solution 2 (1x concentration) were prepared form 10 x concentration. 1 ml of pretreatment solution 1 (1x concentration) was added to the aliquoted TE buffer tube-3 and vortexed vigorously. The mixture was incubated at room temperature (18 – 28ºC) for 5 minutes and vortexed vigorously for 10s at one minute intervals. The content was centrifuged at 13000 rpm for 3 min and supernatant was discarded. 1ml of pretreatment solution 1 was then added and mixed well to re-suspend the pellet. The suspension was transferred to a 1.5ml micro centrifuge tube, incubated at room temperature for 5 minutes, and centrifuged at 13000 rpm for 3 min. 1ml of pretreatment solution 2 was added to the pellet and vortexed vigorously for 10 seconds followed by centrifugation 13000 rpm for3 min. The supernatant was discarded and the step was repeated once more. 50 µl of extraction buffer was added to the pellet and mixed well by pipetting. The mixture was heated at 100ºC for 20 minutes, followed by centrifugation at 13000 rpm for 1 min and the vortexing for 10 seconds. After a final centrifugation step at 13000 rpm for 3 min, 10 µl of supernatant was aspirated and stored at -20°C until PCR testing.

Spectrophotometry:
DNA concentration and purity were estimated by noting the absorbance at 260 nm (A260) and by A260/A280 ratio, respectively, in Eppendorf spectrophotometry instrument.

Culture
To check that the bacteria had been inactivated, 100 µl of the heat killed suspension (Boiling extraction method) was used to inoculate each of two slants of Lowenstein-Jensen medium and incubated at 37°C for 10 weeks.

PCR
All stored extracted DNA were further processed for PCR using MIRU 20 primer (forward:5'TCGGAGAGATGCCCTTCGAGTTAG3', Reverse:3'GGAGACCGCGACCAGGTACTTGTA5'). PCR mixture: Milli Q- 25.8 µl, 10x PCR buffer (15mM MgCl2)-5.0µl, 5x Q solution-10µl, dNTPs (5mM each)-2 µl, forward primer-1 µl, reverse primer -1 µl, Taq polymerase 5 U/µl-0.2 µl, total volume -45 µl. 5 µl of extracted DNA. PCR condition, 95ºC for 15 minutes, 95ºC for 1 min, 59ºC for 1 min, 72ºC for 1.30 seconds, 72ºC for 10 minutes, final 4ºC infinity up to 30 cycles.

Gel electrophoresis
2µl of the PCR amplified product was loaded into the agarose gel and was run with the settings compatible to the used system for 15-Minutes at 90 volts. Base-ladder with DNA fragments of 50 bp or 100 bp range was also run in parallel.

Statistical analysis: All data were arranged in Excel spread sheets. Mean and standard deviation were calculated by using Excel software. ANOVA and Paired t-test was calculated by using SPSS 2.0 statistical software.

RESULTS
A total of 104 *M. tuberculosis* isolates were included for extraction. Overall, the three DNA extraction methods showed significant differences in DNA yield; the highest DNA concentration resulted from the Lyte star TB extraction method (values 89.09 µg/ml ± 14.69), while the lowest one resulted from the CTAB method (value 35.62 µg/ml ± 8.4). There is a significant difference (p=<0.00001) found among three extraction methods. (Table 1). The yield by the boiling method (51.67 µg/ml ± 9.21) was higher compared to the CTAB method but it was not statistically significant (p =0.3517). Paired t test was calculated between individual two extraction methods. There was significant differences among boiling method to Lyte star TB kit (p =<0.00001) and CTAB method to Lyte Star TB kit (p=<0.00001).

However, spectrophotometric analysis for A260/280 ratio shows that purity of DNA was high in CTAB method compared to remaining two extraction methods. These difference shows statistically significant (p=<0.00001)(Table 2).

Table 1: Comparison of the DNA yield (in µg/ml) between the three extraction methods.

Extraction method	MEAN	STDV	STATISTICS
BOILING	51.67	±9.21	P = 0.3517
CTAB	35.62	±8.4	
BOILING	51.67	±9.21	P=<0.00001
LYTE STAR TB	89.09	±14.69	
CTAB	35.62	±8.4	P=<0.00001
LYTESTAR TB	89.09	±14.69	

Table 2: Comparison of purity of DNA for three extraction methods.

PURITY OF DNA A260/280 RATIO			
	MEAN	SD	P VALUE
BOILING	2.28	±0.28	P=<0.00001
CTAB	1.77	±0.12	
LYTE STAR TB	1.499	±0.28	

The efficacy of heat killing of mycobacterium by boiling extraction method was tested by culture of extracted material from two difference sites of the eppendorf tubes. One was from extraction tube cap site and second from lower portion of extracted tube and was cultured on Lowenstein Jensen medium and incubated for 10 weeks at 37ºC temperature. 4.8% of isolates survived in cap after 90ºC heat treatment for 20 minutes and 3.8% of isolates survived in lower portion of extracted tube respectively. There was no statistically significant difference (p= 0.6090) found between the isolation rates from the two sites (cap site and lower portion) (Table 4).

Table 3: Culture results of *M.tuberculosis* after extraction by boiling method.

LJ MEDIUM	CAP SITE	LOWER PORTION
GROWTH	05 (4.8%)	04 (3.8%)
CONTAMINATED	4 (3.9%)	4 (3.9%)
NO GROWTH	95 (91.3)	96 (92.3%)

PCR was performed for all extracted DNA by using MIRU 20 Primer. All 104 extracted DNA could be amplified by PCR and showed distinct bands in Gel Electrophoresis (Figure 1).

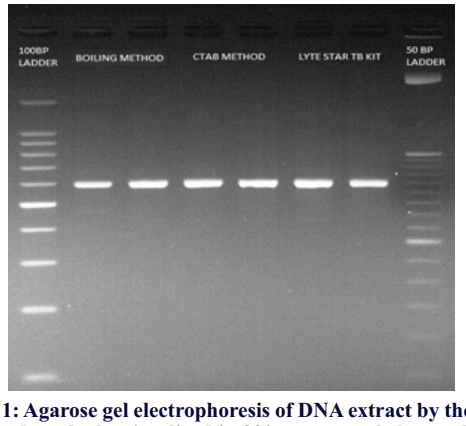

Figure 1: Agarose gel electrophoresis of DNA extract by the three compared methods, visualized in 2% agarose gel electrophoresis after PCR.

DISCUSSION:
Molecular work with organisms relies heavily on the extraction of nucleic acids by multiple means. DNA isolation works often use detergents to solubilize the cell wall; a proteolytic enzyme such as proteinase K to digest proteins; and EDTA to chelate the divalent cations needed for nuclease activity (11). This lysate can be used directly or used after extraction with phenol-chloroform-isoamyl alcohol and precipitation of nucleic acid by ethanol. These additional steps remove proteins and traces of organic solvents and concentrate

the specimen (12). However the classic alkaline lysis and the traditional extraction method mentioned earlier and although less expensive then commercial methods are not the method of choice in diagnostic laboratories as they are associated with serious practical limitations, including use of toxic chemicals and the time required for processing a large number of samples (13). However, these and other methods like simple boiling, particularly if the organisms are from pure culture, still find a place in the research and molecular genetics laboratories to handle small sample volumes (14).

In our study we have evaluated the simple boiling method, CTAB method, and one commercial kit for *M.tuberculosis* DNA extraction from organisms grown on Lowenstein-Jensen medium. The primary aim was to find the suitability of a cheaper method for DNA isolation for genotyping of the isolates. The yield of DNA was highest with the commercial kit (89.09 ±14.69 µg/ml) and was significantly higher compared to the boiling and the CTAB methods ($p<0.00001$). The boiling method yielded 51.67±9.21 µg/ml of DNA which was comparable to the CTAB method. In parallel, comparison of the purity of the DNA extracted by the three methods with the help of spectrophotometric analysis for A260/280 ratio showed that the commercial method produced the most pure DNA. A few studies have compared the boiling method with chemical and other methods and have come out with comparable results. In one study, the boiling method was compared to a conventional, labor-intensive chemical method using lysozyme and silica particles for *M.tuberculosis* DNA extraction from clinical samples (15). The sensitivity was 100 and 92% for the boiling and chemical methods, respectively, and the authors opined that boiling method of DNA extraction is more sensitive and no less specific than a conventional chemical method. In a more recent study, the authors analyzed human oral microbes to compare the performance of three DNA extraction methods: PowerSoil (a method widely used in this field), QIAsymphony (a robotics method), and a simple boiling method using dental plaque material (16). The results indicated that the efficiency of PowerSoil and QIAsymphony was comparable to the boiling method and as per the authors, the boiling method may be a promising alternative because of its simplicity, cost effectiveness, and short handling time. They also mentioned that based on these findings; there is no "gold standard" for DNA extraction. Miyata et al (17) compared the quality of DNA extracted from *M.tuberculosis* by thermolysis method and the CTAB method for molecular identification and genotyping by spoligotype analysis. Their 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 and the quality of the DNA obtained by thermolysis was sufficient to allow molecular identification by PCR and genotyping by spoligotyping. In our study, we amplified the extracted DNA by MIRU 20 primer for all 104 isolates, and consistently got distinct bands with the DNA obtained by boiling method, and its sharpness was comparable to the DNA extracted by CTAB and the commercial system (Fig 1). It shows that the integrity of *M. tuberculosis* genomic DNA is conserved by the boiling method.

Viability of *M.tuberculosis* has always been an issue with the heat method, and there are conflicting reports regarding the same. In our study, a small proportion of the samples showed growth after the heat extraction procedure (Table 3). Experiments performed to investigate a laboratory case of tuberculosis contamination showed that not all tubercle bacilli are inactivated at 80°C, even after lysozyme and proteinase K treatment (9). On the other hand, one study showed that heating of cultures at 100°C for at least 5 min is sufficient to inactivate *M. tuberculosis* and that the heating time is of more importance than the heating temperature in preserving the integrity of the *M. tuberculosis* DNA (8). Safety concerns were also examined in another study where 74 isolates were heated in a water bath at 80°C for 20 minutes and none of the heat killed *M tuberculosis* suspensions produced visible colonies or gave a positive growth signal from liquid culture. This method did not affect the integrity of the DNA for subsequent molecular investigations (6).

In conclusion it can be said that the boiling method for DNA extraction is a cheap and rapid procedure which produces sufficient quantum of DNA and also preserves the integrity of the DNA. The DNA is suitable for genetic analysis as shown in an earlier study (17) as well as our study for spoligotyping and MIRU-VNTR analysis respectively.

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