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Comparison of Different DNA Extraction Protocols for Molecular Diagnosis of Mycobacterium Tuberculosis

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Mycobacterium tuberculosis; Polymerase Chain Reaction (PCR); *IS6110*

ABSTRACT:

Tuberculosis remains a global epidemic, especially in developing countries; early diagnosis plays a critical role in controlling the disease. Traditional method is not reliable because isolation and identification of mycobacteria may take up to several weeks or more in achieving results. The aim of this study is to compare the efficacy of different protocols of mycobacterial DNA extraction in order to standardize a trustable DNA extraction protocol providing high quality DNA for molecular diagnosis. DNA was extracted using six different protocols. PCR was performed using primer of *IS6110* that specific for all the members of *M. tuberculosis complex*. The highest DNA extraction efficiency (68.75%) was observed using the protocol number 3, and DNA extraction was proved to give a higher accuracy to *IS6110* PCR in comparison with the other protocols. It could be concluded that most of DNA extraction kit dependent protocols are costly but of high quality DNA extraction, otherwise some of the classical methods are easy, low cost and simple but the purity of the DNA are of low quality.

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1. INTRODUCTION

Despite global efforts to control tuberculosis, it remains a public health problem worldwide, and for optimal control, early diagnosis is necessary (Dye et al., 1999; Drobniewski et al., 2004; Cox et al., 2003; Yang et al., 2008; 2010)

The most widely used routine methods for diagnosis of tuberculosis are microscopic examination of direct smear for acid-fast bacilli and culturing of *Mycobacterium tuberculosis (MTB)*. The former method has the disadvantage of low sensitivity and the latter method has to be incubated for more than one month before a final diagnosis **(**Soini et al., 2001). So, the use of Polymerase Chain Reaction (PCR) enabled a fast and sensitive diagnostic method for identification of *M.Tuberculosis* especially of samples of low bacilli load (Haldar et al., 2007).

A major problem facing the early diagnosis of TB caused by the conflicting data of DNA extraction protocols. In literature, different PCR techniques, Mycobacterial cell lysis buffers and DNA extraction protocols are available for TB diagnosis (Yazdani et al., 2008; Arjomandzadegan et al., 2011).

Many workers evaluated these protocols in order to obtain pure and high quality Mycobacterial DNA with minimal efforts costs, amount of needed samples and risk of missing Mycobacterial DNA (during processing prior to DNA isolation)(Espasa et al., 2005; Elbir et al., 2008). So, the aim of present work is to compare and evaluate six protocols of extracting *M. tuberculosis* DNA in order to improve the molecular diagnosis of *MTB.*

2. MATERIALS AND METHODS:

2.1. Tissue Samples (lymph nodes and other organ)

Each sample was divided into two parts, one for decontamination processing for bacterial isolates and the second for direct DNA extraction from tissue.

2.2. Bacterial Isolates

Samples were decontaminated, homogenized and cultured on Lowenstein-Jensen (LJ) medium using Petroff technique (Farnia et al, 2009). By adding an equal volume of 4% NaOH, centrifugation, the supernatants were discarded, and pellets (sediment) were used for DNA isolation. The cultures were incubated at 37ºC for up to 12 weeks, the tubes checked weekly for

observation of the growth of the colony (OIE 2004).

1.3. Protocols of DNA extraction:

In the present work six protocols of DNA extraction were used depend on direct DNA extraction from tissue or DNA extraction from grown mycobacterium isolate on LJ media.

1.3.1. 1st protocol (using QIAamp Blood and Tissue Kit)

DNA was extracted from the tissue samples (Lymph node, lung and liver) with and without visible lesions from tuberculin reactors and which were collected from slaughtered animal.

Using a modified QIAamp Blood and Tissue Kit (Qiagen, Germany). A small piece of tissue (25 mg) was macerated and placed in a 1.5 ml micro centrifuge tube. It was suspended in 180 µl of suspension buffer then 20 µl of proteinase K were added and incubated for 2 h at 56°C, in order to improve the process of bacterial lysis. DNA was eluted from the QIAamp mini spin columns. DNA was eluted with 100 µl of the buffer for increase the concentration of the DNA.

1.3.2. 2nd protocol (Using of the sediment and QIAamp Blood and Tissue Kit)

Using of the pellet (sediment) which is the end product of tissue processing for bacteriological culture. Apart of the sediment in 1ml of Normal saline (0.85% NaCl) was taken. The tube was then centrifuged at 14000 for 1 min. take (25 mg) of the pellet (sediment) in 1.5 ml micro centrifuge tube. The pellet was suspended in 180 µl of suspension buffer then 20 µl of proteinase K were added and incubated for one hour at 60°C, in order to improve the process of bacterial lysis. DNA was eluted from the QIAamp mini spin columns. DNA was eluted with 100 µl of the buffer to increase the concentration of the DNA.

1.3.3. 3rd protocol (Using of QIAamp Blood and Tissue Kit with prepared enzymatic lysis buffer as pretreatment in case of grown mycobacterial isolate).

In this protocol the use of the prepared enzymatic lysis buffer as pretreatment for mycobacterial isolate before using Qiagen blood and tissue kit as the following.

Using the prepared lysis buffer as pretreatment of mycobacterial isolate (180 µl for each sample). The lysis buffer composed of (1% Triton X-100, 0.5% Tween 20, 10mM Tris- HCL (PH 8.0), and 1mM EDTA). 2 µl of lysozyme were added immediately to lysis buffer before using it. Two or three mycobacterial colony was taken from grown mycobacterial isolate, then added to the enzymatic lysis buffer (lysis buffer+lysozyme) and incubate for 30 min at 37C, then 25 µl of proteinase K were added and 200 µl AL purification buffer (without ethanol). Mix by vortexing incubated for 30 h at 60°C, in order to improve the process of bacterial lysis. 200 µl ethanol (96–100%) were added to the sample, and mix thoroughly by vortexing in order to yield homogenous solution. Pipetting the mixture (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube from (QIAamp Blood and Tissue Kit). Centrifuge at (8000 rpm) for 1 min, flow-through and collection tube. DNeasy Mini spin column were Placed in a new 2 ml collection tube, 500 µl Buffer AW1 (washing buffer) was added, then centrifuged for 1 min at (8000 rpm). Discard Flow-through were discarded. Another DNeasy Mini spin column were added in another 2 ml collection tube, 500 µl Buffer AW2 (washing buffer) were added, then centrifuged for 3 min at (14000). DNA was eluted from the QIAamp mini spin columns. DNA was eluted with 100 µL of the buffer for increase the concentration of the DNA.

1.3.4. 4th protocol (Using lysis buffer) (Reischl et al., 1994)

In this protocol the use of prepared lysis buffer only for DNA extraction from grown mycobacterial isolate. A loopful of the bacterial colony was taken in 200 µL of the prepared lysis buffer (1% Triton X-100, 0.5% Tween 20, 10mM Tris-HCL (PH 8.0), and 1mM EDTA) in 1.5 ml of micro centrifugation tube. The tube was left at room temperature for an hour. Put in heat block for 20 min at 95-100c. The tubes were then centrifuged at 14,000 rpm for 5 min. The

supernatant was taken into new micro centrifugation tube. Centrifuged again at 14000 rpm for 5 min. Supernatant containing the DNA was taken and stored it at -20°C.

1.3.5. 5th Protocol (Using boiling method) (AVID, 2007)

In this protocol the use of classical method (boiling method) for DNA extraction from grown mycobacterial isolate. A loopful of the bacterial colony was taken in 100 µl of distilled water in 1.5 ml micro centrifuge tube. Incubated in heat block for 20 min with vortex every 10 min or water bath at 100C for 15 min with gentle shaking. The tubes were then centrifuged at 12000 rpm for 5min. The supernatant was taken into new micro centrifugation tube. Centrifuged again at 12000 rpm for 5min. Supernatant containing the DNA was taken and stored it at -20°C.

2.3.6. 6th protocol (Using of Bacteria DNA Preparation kit of Jena bioscience)

A loopful of the bacterial colony was taken in 500 µl distilled water in 1.5 ml of micro centrifugation tube. The tube was then centrifuged at 10000 rpm for1 min. The supernatant was discarded. The pellet was suspended in 300 µl of suspension buffer. 2 µl lysozyme were added. After mixing well, the tube was incubated at 37C for 1 hour. The tube was then centrifuged at 10000 rpm for 1 min. The supernatant was discarded. Then 300 µl of cell lysis buffer and 2 µl of RNase were added to the pellet. Vortex, 8 µl proteinase K were added and incubated at 60C for 10 min. 300 µl of binding buffer was added. Vortex. The tube was then centrifuged for 5 min at 10000 rpm. DNA was eluted with 50 µl of the elution buffer.

2.4. Calculation of the purity of Extracted DNA

The absorbance at 260 and 280 nm was used for determination of the purity of the extracted DNA. A ratio of A260/A280 was calculated. Pure DNA extracted had an ideal ratio when A260/A280 values ranging from 1.8 to 2.0 this ratio is significantly lower if there is a contamination with protein or any other impurities (Maniatis et al., 1989; Sambroock et al., 2001).

2.5. PCR and electrophoresis

PCR was performed in final volume of (25 µl) reaction mixture containing 5 µl of (5 x) PCR master mix (Jena bio science®), 3 µl of each primer, (Jena bio science®), 12 µl PCR grade water and 2 µl of DNA template.

The most commonly used PCR system is based on primers that amplify segments of the *IS6110* element, particularly targeting 245-bp fragments, the primer used in this study were designed to detect *IS6110* that specific for all members of *Mycobacterium tuberculosis* complex (MTC).

The forward primer is INS1 (5'-CGTGAGGGCATCGAGGTGGC-3') and the reverse primer is INS2 (5'-GCGTAGGCGTCGGTGACAAA-3') (Hermans et al., 1990).

Amplification was carried out for 30 cycles, each consisting of initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 1 min, annealing at 68ºC for 1 min and extension at 72ºC for 1 min, with a final extension at 72ºC for 7 min. PCR products were checked by electrophoresis on 1.5% agarose gels stained with ethidium bromide (Figueiredo et al. 2009; 2010). The amplification product should be visualized at 245 bp.

3. RESULTS AND DISCUSSION

Early diagnosis of *M.tuberculosis* plays a critical role in controlling the spread of the disease. In the last decade, PCR became the most commonly used diagnostic techniques in TB specialized labs worldwide due to its potential to detect *MTB* directly from clinical samples (Richardson et al., 2009).

The sensitivity of PCR depends to a large extend on the quality of extracted DNA as the lysis of mycobacterial cell wall is difficult. Therefore, classical methods of DNA extraction of Gram positive bacteria produce poor quality DNA which negatively affects the PCR sensitivity.

To overcome this obstacle, a wide range of DNA extraction protocols were tested with varying levels of success.

In the present work, the efficiency of six DNA extraction protocols was evaluated. For this purpose, 13 known positive lymph node samples having occasional acid fast bacilli (AFB), were subjected to the different DNA extraction protocols followed by their examination with PCR using specific primer of the *IS6110* element (table 2).

Amplicons intensity based on gel electrophoresis, the PCR amplicons quality differed in different extraction protocols. The best results were obtained by protocols 3 and 4 (figures 1-C and 1-D). Bands of moderate intensity were obtained by protocols 2, 5 and 6 (Figure 1-B, 1-E and 1-F). The lowest PCR quality was seen when using protocol (1) (figure 1-A). The faint band produced by protocol (1) could be attributed either to the low bacterial load of the tissue or the presence of inhibitors or impurities in the tissue residues.

Based on spectrophotometer measurement, the protocol number 3 using QIAamp Blood and Tissue Kit with prepared enzymatic lysis buffer as pretreatment in case of grown mycobacterial isolate (figure 1-C) had the highest DNA purity (table1) compared with the other five protocols. These results proved that the use of the QIAamp Blood and Tissue Kit (Qiagen) overcomes extraction problems.

The QIAamp Blood and Tissue Kit (Qiagen) depends on solid-phase purification, which gives quick and efficient purification compared to classical methods (Esser et al., 2005). As many problems that are associated with liquid phase extraction such as incomplete separation phase (Gjerse et al., 2009).

Solid phase system can absorb DNA during the process of extraction depending on the pH and salt concentration of the buffer. The absorption process is based on hydrogen-binding interaction with a hydrophilic matrix, ionic exchange and affinity and size exclusion mechanisms. As the desired nucleic acid can absorb to the column due to presence high concentration of pH and salt in the binding solution (Gjerse et al., 2009).

Solid-phase purification can be performed using a spin column, operated under centrifugation force with aid of washing and elution buffers that help in contaminant removal and release of desired DNA (Gjerse et al., 2009).

So this method is able to purify DNA producing high quality templates suitable for amplification rather than the conventional methods. The good amplification of PCR products can be attributed to the efficient elimination of unwanted inhibitors, as eukaryotic DNA or blood originated inhibitory substances such as hemoglobin, lactoferrin and different salts (Liebana et al., 1995; Ward et al., 1995; Zanini et al., 2001; and Araújo et al., 2005; Cardoso et al., 2009).

The highest A260/A280 ratio appeared in protocol number 3 Using of QIAamp Blood and Tissue Kit with prepared enzymatic lysis buffer as pretreatment in case of grown mycobacterial isolate) and lowest A260/A280 appeared in protocol 4 using of the lysis buffer in spite of the presence of concentration of extracted DNA, indicating the presence of impurities in the DNA. Which led to many nonspecific bands rather than the expected ones (figure 1-C, table 1).

Despite the high purity of DNA as in protocol numbers 1, 2 and 3 (Table 1), it is a possible that the presence of traces of impurities present in the extracted DNA blocked the amplification process (Liebana et al., 1995; Cardoso et al., 2009). So that, the effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of nucleases and away from contamination is needed for successful DNA purification (Doyle, 1996).

The quality and integrity of the extracted mycobacterial DNA will directly affect the PCR amplification quality (Cseke et al., 2004; Buckingham et al., 2007).

On the other hand, the application of the classical method such as lysis buffer and boiling can be used for the detection of mycobacterial DNA (figures 1-D and 1-E) although their low purity

and quality of DNA template (Table 3 and 4). They need optimization of heat condition to weak the mycobacterial cell wall, that heating is necessary for weakening the linkages between the lipid contents of mycobacterial cell wall, to release of chromosomal DNA in solution (Sjobring et al., 1990; Buck et al., 1992; Cormican et al., 1992; Fiss et al., 1992).

Further improvement of lysis could be achieved by use of suitable chemical as Triton X-100 (protocol 3 and 4) giving good yield in detection of Mycobacterial DNA (Table 2 and 3) (Banavaliker et al., 1998).

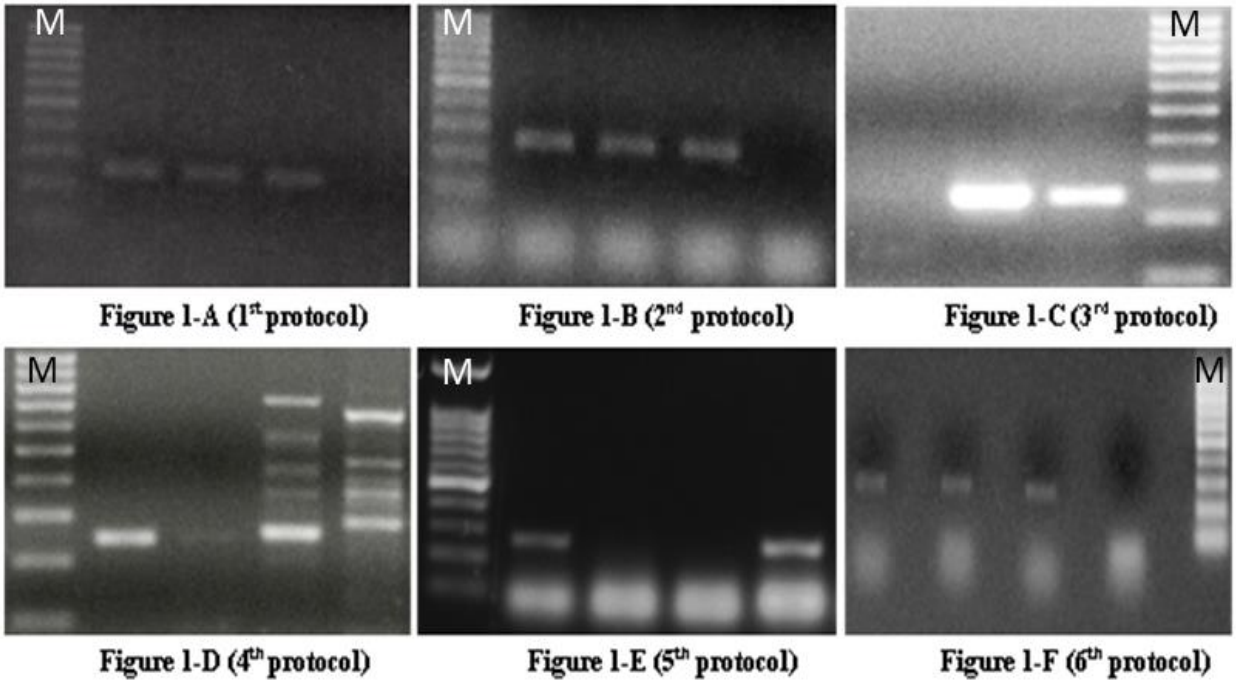

Fig. 1(A ~ F). PCR products obtained by partial amplification of *IS6110*. The amplicon size is 245 bp in various DNA extraction protocols. (1-A) faint band produced by protocol 1, (1-B, 1-E and 1-F, bands of moderate intensity were obtained by protocols 2, 5 and 6, (1-C) had the highest DNA purity, (1-D) indicate presence of multiple nonspecific bands rather than the expected ones obtained by protocol 4. M= 100 bp (Jena bio science®) DNA ladder.

Table (1) Results of DNA purification and the degree of band intensity on various extraction protocols.

Protocol NO.	A260/A280	Intensity of the band on agarose gel 1.5%
1	(1.5)	+
2	(1.85)	++
3	(1.92)	+++
4	(0.82)	+++
5	(0.85)	++
6	(1.42)	++

Table (2). PCR results of 13 known positive lymph node samples having occasional acid fast bacilli (AFB), using specific primer of the *IS6110* element that were subjected to DNA extraction using six different protocols.

Sample No	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Protocol 6
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	_	+	+	+	_
6	+	+	+	+	+	+
7	+	_	+	+	_	_
8	+	_	_	+	_	_
9	+	_	_	+	_	_
10	+	_	+	+	_	_
11	_	_	+	_	_	_
12	_	_	+	_	_	_
13	_	_	+	_	_	_

+: Possitive PCR product -: Negative PCR product

Table (3). Proportion of extraction efficiency to *IS6110* PCR in various protocols.

Protocol No.	PCR +ve (%)	PCR –ve (%)
1	62.5 %	37.5%
2	31.25 %	68.75 %
3	68.75 %	31.25 %
4	62.5%	37.5 %
5	37.5 %	62.5%
6	31.25 %	31.25 %

Table (4). Advantages and dis advantages of Various DNA Extraction protocols

Protocol No.	Quality	Amount of DNA
1	High	Low (100 µl).
2	High	Low (100 µl).
3	High	Low (100 µl).
4	Moderate	High (200 µl).
5	Poor	Moderate (150 µl).
6	High	Very low (40-50 µl).

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