ORIGINAL ARTICLE

A PCR Referenced Comparative Study for Evaluation of Different *Mycobacterium tuberculosis* DNA Extraction Methods Directly from Sputum and from LJ Culture Isolates in Egypt

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ABSTRACT

Background: *Tuberculosis is a critical infectious disease primarily affecting the lungs and is more common in developing countries. In the 21st century, it forms a significant problem for world public health especially with the emergence and rising of drug resistant TB. Microbiological methods are the clue for the laboratory diagnosis. The ordinary methods for TB identification showed either weak sensitivity as in microscopy or lateness for many weeks as in culture. The evolution in molecular biology gives a chance for fast diagnosis of Mycobacterium tuberculosis helping start proper treatment early and holding its spread. The initial critical step in PCR is DNA extraction.* ***Objective:*** *The aim to evaluate different extraction methods of Mycobacterium tuberculosis retrieved directly from sputum samples and from LJ isolates from same patients and comparing DNA yield using conventional PCR.* ***Methodology****: DNA from 32 sputum samples from TB patients extracted by solid, digestion and phenol extraction methods, DNA from 40 LJ isolates extracted by solid, boiling and Cetyl trimethylammonium bromide methods. Extracted DNA was evaluated by conventional PCR.* ***Results****: Among 32 sputum samples, the extracted DNA by phenol method was 21/32 (65.62%) with highest DNA yield, digestion method 14/32 (43.75%) and solid phase method 1/32 (2.5%) with least DNA yield. From 40 MTB LJ culture isolates, the extracted DNA by boiling method was 28/40 (70%), CTAB method 18/40 (45%) and solid phase method 2/40 (5%).* ***Conclusion****: Phenol method was the best method (mean rank 2.34) for DNA extraction from sputum samples, while the easy and economic boiling method was the best method (mean rank 2.45) for DNA extraction from LJ culture isolates. The worst method of DNA extraction from both sputum and culture was phase solid method. A greater and easier yield of DNA was obtained from MTB LJ Culture than sputum.*

INTRODUCTION

Tuberculosis (TB) is a critical infectious disease that primarily affects the lungs and is more common in developing countries[1]. In the 21st century, it forms a significant problem for world public health especially with the emergence and rising of drug resistant TB[2]. In Egypt, It is one of the major health concerns, as per WHO global report issued in 2013; prevalence of TB in Egypt was 29/100,000 of population[3]. Globally, WHO report in 2012 showed that there were new 8.6 million cases of TB, 20 % of them were extra-pulmonary TB, about 1.3 million deaths from TB and 70 million TB deaths are expected in the next 20 years[4].

The cornerstone of holding the spread of TB includes quick diagnosis, proper case finding, immediate initiation of effective therapy and contact tracing to arrest further transmission. Recent advances in molecular biology methods have led to rapid identification of mycobacterial DNA[5].

Microbiological methods are the clue for the laboratory diagnosis of TB. The ordinary methods for identification of TB showed either weak sensitivity as in microscopy or lateness for many weeks as in culture. The evolution in molecular biology gives a chance for fast diagnosis of *Mycobacterium tuberculosis* (MTB) infection which is substantial to start treatment early and holds its spread[6]. The efficacy of these methods relies on the type of sample, processing method and the PCR steps. The initial critical step in PCR is the DNA extraction from mycobacterial cells, giving a sufficient and pure DNA for effective PCR test[7].

Several molecular techniques for the diagnosis of TB are suggested and advocated by several studies[8].

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The basic interest of these studies is to establish a simple, precise and cheap technique[9]. In addition, the convenient PCR depends on the selection of the most suitable extraction method and the target to be amplified[10].

There are many restricting factors facing the success of mycobacterial DNA extraction techniques from clinical samples such as the slow generation time resulting in a few numbers of organisms, rigid cell wall rich in lipids that interfere with cell wall lysis and finally the intracellular presence of the pathogen[11].

Also, the purification of mycobacterial DNA is hindered by the compound lipids and the plenty of polysaccharides in the wall of mycobacteria. The positive aspect of polysaccharides is that it helps the isolation of intact bacteria from contaminating material[12].

Various physical and chemical methods are used for DNA extraction from MTB. Boiling is one of the physical methods that have been used to extract mycobacterial DNA as heating to 100 ◦C in a suitable buffer disrupts the bonds between the cell wall lipids resulting in adequate DNA extraction. It is also a simple, easy and cheap method for DNA extraction from culture, but not from clinical specimens[13]. On the other hand, phenol extraction is an example of chemical methods. Phenol is a strong proteolytic, corrosive and caustic agent that dissolves the cell wall of mycobacteria by its solubilizing and denaturing effects on both proteins and lipids. In addition, the chloroform used in this method augment the effect of phenol as it has the same action[14].

The aim of this study was to compare the yield of MTB DNA extraction from direct sputum samples and from LJ culture isolates by different extraction methods.

METHODOLOGY

Clinical Samples:

32 positive morning sputum samples and 40 positive LJ culture specimens from the same TB confirmed patients were collected from Mansoura University Hospital, Chest Department, from January 2017 to June 2017. Each of direct sputum samples and LJ culture MTB isolates were subjected to DNA extraction by different extraction methods.

Methods of DNA extraction from sputum

Preparation of sputum for DNA extraction: First, decontamination of sputum samples was done by adding equal volume of N-acetyl-L-cysteine- 2 % NaOH to it for 30 minutes at 37◦C with recurrent vortexing. After that, this mixture was centrifuged. The second step was processing of the resulting pellet by washing two times with sterile distilled water by centrifugation and then, 1.5ml of Tris-EDTA buffer (TE) was added to make a homogenous suspension. The last step was to divide this suspension into three parts for DNA extraction by different methods[13].

Solid phase absorption method:

Using QIAamp DNA Mini Kit as mentioned by manufacturer's instructions (QIAGEN, Hilden, Germany).

Digestion buffer method:

2-3 ml of digestion buffer [500 mM Tris HCl with pH 9.0, 20 mM EDTA, 10 mM NaCl and 1% Sodium dodecyl sulfate (SDS)] was aseptically added to 0.5 ml of the prepared pellet in a 10 ml tube, incubated overnight at 60°C, and then vortexed for 20 seconds then 0.5 ml phenol was added to 0.9 ml of decontaminated sample, vortexed for 20 seconds and centrifuged for 5 min at maximum speed (14.000 x g). The aqueous solution containing target DNA was transferred to a fresh tube, containing 0.5 ml phenol, vortexed for 20 sec and centrifuged for 5 min at maximum speed (14.000 x g). Again the aqueous solution (approximately 350 µl) was transferred to a fresh tube containing 35 µl 3 M Na acetate and 800 µl absolute ethanol, mixed and incubated for 20 min at -20°C and then centrifuged for 30 min at room temperature with maximum speed. The supernatant was discarded and the pellet was washed with 500 µl 70% ethanol and centrifuged for 5 min at maximum speed. The DNA pellet was re-suspended in 50-200 µl 1x TE and stored at -20°C until further use[15].

Phenol-chloroform-isoamyl alcohol method:

25 µL of lysozyme solution (final concentration of 2.5mg/ml) was added to 75 µL of prepared pellet and incubated for 30 min at 37oC. 3.0 µL of proteinase K (final concentration of 150 µg/ml) and 20 µL of 10% SDS (final concentration of 1%) were added and completed to a final volume of 200µL with TE, incubated at 65 oC with recurrent agitation. Extract DNA with 300µL of phenol/chloroform / isoamyl alcohol (25:24:1), centrifuged at 14,000 x g for 5 minutes, and the aqueous phase was transferred to a clean microcentrifuge tube with the addition of 30 µL of sodium acetate 3M with pH 4.8. DNA was precipitated with 1 volume isopropanol (300 µL), agitated manually, and centrifuged at 14,000 x g for 15 min. The supernatant was discarded and cold ethanol 70% (300 µL) was added to the pellet and centrifuged at 14,000 x g for 5 min. The pellet was dried at room temperature and then re-suspended in 100 µL TE buffer[16].

Methods of DNA extraction from LJ culture:

From Culture on LJ media all collected samples were subjected to decontamination and concentration according to what mentioned in[17] and cultured on LJ solid medium, slopes were inspected weekly for up to 8 weeks and suspected growth was confirmed by ZN stain.

Solid phase absorption:

Using QIAamp DNA Mini Kit as mentioned by manufacturer's instructions (QIAGEN, Hilden, Germany).

Boiling method:

The simplest way of DNA release from mycobacterial suspension is boiling for 10 to 15 min in distilled water then centrifugation was done at 12000g for 3 minutes, 5µl of the supernatant was used for the PCR after that where it was kept in clean microcentrifuge tubes and stored at – 20 ° C until used[5].

Cationic cethyl-tri-methyl-ammonium bromide (CTAB) method:

At least one loopfull of colonies was transferred into microcentrifuge tube containing 400 µl of 1xTE buffer, then heated for 20 min at 80°C to kill the cells, and cooled to room temperature. 50 µl of 10 mg/ml lysozyme was added, vortexed and incubated at least 1 hour at 37°C. 75 µl of 10 % of SDS/proteinase K solution (5 µl proteinase K, 10 mg/ml and 70 µl 10% SDS) were added, vortexed shortly and incubated 10 min at 65°C. Then, 100 µl l5M NaCl and 100 µl CTAB / NaCl solution (4.1 g NaCl and 10 g CTAB in 100 ml distilled water) were added after pre-warming at 65°C,vortexed until the liquid content becomes white and incubated for 10 min at 65°C. After that 750 µl of Chloroform / isoamyl alcohol (24:1) was added, vortexed for 10 seconds and centrifuged at room temperature for 5 minutes at 12,000 g. The aqueous supernatant was transferred to a fresh microcentrifuge tube and 450 µl isopropanol was added, incubated 10 min on ice and centrifuged 15 min at room temperature. The last step was to discard the supernatant and wash the pellet with 1 ml of 70% ethanol and centrifuged for 5 min at room temperature, then the pellet was dried and dissolved in 100 µl of 1x TE buffer[18].

Evaluation of Extracted DNA

To test for the purity of the extracted DNA (should be between 5 to 100 ng/µL) was tested by measuring the absorbance using spectrophotometry (SpectraMax Plus) at 260 and 280nm. For pure DNA extract, the ratio of A260/A280 should be between 1.8 and 2.0[19].

Conventional PCR amplification protocol and gel electrophoresis:

Extracted DNA was identified by amplification of a fragment of the insertion sequence IS*6110*, which is specific for the *MTB* by using a pair of universally accepted primers[20]. For the PCR reaction, 24µL of 1x PCR-Master Mix (Fermentas™, USA), 3.0µL of each primer:
(INS-15'-CGTGAGGGCATCGAGGTGGC-3' and INS-2 5'GCGTAGGCGTCGGTGACAAA-3') and 6.0µL of genomic DNA were mixed with 14.0µL of sterile nuclease free water. After this, the reaction was performed in the thermo cycler (Thermo-fisher, USA) under the following conditions: initial denaturation cycle of 95ºC for 4 minutes, followed by 30 cycles of denaturation at 95°C for 30 minute, primer annealing at 55°C for 30 seconds and finally extension at 72°C for 1 minute and a final cycle of 72°C for 10 minutes. Subsequently, for detection of amplified DNA, 5µL of amplified product (amplicon) was loaded on 1.5% agarose gel along with the molecular weight marker (DNA ladder) and stained with ethidium bromide and then separated by electrophoresis[21].

Compliance with ethical standards:

Informed consent was obtained from all patients involved in this study. The study was approved by Ethical Committee of Mansoura University.

Statistical Analysis:

Data analysis was performed using SPSS software program for Windows, version 21, USA). Normal distribution of variables was tested with the Shapiro Wilks test. The results were expressed as number (percentages) for categorical variables. Chi-square test was done for different variables. The difference between the used methods for both sputum and culture was analyzed by Friedman Chi-Square test and Kendall's W tests which are significant in the present study, then Wilcoxon signed-ranks test was conducted to identify where the specific differences lie.

RESULTS

Thirty two positive sputum samples and 40 culture isolates from the same TB infection confirmed patients were collected from Mansoura University Hospital, Chest Department, from January 2017 to June 2017. In table 1, DNA extraction methods (solid phase absorption**,** digestion buffer method and phenol-chloroform-isoamyl alcohol method) from 32 positive sputum samples revealed that the number of DNA extraction by phenol-chloroform-isoamyl alcohol method was 21/32 (65.62%), followed by digestion buffer method 14/32 (43.75%) and the least one was solid phase method 1/32 (2.5%). The number of positively extracted DNA were significantly ($P < 0.05$) lower than the number of negative DNA extract obtained by solid method. On the other hand, the positively extracted DNA didn't differ significantly from the negative results for extraction by using phenol method.

Table 1: DNA extraction methods from 32 Sputum Samples

Extraction Method	Sputum Samples (n = 32)		
	Solid Phase	Digestion Buffer	Phenol
Positive No (%)	1(3.12 %)	14 (43.75 %)	21 (65.62 %)
Negative No (%)	31 (96.88%)	18 (56.25 %)	11 (34.38 %)
Chi-square	36.100	3.6	0.1
P-value	0.001	0.058	0.752

As shown in table 2, among DNA extraction methods (solid phase method, boiling method and CTAB method) from 40 culture isolates, the number of DNA extracted cases by boiling method was 28/40 (70%), followed by CTAB method 18/40 (45%) and the least one was solid method 2/40(5%). The number of positively extracted DNA were significantly ($P < 0.05$) higher than the number of negative DNA extraction cases by boiling method, while, the number of positively extracted DNA was significantly lower than the number of negative DNA extraction cases by solid phase method. For CTAB method, the positively extracted DNA cases didn't differ significantly from the negative extraction cases.

Table 2: DNA extraction methods from 40 LJ culture isolates

Extraction Method	LJ culture isolates (n = 40)		
	Solid Phase	Boiling	CTAB
Positive No (%)	2 (5 %)	28 (70 %)	18 (45 %)
Negative No (%)	38 (95 %)	12 (30 %)	22 (55 %)
Chi-square	32.4	6.4	0.4
P-value	0.001	0.011	0.527

In table 3, the Friedman test which evaluated the differences in medians among the three methods of DNA extraction from both sputum and culture was significant ($p < 0.05$). Then the Kendall's W test was 0.351 for sputum and 0.391 for culture, indicating fairly strong differences among the three DNA extraction methods for both sputum and culture. In the current study, the Wilcoxon test was conducted to evaluate the comparisons between pairs of medians for both sputum and culture DNA extraction. For sputum, three comparisons (digestion-solid, phenol-solid and phenol-digestion) were significant at the 0.05 alpha levels. For culture, three comparisons (boiling-Solid, CTAB-solid and CTAB-boiling) were significant at the 0.05 alpha levels.

Table 3: Mean rank of extraction methods (solid, digestion and phenol) in sputum samples and (solid, boiling and CTAB) in LJ culture isolates.

Sputum samples		LJ Culture isolates	
Methods	**Mean Rank**	**Methods**	**Mean Rank**
Solid (n = 32)	1.59 [a]	Solid (n = 40)	1.48 [a]
Digestion (n = 32)	2.08 [b]	Boiling (n = 40)	2.45 [b]
Phenol (n = 32)	2.34 [c]	CTAB (n = 40)	2.08 [c]
Friedman Chi-Square = 28.091 Kendall's W = 0.351 P-value = 0.001		Friedman Chi-Square = 31.273 Kendall's W = 0.391 P-value = 0.001	

a, b, c Superscript letters in the same column were significantly different at $p \leq 0.05$

All these data depends on detected MTB sequence of insertion sequence IS6110 detected by conventional PCR test as shown in figure 1

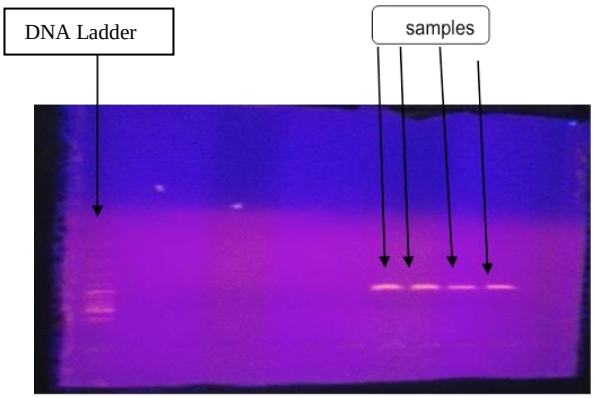

Fig 1: Conventional PCR for detection of insertion sequence IS6110 showing positive DNA extracts on the right side and DNA ladder on the left side

DISCUSSION

Continuous efforts are directed to evolve a rapid, simple, precise and cheap method to extract mycobacterial DNA; however, there is no established reference method. So, most of the laboratories have developed their own extraction procedures after a prolonged time of evaluation of different methods[22]. A molecular method as PCR enables quick detection of MTB which is substantial to start treatment early and hold its spread. The initial step in PCR is DNA extraction[23]. The mycobacterial DNA extraction techniques are very difficult due to the presence of complex cell wall rich in lipids and the intracellular presence of pathogen[11].

In this study, direct DNA extraction methods of MTB from sputum samples were done by 3 methods solid phase absorption, digestion buffer and phenol-chloroform-isoamyl alcohol methods. Among 32 positive sputum samples, 21 (65.62%), 14 (43.75%) and 1 (2.5%) DNA extracts were obtained by phenol-chloroform-isoamyl alcohol method, digestion buffer method and solid phase method respectively. The number of positively extracted DNA cases were significantly (P<0.05) lower than the number of negative DNA extraction cases obtained by solid phase method. However, for phenol method, the positively extracted DNA cases didn't differ significantly from the negative DNA extraction cases. As a consequence, the best method for DNA extraction from sputum in the current study was phenol-chloroform-isoamyl alcohol method (mean rank 2.34).

The phenol-chloroform-thiocyanate guanidine method for mycobacterial DNA extraction was superior over other methods owing to the good solubilizing and denaturing effect of both phenol and chloroform, helping lysis of the cell envelope and the removal of lipid and protein layers of the cell wall[24]. The other characteristic of this method is the adjuvant effect of the use of SDS for cell wall lysis and proteinase K for dissolving proteins. On the other side, the drawbacks of this method included the toxicity of phenol-chloroform and the multiple steps[25].

Successful PCR is dependent on DNA purification after extraction by excluding inhibitors from the sputum samples. In order to achieve this, cell lysis was done by both physical and chemical steps with the addition of proteinase K and lysozyme. Also, ethanol was used for precipitation of extracted DNA and removal of organic contaminants which supports the inconvenience of Solid phase absorption method for DNA extraction from sputum samples[13].

Mycobacterium tuberculosis extraction from culture isolates was done by solid phase, simple boiling and CTAB methods in the current study. Among the 40 LJ culture isolates, 28/40 (70%), 18/40 (45%) and 2/40 (5%) DNA extracts were obtained by boiling, CTAB and solid phase methods respectively. The number of positively extracted DNA were significantly (P<0.05) lower than the number of negative DNA extraction cases obtained by solid method, meanwhile, the number of positively extracted DNA were significantly (P<0.05) higher than the number of negative DNA extraction cases obtained by boiling method. But for CTAB method, there was no significant difference between positively extracted and negative DNA extraction cases. According to our results, the best method for extracting DNA from culture isolates was simple boiling method (mean rank 2.45)

Boiling extraction method is ideal especially in developing countries as it is simple, used familiar and cheap, but it doesn't include purification and precipitation steps which leads to poor quantity and quality of DNA extract when used to extract DNA from clinical samples but it will be ideal for DNA extraction from cultured isolates[13]. That what we proved in our study, a result matching what Cao et al[26], showed in their study where they concluded that best methods for MTB DNA extraction and amplification were boiling and the phenol-chloroform methods. Also, our results agreed with De Almeida et al[19] and Pan et al[27] results. Amaro et al[28] results are partially consistent with our data regarding CTAB method. Disagreeing results were shown by Ruqaya et al[13] in their study where CTAB method yielded more DNA extract than boiling method and solid phase method didn't yield any DNA. Where boiling method showed a poor A260/A280 ratio and weak band on agarose gel electrophoresis denoting the presence of DNA contaminants, while CTAB method yielded pure DNA. This variation may be due to technical differences between studies and methodologies used Castro et al[29] but generally we can recommend boiling method as a suitable method for MTB DNA extraction from cultured isolates especially in developing countries with high number of samples to be done.

We used IS6110 primers for amplification of extracted MTB DNA which is the most commonly used sequence to detect MTB as it has multiple copies that increases the sensitivity of PCR test to detect the sequence Wildner et al[30]. The IS6110 target sequence is a MTB complex repetitive sequence of 1350 PB that differs in number of copies inserted in chromosomal sites of different species Goldsborough and Bates[31]. Sattar et al[32] said that false negative PCR cases may be due to absence of the IS6110 in some species.

CONCLUSION

Phenol-chloroform-isoamyl alcohol method was the best method (mean rank 2.34) for DNA extraction from sputum, while the easy and economic boiling method was the best method (mean rank 2.45) for DNA extraction from culture isolates. The worst method of DNA extraction from both sputum and culture was solid method. A greater and easier yield of DNA was obtained from MTB Culture than sputum.

- The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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