

Qualitative Evaluation of Mycobacterial DNA Extraction Protocols for Polymerase Chain Reaction

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Abstract

We have compared the efficacy of various reported protocols of mycobacterial DNA extraction for detection of mycobacterial DNA by PCR assay. Seven DNA extraction protocols were tested for their quantitative as well as qualitative yield of mycobacterial DNA in 15 known positive sputum samples having occasional acid fast bacilli (AFB). DNA samples obtained by various methods were amplified in uniform standard conditions and analysed on 3% agarose gel. Protocol 6 and 7 showed 100% detection sensitivity with strong bands on agarose gel. Protocols 1-5 were found to be unsatisfactory because they yielded either low quantity or poor quality of DNA or were unable to remove inhibitors of DNA amplification. We conclude that a strong physical treatment, use of a detergent and enzyme for lysis, treatment with proteinase K, DNA purification step with or without phenol and DNA precipitation in ethanol or isopropanol are essential steps for extraction of mycobacterial DNA from clinical samples. Protocol 6 is standard in our laboratory and we have found reproducible results with this method. Purification with phenol followed by chloroform treatment was not found to have any inhibitory effect on amplification. A more extensive evaluation of this protocol in samples with lower bacterial load may be necessary.

Introduction

Despite promising results of numerous published reports, the practical application of Polymerase Chain Reaction (PCR) in a clinical laboratory setting for routine diagnosis is impeded by problems of contamination and complex procedures required for sample preparation, DNA extraction and amplification methods. The sensitivity of PCR is largely dependent on the efficiency of the DNA extraction procedure. Because of complex structure and impermeability of the cell wall, lysis of mycobacterial cells is difficult. Therefore 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. Various studies with different clinical specimens have reported a wide range

of sensitivity and specificity of the PCR technique for diagnostic purposes. The DNA extraction protocol (lysis method of mycobacterium and DNA extraction procedure), the target nucleic acid sequence, selection of primer pair, detection systems of amplified products and the number and type of clinical samples used differ from one another, making the reported sensitivity and specificity difficult to compare (Boddinghaus *et al.*, 1990; Theyry *et al.*, 1990; Kaneko *et al.*, 1990; Fries *et al.*, 1991; Piere *et al.*, 1991 and Kolk *et al.*, 1992). Most of the methods are difficult, time consuming and costly. To the best of our knowledge, there is no published report of comparative evaluation of mycobacterial DNA extraction protocols.

Hence, the present study was undertaken to compare seven reported protocols of mycobacterial DNA extraction protocols reported from time to time by various workers, including one standardised in our laboratory. We noted a marked difference in quantity as well as quality of DNA extracted by various methods.

Results and Discussion

In order to test the sensitivity of *in vitro* amplification of mycobacterial DNA with primers based on detection of IS6110 sequence, different concentrations of *M. tuberculosis* DNA (0.5 pg to 1.0 fg) were amplified. The results presented in Figure 1 show that these primers enable detection up to 10 fg of DNA on ethidium bromide stained agarose gels. This amount of DNA corresponds to about three genomic equivalents (Theyry *et al.*, 1990). Various other workers (Boddinghaus *et al.*, 1990; Theyry *et al.*, 1990) reported similar results. According to their findings, the sensitivity was found to increase when specific probes were used for detection.

It was found that protocols 1, 2, 4 and 5 yielded low quantity of DNA compared to protocols 3, 6 and 7 (Table 1). To check the reproducibility all the experiments were run in triplicate and values were found to be comparable. Mean absorbance of three experiments as noted in all the protocols is shown in Table 1.

10 fg of the standard strain DNA extracted by all seven protocols was used for amplification by PCR in standard uniform conditions. A sharp band of 123 bp was detected in lanes with PCR product using DNA template extracted by protocols 3, 4, 6 and 7. PCR products of DNA templates extracted by protocols 1, 2 and 5 showed a faint band (Figure 2) in ethidium bromide stained agarose gel.

The quality of the DNA extracted for PCR analysis was also 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 were able to detect only a faint band on agarose gels. Protocols 3 and 4 show a poor A260/A280 ratio in spite of high concentration of extracted DNA, signifying the presence of impurities in the DNA. No

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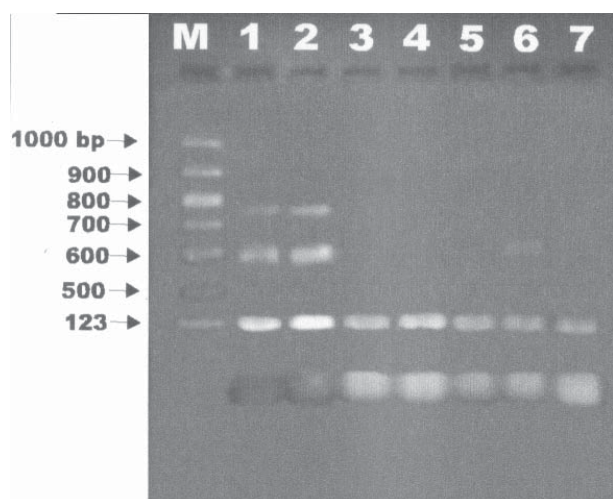


Figure 1. Sensitivity of *M. tuberculosis* DNA detection by PCR. Dilutions of *M. tuberculosis* DNA were amplified with primer pair based on detection of IS6110 sequence to amplify a 123 bp fragment. Lanes 1-7; template DNA concentrations 10ng, 1ng, 100 pg, 10pg, 1pg, 100 fg and 10 fg, Lane M-molecular weight marker.

purification steps were used in these protocols. Protocols 6 and 7 yielded pure DNA (Table 1). There is a possibility that the light intensity (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.

DNA samples extracted from all the sputa by all the methods were subjected to amplification by PCR in standard uniform conditions. Amplified products were analysed on ethidium bromide stained agarose gel. It was found that 6 (40%) samples in protocol 1, 5 (33.4%) samples in protocol 2, 3 (20%) samples in protocol 4 and 4(26.7%) samples in protocol 5 showed a faint band on the agarose gel. Protocol numbers 6 and 7 showed a strong band in 100% samples. In protocol 3, 20% (3) samples showed negative result. On testing, some unknown inhibitor(s) were found to be present in these three samples (Table 2).

Successful detection of DNA by amplification methods depends on purity and quality of DNA template. Our results indicate that successful extraction of mycobacterial DNA from sputum samples need each of the following steps:

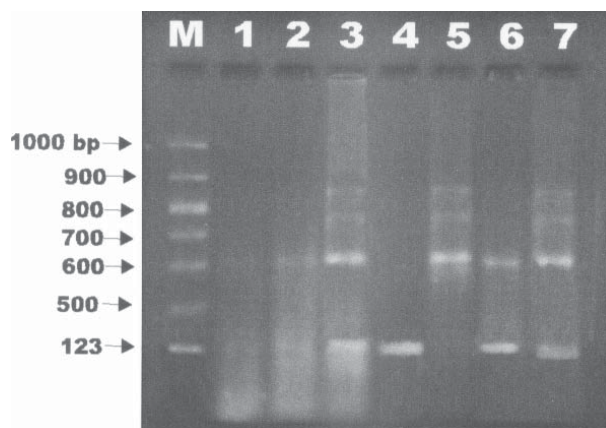


Figure 2. Amplified product of *M. tuberculosis* DNA detected by various protocols. Lanes 1-7; Amplified product of DNA extracted by protocols 1-7 respectively, Lane M-molecular weight marker.

1. Harsh physical treatment to weaken the mycobacterial cell wall
2. Chemical treatment to lyse the mycobacterial cell wall
3. DNA purification steps including treatment with proteinase K to remove proteins
4. DNA precipitation from solution

Harsher physical treatments like freezing at -20°C and boiling of bacterial suspension for 10 min in suitable buffer were helpful in extraction of mycobacterial DNA. Protocol 1 does not include heating or freezing the samples prior to lysis. Low DNA yield from H37Rv and a faint band in 40% AFB positive sputa were noted for this protocol (Tables 1 and 2). Heating is necessary for weakening the linkages between the lipid contents of mycobacterial cell wall, resulting in release of chromosomal DNA in solution. Sjobring *et al.* (1990) and Buck *et al.* (1992) reported that combination of sonication and boiling was found to be suitable for achieving lysis. However, cell disruption by sonication is impractical in clinical laboratories. A few other studies suggested that heating the sample to 100°C in a suitable buffer could adequately extract mycobacterial DNA for use in PCR (Fiss *et al.*, 1992; Cormican *et al.*, 1992). Freezing at -20°C was not used in protocols 2 and 6 also. Strong bands in all samples were noted in protocol 6.

Table 1. Quantitative Analysis of Mycobacterial DNA Extracted by Various Protocols

Protocol No.	Mean absorbance at 260 nm*	Concentration of DNA obtained/ml	Purity of DNA A260/A280	PCR results (intensity of band)
1.	0.003	72 μg	1.50	Faint
2.	0.006	144 μg	1.50	Faint
3.	0.019	456 μg	0.82	Strong
4.	0.013	312 μg	0.64	Strong
5.	0.012	288 μg	1.70	Faint
6.	0.024	576 μg	1.84	Strong
7.	0.017	408 μg	1.70	Strong

* values are mean of three readings.

Table 2. Results of PCR Analysis in Sputum Samples

Protocol No	PCR Positive (%)	Very faint or unclear band (%)	PCR Negative (%)
1.	9 (60)	6 (40)	NIL
2.	10 (66.60)	5 (33.41)	NIL
3.	12 (80)	NIL	3 (20)*
4.	12 (80)	3 (20)	NIL
5.	11 (73.30)	4 (26.71)	NIL
6.	15 (100)	NIL	NIL
7.	15 (100)	NIL	NIL

*These three samples contain PCR inhibitors

Freezing can be an optional step, but we have found satisfactory results without freezing. Some other workers omitted the freezing and have reported sensitivity ranging from 86% to 97% (Clarridge *et al.*, 1993; Querol *et al.*, 1995).

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 mycobacterial cell wall was found to be essential by most of the workers (Baes *et al.*, 1974; Banavaliker *et al.*, 1998). Methods included in our study used either sodium dodecyl sulphate (SDS) or Triton X-100. Protocols 6 and 7 used triton X-100 and SDS respectively. Both the methods reported good yield and 100% detection of mycobacterial DNA. Hence, use of any one of detergents does not interfere with the results.

Lysozyme is a frequently used enzyme in the lysis buffer of various reported methods for the complete lysis of mycobacterial cell wall. Protocols 2, 3 and 6 did not use lysozyme for cell wall digestion but 3 and 6 gave a good DNA yield. Regarding detection of DNA from clinical samples, protocol 6 had detected 100% of the samples. Therefore, use of lysozyme also depends on the choice of worker. Our protocol (6) does not include lysozyme in lysis buffer, but both quality and quantity of DNA is good

DNA purification is one of the most important steps to remove inhibitors from the sputum samples. Protocol 3 did not use any purification and precipitation step. Though quantity of DNA extracted by this method was high, the presence of inhibitors in 20% of the clinical samples was noted. Boiling the bacterial pellet in lysis buffer could be an easy and economical solution for extracting DNA from culture, but for clinical samples this is not recommended. Use of proteinase K was found to be helpful in removing DNA bound proteins resulting in improvement in quality of template DNA. Protocol 2 and 5 did not use proteinase K. Both protocols reported poor quality and quantity of DNA resulting in faint bands in a number of the samples, although they used all other steps used by protocols 6 and 7.

Extraction of DNA using phenol:chloroform:iso-amyl alcohol improved the quantity as well as the quality of target DNA. Every protocol used in our study except 3 and 4 used phenol and/or chloroform for extracting DNA. Results with protocol 3 and 4 were not found to be satisfactory. Noordhoek *et al.* (1995) observed that phenol extraction of DNA removes inhibiting substances from those samples in which inhibitors were present even after DNA extraction with guanidinium thiocyanate (GuSCN) and silica particles. Querol *et al.* (1995) achieved 97% PCR positivity by using

phenol:chloroform:iso amyl alcohol extraction followed by isopropanol precipitation of DNA. Similarly, some workers (Sjobring *et al.*, 1990; Boddington *et al.*, 1990) reported that the use of phenol and chloroform for extraction and ethanol and/or isopropanol for precipitation of DNA surely improves the yield of the purified target DNA, which finally results in increased sensitivity of PCR. However Brisson-Noel *et al.* (1991) estimated that 5% of clinical specimens contain some inhibiting component(s), which are not removed by phenol extraction of DNA. Protocol 7 did not use phenol at all but gave good yield.

Removal of residual phenol is necessary because it may act as a potent inhibitor of Taq polymerase (Clarridge *et al.*, 1993). An additional step of extracting DNA with chloroform was found to be helpful in removing residual phenol in our protocol 6. Chloroform is also helpful in denaturation of proteins and facilitates the separation of aqueous and organic phases. The role of the extraction step in removal of various unknown inhibitors of Taq polymerase is also well established. We did not attempt to identify the nature of inhibitors, but it has been suggested that substances such as phenol, heparin, haemoglobin and SDS may be potent inhibitors of PCR (Gelfand *et al.*, 1989; Pannaccio *et al.*, 1991; Holodny *et al.*, 1991).

Precipitation of DNA by ethanol or isopropanol was found to be another alternative approach for removal of inhibitors from the target DNA. Ethanol precipitates the DNA and RNA while isopropanol selectively precipitates DNA leaving RNA and polysaccharides in the solution. Protocols 6 and 7 use ethanol and isopropanol respectively. We found comparable results by both methods. No doubt ethanol plays a vital role in DNA precipitation, but special care has to be paid to the removal of ethanol after precipitation. It has been reported that any residual ethanol may hinder the polymerase chain reaction as an inhibitor.

Thus, we suggest that use of both physical and chemical steps for cell lysis, use of proteinase K and phenol:chloroform for DNA purification and ethanol or isopropanol precipitation, are essential steps for extraction of mycobacterial DNA from clinical samples. Methods lacking in one or more steps (1, 2, 3, 4 and 5) are not found to be useful for clinical use. Rest of the methods (6 and 7) need to be evaluated at a larger level especially in samples with lower bacterial load.

Table 3. Summary of Various DNA Extraction Protocols

Protocol No.	Physical Treatment		Chemical Lysis				DNA Purification		DNA precipitation		
	-20*	100^	SDS	TX	LZ	PK	Phel	Chlor	IAA	ETH	IP
1	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No
2	No	Yes	Yes	No	No	No	Yes	Yes	No	Yes	No
3	Yes	Yes	No	Yes	No	No	No	No	No	No	No
4	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes
5	Yes	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	No
6	No	Yes	No	Yes	No	Yes	Yes	Yes	No	Yes	No
7	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes

* 30 min. at -20°C

^10 min. at 100°C

SDS (sodium dodecyl sulphate), TX (triton X-100), LZ (lysozyme), PK (proteinase K), Phe (Phenol), Chlor (Chloroform), IAA (iso-amyl alcohol), ETH (ethanol), IP (isopropanol),

Experimental Procedures

Sample Preparation

Fifteen sputum samples which were AFB positive (2-9 bacilli/smear) were randomly selected from samples referred to Department of Microbiology, King George's Medical College, Lucknow. These samples were culture positive on LJ media. Growth was identified as *M. tuberculosis*. Saliva samples from ten healthy persons were included as negative controls.

Preparation of Clinical Samples for DNA Extraction

A total of 3 ml of all positive and negative samples were first treated with equal volume of 4% NaOH at 37°C for 30 min with intermittent vortexing. Samples were then centrifuged at 6000 g and pellets were washed twice with sterile distilled water by centrifugation (Vestal *et al.*, 1977). The pellet was suspended in 1.5 ml of TE buffer to make a homogenous suspension and finally was equally distributed in seven sterile tube (200 µl each) for DNA extraction by seven different protocols.

In order to avoid contamination, samples were processed in a separate bio-safety cabinet, which was not used for DNA amplification or detection. All the plastic wares used for DNA extraction were DNase free, disposable and not reused throughout the experiment. Different sets of micro-pipettes were used at each step i.e. for sample processing, DNA extraction, PCR mix preparation and electrophoresis.

Methods of DNA Extraction

Seven protocols were used for extracting DNA according to previous published reports including protocol number six, which is standardised in our laboratory. This protocol is a combination of various steps already reported. A comparison of all seven protocols is given in Table 3.

Protocol 1

Sample pellet was dispersed in 10 mM Tris-1mM EDTA buffer containing 0.1 % Tween 80 and 2 mg/ml lysozyme. The tube was incubated for 2 hr at 37°C with intermittent shaking and centrifuged. The pellet was lysed by re-dissolving in TE buffer containing 100µg of Proteinase K/ml and 1% (w/v) sodium dodecyl sulphate and incubated for 1 hr at 37°C. DNA was extracted by adding an equal volume of TE saturated phenol: chloroform: iso-amyl alcohol (25:24:1 v/v/v). The aqueous phase was transferred to another tube and 0.1 volume of cold 3 M sodium acetate (pH 5.2) was added. Sample was mixed by inversion and placed on ice for 10 min before centrifugation for 10 min. The supernatant fluid was transferred to another tube and DNA was precipitated by the addition of 20 µg of acrylamide/ml, 0.05 volumes of 3M sodium acetate and 2.5 volume of ethanol, washed, dried, dissolved in 25 µl sterile triple distilled water (Boddinghaus *et al.*, 1990).

Protocol 2

Sample deposit was incubated with 0.1 M sodium hydroxide containing 2 M sodium chloride and 0.5 % sodium dodecyl sulphate for 15 min at 95°C. Sample was extracted twice with phenol: chloroform (24:1), after which the DNA was precipitated with ethanol and dissolved in 25 µl of sterile triple glass distilled water (Brisson-Noel *et al.*, 1989).

Protocol 3

NaOH treated centrifuged deposit of the sample was suspended in 100 µl of sample buffer consisting of 10 mM Tris (pH 8.0), 1 mM EDTA and 1 % Triton X-100 (TET) and the mixture stored at -20°C. The specimen was thawed and then placed in heat block at 100°C for 30 minutes. The lysate was centrifuged for 2 minutes to pellet debris and 5-10 µl of the supernatant was directly used for the PCR assay (Nottle *et al.*, 1993).

Table 4. Merits and Demerits of Various DNA Extraction Protocols

Protocol No.	Merits and Demerits	Remark
1	Quality and quantity of DNA poor	UNSATISFACTORY
2	Quality and quantity of DNA poor	UNSATISFACTORY
3	Quantity of DNA good, Quality of DNA poor,	UNSATISFACTORY
4	Quantity of DNA good, Quality of DNA poor,	UNSATISFACTORY
5	Quantity of DNA good, Quality of DNA poor,	UNSATISFACTORY
6	100% positivity. Good quality and quantity of DNA	SATISFACTORY
7	100% positivity. Good quality and quantity of DNA	SATISFACTORY

Protocol 4

Clinical sample in TE buffer was subjected to freeze thaw cycles (30 min at -70°C, 10 min at 100°C), followed by treatment with lysozyme (40µg/ml), sodium dodecyl sulphate (0.05%) and proteinase K (50mg/ml) at 37°C for 2hr. DNA was precipitated by 0.6% (v/v) of isopropanol and pellet was air dried and suspended in 25 µl of sterile distilled water for PCR analysis (Banvaliker *et al.*, 1998).

Protocol 5

Decontaminated pellet of clinical sample was suspended in 200µl of Tris-EDTA buffer (50mM Tris and 5mM EDTA pH 8.0) and frozen at -20°C. Frozen aliquot was digested with lysozyme (1 mg/ml) at 37°C for 2hr. Specimen was lysed by heating at 95°C with an alkaline sodium dodecyl sulphate solution (10% w/v), followed by neutralisation with Tris-HCl buffer. Finally DNA was extracted once with phenol: chloroform (24:1) and then precipitated with 2.5 volume of ethanol. DNA pellet was suspended in 25µl of sterile distilled water for PCR analysis (Yuen *et al.*, 1993).

Protocol 6

200µl of pre-treated sample in TE buffer was placed in boiling water bath at 100°C for 10 min. It was followed by incubation at 56°C for 2-3 hr after addition of equal amount of lysis buffer (Tris 10mM, EDTA 2mM, NaCl 0.4M and Triton X-100 0.5%) (pH 8.0) and 10µl of Proteinase K (10mg/ml). The sample was then vortexed and boiled at 100°C for 10 minutes to inactivate proteinase K. DNA purification was done by addition of equal volume of Phenol: Chloroform (24:1) followed by chloroform only. The aqueous phase was finally transferred in 2.5 volume of chilled ethanol and sodium acetate (0.3M final conc.) was added. Tubes were kept at -20°C overnight. The sample was centrifuged at 10,000 rpm for 10 minutes and the DNA pellet was washed with 70% chilled ethanol by centrifugation. The pellet was allowed to air dry and finally suspended into 25µl of T.D.W. (sterile) for PCR analysis.

Protocol 7

To 200µl of pre-treated sample, 200µl of TE buffer (10mM Tris, 1mM EDTA, pH 8.0) was added and boiled at 85-90°C for 10 minutes and immediately frozen at -20°C for 15 minutes. Lysozyme 40µl (20mg/ml) was added and vortexed before incubation at 37°C for 2hr. The bacterial

membrane was disrupted by increasing the temperature to 65°C and adding Proteinase K and SDS to a final concentration of 250 µg/ml and 1% respectively for 30 minutes with shaking. A mixture of CETAB and NaCl (CETAB 10%; NaCl 0.7M) was added to a final concentration of 1%. Finally, the suspension was incubated once more at 65°C for 20 minutes. DNA was extracted with chloroform: iso-amyl alcohol (24:1) precipitated with 0.6 volumes of isopropanol (Baes *et al.*, 1974).

Quantitation of Mycobacterial DNA

DNA from equal numbers of *M. tuberculosis* (H37Rv) organisms was extracted by all the seven protocols in triplicate to check the reproducibility of the procedure. Five large colonies of *M. tuberculosis* were suspended in 5 ml of TE buffer after washing twice with sterile distilled water. A homogenous suspension was made by vortexing this suspension for 5 min after addition of six sterile glass beads. The tube was allowed to stand for 5 min. allowing the larger 'clumps' to settle. Then 200 µl of this suspension was distributed into 21 tubes, followed by extraction by seven protocols in triplicate. Quantitation of DNA was done spectrophotometrically by measuring absorbance at 260 nm for comparative quantitative analysis of DNA (Sambrook *et al.*, 1989).

Serial dilutions of DNA extracted from all protocols were prepared ranging from 0.5 µg to 1.0 fg. All of these dilutions were amplified to compare the quality as well as to find out the minimum amplifiable quantity of DNA.

Purity Check 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. If there is a contamination with protein or phenol the ratio is significantly lower (Maniatis *et al.*, 1989).

Amplification of Mycobacterial DNA by PCR

DNA amplification reaction was performed in a total volume of 20µl in 10mM Tris-HCl (pH8.3), 1.5mM MgCl₂, 50mM KCl, 50 pico moles of each primer (5'- CCTGCGAGCGT AGGC GTCGG and 5'- CTCGTCCAGCGCCGCTTCGG, Bangalore Genei, India), 0.25 mM of each deoxy nucleoside triphosphate (dATP, dGTP, dTTP, and dCTP)

and 0.5 unit of *Thermus aquaticus* DNA polymerase (B. Genei). 2 µl of extracted DNA was added to 20 µl of PCR mixture with a positive displacement pipette. Amplification mixtures were subjected to 40 cycles of amplification in an automated thermal cycler (TECHNE, Progene). Amplification cycles were as follows: incubation for 5 minutes at 94°C for initial denaturation, followed by 40 cycles of denaturation, annealing and polymerisation (94°C for one minute, 60°C for one minute, and 72°C for one minute respectively). Seven minutes of final extension at 72°C was done.

Each set of amplification tubes included a positive control of *M. tuberculosis* (H37Rv) DNA as well as a negative control of DNA samples extracted from saliva of normal healthy persons.

Analysis of PCR Products

An aliquot (5µl) from PCR product of each sample was analysed by gel electrophoresis in 3% agarose gels at constant voltage (30V), along with the molecular weight marker and PCR products of positive and negative controls. A sharp band of 123 bp of amplified DNA was visualised under UV light in positive samples by staining the gels with ethidium bromide (0.5 µg/ml, Sigma) for 15 minutes and de-staining with distilled water. The presence of a distinct band of 123 bp (IS 610) was considered as a positive PCR result for *M. tuberculosis* DNA.

Evaluation of PCR Inhibitors

All clinical samples, which were found negative by PCR, were also examined for the presence of PCR inhibitors. This was achieved by performing a second PCR with DNA from the clinical samples under the conditions described above, but with the addition of known template and primers. Detection of the product was performed by agarose gel electrophoresis.

Acknowledgements

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