# Comparison between different methods for DNA Extraction for detection of Mycobacterium tuberculosis from sputum using Polymerase chain reaction

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## Abstract

Deoxyribonucleic acid (DNA) extraction methods from Mycobacterium tuberculosis using sputum samples were evaluated including boiling, Soled phase absorption, Triton and N- cetyl-N,N,N-Trimethial ammonium bromide (CETAB). CTAB method showed 70 % detection sensitivity with strong band on agarose gel. Boiling and Triton methods to be unsatisfactory because they yielded either low quantity or poor quality of DNA or were unable to remove inhibitors of DNA amplification.

**Key Words:** PCR, DNA extraction, Mycobaterium tuberculosis**.**

# مقارنه بين طرق مختلفه لاستخلاص الدنا من القشع لغرض تشخيص عصيات السل البشري بأستخدام تفاعل البلمره المتسلسل .

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## الخلاصة

تم تقييم طرق استخلاص الحمض النووي لعينات البلغم والتي تشمل في ذلك الغليان، وأستخام عده جاهزه ، والتريتون و (CETAB). وأظهرت طريقة CTAB 70٪ حساسية الكشف مع ناتج ذو قوه عاليه على هلام الاكاروز. وأظهرت طريقه الغليان والتريتون نتائج غير مرضية لأنها أسفرت إماعن تراكيز منخفضة أو عدم نقاوه الحمض النووي مع عدم أمكانيه إزالة مثبطات تضخيم الحمض النووي **وبالتالي الحصول على نتائج غير واضحه عند الترحيل على هلام الاكاروز .**

**الكلمات الداله :** تفاعل البلمره المتسلسل ، طرق مختلفه لاستخلاص الدنا ،عصيات السل البشري**.**

## Introduction

Mycobaterium tuberculosis is the etiological agent of tuberculosis (TB), this disease affecting 9.2 million people in 2010 and 20% died within 10 years (1, 2).This world health problem has been exacerbated by increasing numbers of susceptible population due to spread of Human Immunodeficiency virus (HIV), malnourishment, immunosuppressant environmental conditions and M. tuberculosis multidrug resistant (3). Key issues in TB management to stop chain of transmission are quick diagnosis, adequate treatment and location of contacts (4).

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The diagnosis method is one of the most important aspects; effective molecular methods have been developed to characterize the *M. tuberculosis* and non tuberculosis mycobecteria. In addition to reduce the time for diagnosis (5). Polymerase chain reaction (PCR) has been widely used for rapid diagnosis of *M. tuberculosis* from clinical samples, the sensitivity of this method affected by the DNA extraction procedure, target region and probe using (6). Because of complex structure and impermeability of the cell wall of this genus, lysis of mycobacterial cells is difficult. Therefore the commonly employed methods of isolation DNA yield either low quantity (due to incomplete lysis of bacterial cell wall) our poorless quality of mycobacterial DNA, resulting in low sensitivity of the PCR method (7). There have been many kinds of in-house PCR methods and commercial detection kits used with the aim of routine diagnosis (8).

The aim of this study has been carried about using 4 DNA extraction techniques from *M. tuberculosis* in sputum samples collected from the smear positive patients with pre diagnosed with tuberculosis to investigate the performance of PCR method in detecting positive results.

## Materials and methods

### Samples preparation

Sputum samples were collected from 50 untreated patients, with pulmonary tuberculosis were detected by clinical and radiological findings. The patients randomly selected from Institute of Chest and Respiratory Diseases in Baghdad during April /2010. The sputum specimens dilution to $10^6$ , $10^4$,$10^2$ and $10^1$ fu/ml density .

These samples were decontaminated with 2% NaOH and culture on Lowenstein - Jensen (LJ) media and used for Ziehl Neelsen(ZN) staning, then incubated at 37 $^{\circ}$C for 8 weeks, use culture of *Mycobacterium tuberculosis* H37Rv as a positive control. The identification of *M. tuberculosis* was made by Nitrate reduction; Niacin, Tiofen 2 carboxylicacide hydrazid(TCH) and Paranitrobenzoic acid (PNB) test (9).

### Preparation of clinical samples for DNA extraction

A total of 3 ml from each sputum samples were decontaminated with equal volume of 4% NaOH for 30 min at 37 $^{\circ}$C with interrupted vortexing. Then samples were centrifuge at 6000 rpm and pellets were washed twice with sterile distilled water by centrifugation (9). The pellet was suspended in 1.5ml of Tris-EDTA buffer (TE) to make homogenous suspension and finally was equally divided to four sterile tubes (400µl for each) for DNA extraction by the four different methods.

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## Methods of DNA Extraction

### 1. Boiling extraction method

A 400 µl of TE buffer (10mM Tris HCL {PH8.0}, 1mMEDTA) was added, to the mixture was mixed vortex. The suspension was placed in boiling water bath for 15 minute to lysis any mycobacteria and then centrifuged at 16000 rpm for 5 minute. Then 100 µl of supernatant was transferred to a sterile microcentrifuge tubes and stored at -20 °C (10).

### 2. Soled phase absorption

(DNA- Sorb-B, DNA Extraction kit, Sacace biotechnologies / Italy) was (performed according to the instructions of the Manufacture).

### 3. Triton method

Four hundred µl of lysis buffer (100mM NaCl,10mM Tris –HCl ,PH8.3;1mMEDTA,PH 8.0;1%Triton X-100) was added to an equal volume of working cell suspension were boiled for 15 minute and allowed to cool at room temperature and stored at -20 °C(11).

### 4. N- cetyl-N,N,N-Trimethial ammonium bromide (CETAB) method

To 400µl of pre-treated sample, 200µl of TE buffer (10mMTris, 1mM EDTA, pH 8.0) was added and boiled at 80°C for 30 minutes then immediately frozen at -20°C for 15 minutes. Lysozyme 30µl (50mg/ml) was added and vortexed before incubation at 37°C for overnight. The following morning, 70µL 10% SDS and 10µL 20 mg / ml proteinase K were added before incubation at 65°C for 10 minutes. Then, 100µL of 5M NaCl 100µl and CTAB / NaCl (pre-warmed to 65°C) were added and incubated at 65°C for 10 minutes. 750µL chloroform / isoamyl alcohol (24:1) was added and the tubes were mixed by inversion. The tubes were centrifuged at 14,000 rpm for 15 minutes and the upper aqueous phase containing the DNA was removed and added to 450µL of ice-cold isopropanol. These tubes were mixed gently before chilling at –20°C over night. The following morning, tubes were centrifuged at 10,000rpm for 10 minutes at room temperature, the supernatant was removed and the DNA pellet was washed with 1mL ice-cold 70% ethanol. The tubes were centrifuged at 10,000rpm for 5 minutes at room temperature, the supernatant was removed and the pellet was allowed to air-dry. The pellet was resuspended using TE buffer and DNA preparations were stored at -20°C until used (12).

## Purity of Extracted DNA

The absorbance at 260 and 280nm was used to check the purity of the extracted DNA. A ratio of A260/A280 was calculated. Pure preparation of DNA had A260/A280 values ranging between 1.8 and 2.0.

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**Amplification of Mycobacterial DNA by PCR**

DNA amplification reaction was performed in a total volume mixture was prepared with 25 µl of PCR Master (QIAGEN$^{R}$ ), 18.5µ l of water , 2.5 µl of dimethyl sulfoxide, 1 µl of each primer (*Cfp32*) at 20 µM F 5' ATG CCC AAG AGA AGC GAA TAC AGG CAA 3', R 5' CTA TTG CTG CGG TGC GGG CTT CAA 3' , and 2µl of DNA. The PCR programmed used as initial denaturation of 15 min at 95°C followed by 25 cycles of 1 min at 94 °C.1 min at 60°C, and 1 min at 72°C, and ending with a final extension step for 10 min at 72°C. Each set of amplification tubes included a positive control of *M. tuberculosis* (H37Rv), and negative control containing 2 µl of sterile distilled water.

**Analysis of PCR Products**

An aliquot (5µl) from PCR product of each sample was analyzed by gel electrophoresis in 1.5 % agarose gels at constant voltage (110V), along with the molecular weight marker and PCR products of positive and negative controls. A sharp band of 100 bp of amplified DNA was visualized under UV light in positive samples by staining the gels with ethidium bromide for 15 minutes and de-staining with distilled water. The presence of a distinct band of 786bp was considered as a positive PCR result for *M. tuberculosis* DNA.

## Results

To test the sensitivity of in vitro amplification of mycobacterial DNA with primers based on detection of *Cfp32* sequence the results presented in Figure 1 show that these primers enable detection up to 25 ng of DNA on ethidium bromide stained agarose gels (Figure1).

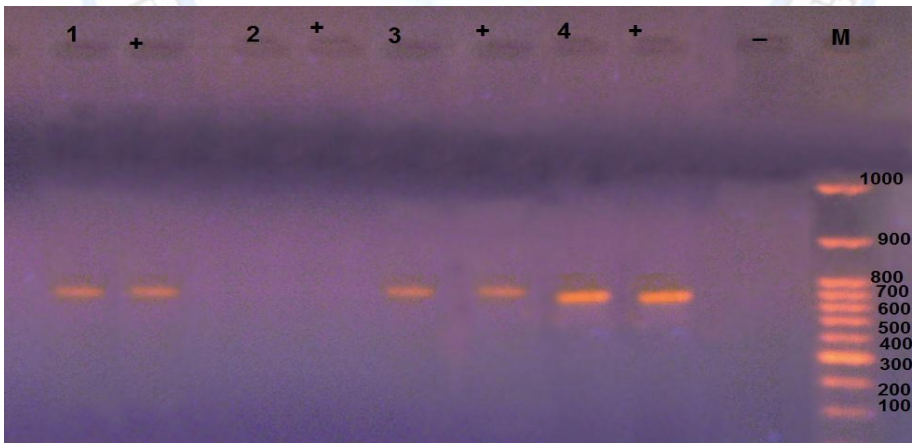


Figure 1: Amplified product of the DNA from samples detected by various method Lanes 1-4 amplified product of DNA extracted by method 1-4 respectively, lane (+) control positive to each method and M 100bp marker.

It was found that method one and three yielded low quantity of DNA compared to the method four, while no DNA was yield in method two (Table 1).

Table 1. Concentration and purity Mycobacterial DNA Extracted by Various methods

| DNA extraction method | Mean absorbance at 260 | Concentration of DNA obtained/ml | Purity of DNA A260/A280 | PCR results (intensity of band) |
|---|---|---|---|---|
| Boling | 0.096 | 1.3 µg | 1.5 | Faint |
| Solid phase absorption | - | - | - | Negative |
| Triton method | 0.091 | 1.5 µg | 1.5 | Faint |
| CTAB | 0.600 | 23.863 µg | 1.8 | strong |

The quality of the DNA extracted for PCR analysis was found to be an important factor. We found that sufficient quantity of DNA being obtained by a few of the methods, we were able to detect only a faint band on agarose gels. Method 1and 3 show a low A260/A280 ratio in spite of high concentration of extracted DNA, signifying the presence of impurities in the DNA. No purification steps were used in these methods. Method 4 yielded pure DNA the ratio was 1.8 µg/ml (Table1).

Fifty sputum samples tested in the study, after processed by 4% NaOH method, were evaluate by (ZN) staining. Clinical sample were divided into 4 groups depend on the microscopic evaluation (Table 2).

**Table2: PCR results by the microscopic evaluation of sputum sample**

| | The groups of the samples | | | | |
|---|---|---|---|---|---|
| DNA extraction method | Smear (-) N=6 | Smear (+) N=12 | Smear (++) N =15 | Smear (+++) N=17 | Total N=50 |
| Boling | - | - | 3(20%) | 6(35.29%) | 9(18%) |
| Solid phase absorption | - | - | - | - | - |
| Triton method | - | 1(8.3%) | 7(46.6%) | 10(58.82%) | 18(36%) |
| CTAB | - | 5(41.6%) | 13(86.6%) | 17(100%) | 35(70%) |

Results of PCR were compared to clinical finding and the culture on (LJ) method. This method was positive in the 47 of 50 samples which were prediagnosed from TB with clinical and radiological findings. The samples, whose cultures were negative, were also negative in the smear sample. PCR results of smear negative samples were negative by all 4 extractions

methods. In the group of smear positive, 1 sample by Triton method and 5 samples by CTAB method were positive by PCR. The average rate of the positive detection of 4 extraction methods in all the samples was 18% with boiling method; all samples were negative by Solid phase absorption method, Triton method 36%, and 70% with CTAB method (Table 2). In this study, $10^{6,}$ $10^4$, $10^2$ and $10^1$ dilutions were used in 4 different extraction methods direct and after homogenization and decontamination were processed by standard 4 % NaOH method (Table 3).

In the evaluation made by direct and processed methods, PCR was positive in boiling method, Triton method and CTAB method using the$10^6$ dilution. In $10^4$ dilutions, PCR was found positive in Triton method and CTAB method, while PCR was positive in extraction performed from direct samples by CTAB method in$10^2$. In $10^1$ dilutions, the results of all PCR studies in direct and processed samples by 4 extraction methods were negative.

Table 3: Results of PCR regarding to dilution of samples in the 4 different DNA extraction methods

| DNA extraction method | Dilution of the sample | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^6$ | | $10^4$ | | $10^2$ | | $10^1$ | |
| | Direct | 4% NaOH | Direct | 4% Noah | Direct | 4% NaOH | Direct | 4% NaOH |
| Boling | + | + | - | - | - | - | - | - |
| Solid phase absorption | - | - | - | - | - | - | - | - |
| Triton method | + | + | + | - | - | - | - | - |
| CTAB | + | + | + | + | + | - | - | - |

## Discussion

One third of the world population is infected with tuberculosis and approximately 8 million new cases are reported every year (1). The accurate diagnosis of tuberculosis is maintained by microbiologic methods. The precise diagnosis is delayed because the sensitivity of the microscopy method is low and the incubation period takes 2-8 weeks for the diagnosis by culture. Recently, molecular methods to detect Mycobecteria have been used in some parts of the words to support conventional technique (11). The sensitivity of these methods depend on clinical sample, method for processing this sample and principles of the PCR method. The most important factor among PCR method procedure steps is the DNA extraction from clinical samples. It has to yield an accurate DNA that does not include the inhibitory substances and is purified from cell structures for a successful PCR test(12 ). In our study, the extraction methods widely used for bacterial suspensions and smear positive specimen. It was found that methods 1and 3 yielded low quantity of DNA compared to method 4.

The purity of the DNA extracted for PCR analysis was found to be an important factor. We found that in spite of sufficient quantity of DNA being obtained by a few of the methods, we detect only afaint band on agarose gels. Method 1 and 3 shows a poor A260/A280 ratio, signifying the presence of impurities in the DNA. No purification steps were used in these methods. Methods 4 yielded pure DNA. There is a possibility that the visual of the target band is due to the shielding of the target sequence by some ' carryover – molecules ' which is present in the extracted DNA. It is well-established fact that the sensitivity of PCR purely resides upon the purity of the DNA (13).

Successful detection of DNA by amplification methods depend on purity and quality of DNA template. Our results shown successful extraction of

DNA from sputum using each physical, and chemical or both treatments (method 4). Physical treatments like boiling of bacterial suspension were helpful in extraction of DNA. Method 1 includes heating, low DNA yield from H37Rv and a faint from 18% AFB positive sputum samples. Heating is necessary for weakening the linkages between the lipids contents of mycobcterial cell well, resulting in release of DNA in solution. In previous studies, suggested that heating the sample to 100 $^{\circ}$C in a suitable buffer could adequately extract DNA (14 ).

Further improvement of lysis could be achieved by use of suitable chemical and enzymatic digestion of bacterial cell wall. Use of a detergent for lysis of cell wall was found to be essential by most of the workers (15). Method included in our study used Triton X100 yield low quantity and quality but use this item improves isolate of DNA than the method (1). Used SDS in method 4 reported good yield and 100% detection of DNA.

Lysozyme enzyme is frequently used in the lysis buffer for the complete lysis of cell wall. Methods 1, 2, 3 did not use lysozyme for cell wall digestion thus the quality and quantity is low un like in method 4 when use this enzyme.

DNA purification is one of the most important steps to remove inhibitors from the sputum samples. In methods 1 and 3 did not use any purification and precipitation step. So low quantity and quality of DNA was extracted by these methods. Solid phase absorption method is inappropriate to use routinely for extraction DNA from sputum samples.

Boiling the bacterial pellet in lysis buffer could be an easy and economical solution for extraction DNA from culture, but for clinical samples this is not recommended. Thus, we suggest that use of both physical and chemical steps for cell lysis, use of proteinase K and lysozyme. For DNA purification use phenol: chloroform and ethanol or isopropanol for precipitation, are essential steps for extraction of Mycobacterial DNA from clinical samples. Among these methods, if the rat of positive detection is taken into consideration, in smear positive sample, CTAB method is more proper to use routinely for isolation from clinical samples (16).

## Reference

1. World Health Organization.(2011) Global tuberculosis control: Surveillance, lanning, nancing. WHO Report (WHO/HTM/TB/2011). Geneva: World Health Organization.

2. Edine W. Tiemersma, Marieke J. van der , Martien W. Borgdorff, Brian G. Williams4, Nico J. D.Nagelkerke. (2011) Natural history of tuberculosis duration and fatality of untreated pulmonary tuberculosis in HIV negative patients: A systematic review. *PLoS ONE* 6(4):17601.

3. World Health Organization (2006). Guidelines for the programmatic management of drug-resistant tuberculosis. WHO/HTM/TB/2006.361. Geneva: World Health Organization.

4. Broekmans, J. ( 1994). Control strategies and programme management. In: Porter JD, McAdam PW, editors.Tuberculosis, back to the future. New York, N.Y: JohnWiley & Sons Inc.p. 171-92.

5. Devallois A, Goh K, and Rastogi N.( 1997). Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp*65 gene and proposition of an algorithm to differentiate 34 mycobacterial species. J Clin Microbiol. 35:2969-73.

6. Wade K. Aldous, June I. Pounder, Joann L. Cloud, and Gail L. Woods. ( 2005). Comparison of Six Methods of Extracting Comparison of Six Methods of Extracting *Mycobacterium tuberculosis* DNA from Processed Sputum for Testing by Quantitative Real-Time PCR J Clin Microbiol.43 : 2471–2473.

7. Jain Amita, Tiwari Vandana, R.S. Guleria, and R.K.Verma.(2002). Qualitative Evaluation of Mycobacterial DNA Extraction Protocols for Polymerase Chain Reaction. Molecular Biology Today 3: 43-50.

8. American Thoracic Society Workshop. (1997). Rapid diagnosis tests for tuberculosis. What is the appropriate use? Am J Respir Crit Care Med. 155: 1804-1814.

9. World Health Organization (1998). Laboratory services in TB control part III: Microscopy. WHO/TB/98.258. Geneva: World Health Organization.

10. Brisson-Noel, A., Gicquel, B., Lecossier, D., Levy-Frebault, V., Nassif, X. and Hance, J. (1989). Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet. II: 1067-1071.

11. Notle, F.S., Metchock, B., McGowan, Je., Edwards, A.,Okwunmabua, O., Thurmond, C., Mitchell, P.S., Plikaytis,B. and Shinnick, T. (1993). Direct detection of Mycobacterium tuberculosis in sputum by polymerasechain reaction and DNA hybridization. J. Clin. Microbiol.31: 1777-1782.

12. Van Embden J, Cave M, Crawford J, Dale J, Eisenach K, Gicquel B. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: Recommendations for a standardized methodology. J Clin Microbiol.; 31:406-9.

13. Claudia Castro, Liliana González, Juan Carlos Rozo1, Gloria Puerto, Wellman Ribón. (2009). Biosafety evaluation of the DNA extraction protocol for *Mycobacterium tuberculosis* complex species, as implemented at the Instituto Nacional de Salud, Colombia. Biomédica 29:561-6.

14. Boddinghaus BTA, Wihchelhaus TA, Brade V, and Bittner T. (2001) Removal of PCR inhibitors by silica membranes: Evaluating the Amplicor *Mycobacterium tuberculosis* Kit. J Clin Microbiol 39: 3750 -3752.

15. Cormican, M.G., Barry, T. and Flynn, J. (1992).Use of polymerase chain reaction for early identification of *Mycobacterium tuberculosis* in positive cultures. J. Clin.Pathol. 45: 601-604.

16. Banavaliker, J.N., Bhalotra, B., Sharma, D.C., Goel, M.K., Khandekar, P.S. and Bose, M. (1998). Identification of Mycobacterium tuberculosis by polymerase chain reaction in clinical specimens. Ind. J. Tub. 45: 15-18.