PCR BASED DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* : EFFECT OF SAMPLE PREPARATION

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Abstract. Tests based on the polymerase chain reaction (PCR) for the detection of the *Mycobacterium tuberculosis* complex in clinical samples have a lower sensitivity when compared to culture. This has been attributed to the presence of inhibitors to *Taq* polymerase and/or suboptimal DNA extraction procedures. We tested different methods of processing smear negative culture positive sputum (n = 52) using different detergents, including nonidet P-40 (NP-40), sodium dodecyl sulphate (SDS), tween 20, triton X 100 and N-lauryl sarcosine. The detergents were used in combination with lysozyme and proteinase K enzymes. NP-40 was significantly better than SDS, tween 20 and N lauryl sarcosine ($p < 0.05$). When NP-40 was used as the detergent, 42 out of 52 specimens gave positive results with the standard amplification protocol which amplifies a 245 bp sequence of the insertion element IS 986. The 10 specimens that were negative were further diluted ten fold and/or eluted in sephadex G-50 columns before standard DNA amplification. A further 8 specimens then became positive. Elution in sephadex G-50 was better than ten fold dilution in processing of samples. The two negative samples had very low colony counts ($n < 5$). The study demonstrates that the sensitivity of the PCR is dependent on the sample preparation technique and the amount of target sequence available for amplification.

INTRODUCTION

Tuberculosis is still a major health problem in many parts of the world (WHO, 1990). Early diagnosis of infection and contact tracing are the major strategies of control programs. A presumptive diagnosis of tuberculosis can be made on the basis of clinical history, physical examination, radiological findings and on the presence of acid fast bacilli in clinical specimens. A definitive diagnosis of tuberculosis is made by isolation of Mycobacterium species of the *Mycobacterium tuberculosis* complex.

Although microscopic examination of smears of acid fast bacteria by Ziehl-Neelsen is currently the most rapid method for detection of mycobacteria, it is insensitive and non specific. Isolation of the organism by culture and subsequent identification by biochemical tests is more specific and sensitive but is laborious and time consuming, requiring around 4-8 weeks. Application of immunological methods for detection of the organism has limited value due to poor sensitivity and/or specificity (Daniel and Debanne, 1987; Kadival *et al*, 1987). More rapid identification techniques such as the BACTEC detection system (Peterson *et al*, 1989) and the Gen-Probe identification system (Gonzalez and Hanna, 1978) reduce identification time, but they still require at least a one week culturing period to obtain sufficient numbers of organisms for accurate identification. Therefore these methods are not suitable and sensitive enough for direct detection of pathogenic mycobacteria in clinical samples.

In an effort to overcome these limitations in the diagnosis of tuberculosis, procedures based on the amplification of mycobacterial DNA by use of polymerase chain reaction (PCR) have been developed recently (Brisson - Noel *et al*, 1989; Sjobring *et al*, 1990; Thierry *et al*, 1990; Hermans *et al*, 1990a; Pao *et al*, 1990). These studies have shown that this procedure can be used for the rapid detection of *M. tuberculosis* DNA in clinical specimens.

However the sensitivity of the technique was not found to be as high as theoretically expected (Pao *et al*, 1990; Sjobring *et al*, 1990; Thierry *et al*, 1990; Hermans *et al*, 1990b). This is especially noticeable in the presence of clinical material like sputum and when low numbers of mycobacteria are present in the specimens. The suboptimal sensitivity of the test has been attributed to the presence of substances in clinical specimens that inhibit *Taq* polymerase (Pierre *et al*, 1991) and suboptimal DNA extraction procedures which do not permit the detection of small numbers of mycobacteria (Hermans *et al*, 1990b).

The sensitivity of detection of mycobacteria in clinical samples containing only small numbers of

organisms by standard bacteriological techniques and those based on amplification of mycobacterial DNA have been compared only in relatively few studies. The present study was therefore conducted in order to increase the sensitivity of detection of *M. tuberculosis* from clinical samples when techniques based on DNA amplification are used and to compare such results with those of culture. This was carried out using different sample preparation techniques with a variety of detergents to optimize DNA extraction from *M. tuberculosis* and to reduce the effect of or to remove any inhibitory substances.

MATERIALS AND METHODS

Clinical specimens

52 smear negative culture confirmed specimens of sputum obtained from patients who attended the Central Chest Clinic, Colombo for suspected pulmonary tuberculosis were used as the study material. In our hands direct smear test has 100% sensitivity for sputum specimens that contain > 10,000 organisms/ml.

Bacterial strain and isolation of DNA

Mycobacterial DNA was prepared from *M. tuberculosis* H37Rv strain grown in tween-albumin medium as described previously (Hermans *et al*, 1990b).

Decontamination of sputum specimens

The sputum was concentrated before culture as follows. To 4 ml of sputum obtained in a 50 ml conical centrifuge tube was added an equal volume of a solution containing a mixture of 2% sodium hydroxide, 1.45% sodium citrate and 0.05% N-acetyl L-cysteine. Following vortexing (15 seconds) the specimen was left at room temperature for 15 minutes in a shaker (100 rpm). The centrifuge tube was then filled with sterile distilled water and centrifuged (10,000g/15 minutes/ 0°C). The supernatant was discarded and the pellet resuspended in sterile distilled water and centrifugation was repeated as before. The pellet was then resuspended in 1.0 ml (0.25 volume of the original sputum sample) of distilled water. 250 µl was used for culture on Lowenstein - Jensen medium and the rest was used for different treatment procedures prior to DNA amplification.

Treatment of sputum for PCR

To five 50 µl aliquots of the processed sample were added freshly prepared lyzozyme (1mg/ml) in TE pH 8.0, (TE : 10 mM Tris pH 8.0, 1mM EDTA pH 8.0) and incubated for 1 hour at 37°C. Following lysozyme treatment each specimen was then reacted with 5 different detergents separately in combination with proteinase K (2 mg/ml) at 55°C for 1 hour in order to lyse the organisms and release the DNA. The detergents used were 0.5% tween 20, 1.0% sodium dodecyl sulphate (SDS), 3.5% N-lauryl sarcosine, 0.45% nonidet P-40 (NP-40) and 1.0% triton X-100 in final concentrations. Subsequently proteinase K was inactivated by heating the sample at 95°C for 10 minutes. A 10 µl aliquot of the resultant DNA detergent mixture was directly used for DNA amplification. A 50 µl aliquot of the remaining processed sample was subjected to conventional phenol/chloroform DNA extraction and ethanol precipitation (Manniatis *et al*, 1989) prior to DNA amplification.

Amplification and detection of mycobacterial DNA

The amplification of mycobacterial DNA was performed as previously described (Kolk *et al*, 1992). The final reaction mixture (50 µl) contained 1 unit of Taq polymerase (Perkin Elmer Corp, Norwalk Conn, USA) 50mM KCl, 1.5mM $MgCl_2$, 10mM Tris HCl (pH 8.3), 0.01% (wt/vol) gelatin, 0.2mM each of dGTP, dATP, dTTP, dCTP, 0.4 µM of each primer and 10 µl of sample. The oligonucleotide primers used were based on the repetitive insertion sequence IS 986 which amplifies a 245 base pair fragment. The PCR mixes prepared as above were stored in 40 µl aliquots in reaction vials and left at –20°C. A 10 µl aliquot of the processed sample was added to each vial and topped with 50 µl of mineral oil (Sigma, St Louis, MO, USA).

DNA was amplified in an automated thermocycler (Perkin Elmer Corp, Norwalk Conn, USA) for 40 cycles using the following parameters, denaturation at 94°C for 2 minutes, annealing at 65°C for 2 minutes and primer extension at 72°C for 3 minutes.

The presence of amplified mycobacterial DNA sequences was detected by agarose gel electrophoresis as described (Manniatis *et al*, 1989). Briefly aliquots of the amplified products were electrophoresed in 2% agarose gels containing 0.5 µg/ml ethidium bromide. An amplified fragment of approximately 245 bp was considered a positive result.

Processing of negative samples

The samples that gave negative results by above methods were processed further using two methods in order to remove (if any) inhibitory substances that may be present. An aliquot of the enzyme/NP-40 detergent processed sample was diluted ten fold and 10 µl was used for DNA amplification as described before. A second aliquot of the enzyme/NP-40 detergent mixture was diluted by the addition of 150 µl TE. The diluted sample was then purified by spun column chromatography (Manniatis *et al*, 1989) in a column prepared in a 2.5 ml disposable syringe containing G-50 pre equilibrated in TE (pH 8.0). An aliquot (10 µl) of the 200 µl eluate obtained after centrifugation (2,000 rpm/ 10 minutes) was used for DNA amplification as described earlier.

RESULTS

Detection of mycobacterial DNA by standard amplification

Table 1 summarizes the results obtained when PCR was used to amplify target DNA isolated by the various processing methods. The 245 bp amplified fragment was detected in 42 out of 52 sputum samples when the detergent used was NP-40. 38 samples gave positive results when the detergent used was triton X-100. As expected, when the sample was treated with SDS and N-lauryl sarcosine which are ionic detergents the amplification reaction was inhibited when DNA detergent mixture was directly used for DNA amplification without phenol/chloroform extraction. Fig 1 shows the results of a representative sample. Phenol chloroform extraction and ethanol precipitation of DNA from samples produced a lower number of positives. General yield of the amplified DNA was greater when the amplification was carried out without phenol chloroform extraction as determined by the intensity of the band when the amplified sample in the agarose gel was examined under UV transillumination.

Table 1

Detection of *M. tuberculosis* by DNA amplification after treatment of samples (n = 52) with different detergents.

Detergent	PCR on DNA detergent mixture (%)	PCR after Phenol chloroform extraction of DNA detergent mixture (%)
NP-40	42 (80.7)	38 (73.0)
Triton X-100	38 (73.0)	37 (71.0)
SDS	00 (00.0)	41 (78.8)
Tween 20	25 (48.0)	35 (67.3)
N-lauryl sarcosine	00 (00.0)	35 (67.3)

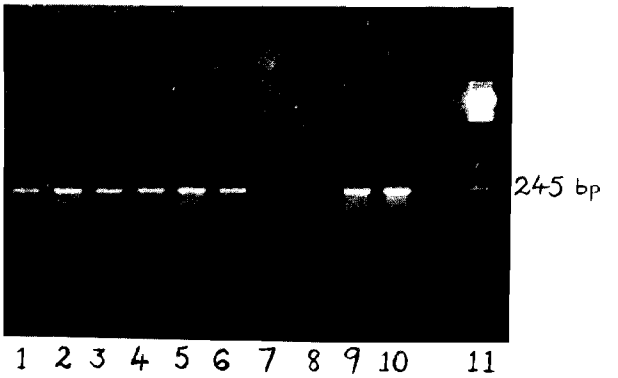

Fig 1–Agarose gel electrophoresis of the amplified product of a representative sample processed using different detergents. Lanes 1 and 6 – 0.5% tween 20, 2 and 7 – 1.0% SDS, 3 and 8 – 3.5% N lauryl sarcosine, 4 and 9 – 0.45% NP-40, 5 and 10 Triton X 100, 11 - DNA/ *Hin* d III, Lanes 1 to 5 contain the phenol/chloroform extracted sample after processing with different detergents.

Evaluation of techniques using dilution and elution in G-50 sephadex columns

The ten specimens that gave negative results (when processed by NP-40) was used for further evaluation. 3/10 produced positive results after a ten-fold dilution before DNA amplification; 6/10 specimens gave a positive result after elution in sephadex G-50 columns. One additional specimen became positive when both methods were combined. There were two specimens which were consistently negative by any of the methods although the culture results from these two specimens were confirmed as *M. tuberculosis.* In these two samples the number of colonies detected from 1 ml of sputum was less then five.

DISCUSSION

We examined the feasibility of using PCR for detection of low numbers of organisms in sputum by

modification of the standard protocol used for preparation of clinical material before PCR. Therefore, as material we used sputum samples obtained from patients which were smear negative but culture positive for *M. tuberculosis*. Therefore these samples had less 10,000 mycobacterial cells per ml of sputum. The low sensitivity of detecting *M. tuberculosis* by the standard amplification procedures have been attributed to several factors. Failure to efficiently extract mycobacterial DNA from sputum have been reported by some workers (Sjobring *et al*, 1990). We have found that this could be overcome by using the appropriate detergents and enzymes in combination.

We have tested different detergents that could be used for processing of clinical material before carrying out DNA amplification on clinical material obtained from patients confirmed as having pulmonary tuberculosis due to *M. tuberculosis*. Out of the detergents tested NP40 gave significantly better results than SDS ($p < 0.001$), tween 20 ($p < 0.05$) and N -lauryl sarcosine ($p < 0.001$) when specimens were directly used for DNA amplification without phenol extraction and ethanol precipitation of mycobacterium DNA. Therefore for sample preparation NP-40 in combination with proteinase K and lysozyme appear to be better than conventionally used detergents such as SDS and tween 20. Treatment of samples with triton X-100 gave results similar to NP-40, although it was technically difficult to use due to its tendency to produce froth during pipetting and mixing.

When the procedure was combined with phenol extraction and ethanol precipitation of DNA, the results appeared to be less satisfactory. There was no statistically significant difference ($p > 0.50$) between the different detergents when the procedure was combined with phenol extraction and ethanol precipitation.

The advantage of the use of NP-40 is that it does not inhibit *Taq* polymerase and therefore the DNA detergent mixture could be used directly for amplification without further purification of mycobacterial DNA by phenol chloroform extraction. Other workers have used SDS which is a strong detergent but a potent inhibitor of *Taq* polymerase. Such methods therefore require careful purificaion of mycobacterial DNA prior to amplification.

Another factor that may be responsible for suboptimal performance of PCR has been attributed to the presence of inhibitor(s) to *Taq* polymerase in some clinical material (Pierre *et al*, 1991). Though dilution of samples could reduce the inhibitory effect, it also may significantly reduce the target molecules in specimens containing low numbers of organisms. Elution of samples using G-50 sephadex columns produced positive results in samples where the standard amplification protocol gave negative results. Elution through sephadex G-50 appeared to be better than dilution of the sample to remove inhibitors, although the difference was not statistically significant ($p > 0.05$).

There were two samples (3.8%) that were consistently negative. Other factors that may have been responsible for the negative results could be the presence of low copy numbers of target sequence or the complete absence of the target sequence IS 986 as recently reported for some isolates (Kolk, personal communication). The two negative samples had a very low colony count on culture (Table 2). It has been found using restriction fragment length polymorphism studies that certain strains of *M. tuberculosis* had only a single copy of the target sequence, IS 986. In a study currently underway 2 out of 20 isolates of *M. tuberculosis* from Sri Lanka had only a single copy of the target molecule IS 986. When the number of target molecules are few, non-specific amplification products (primer dimers) are generated which may compete with the low numbers of target molecules thereby decreasing the sensitivity of the assay.

Table 2

PCR results of negative samples (n = 10) by the standard amplification when processed by ten fold dilution and elution in sephadex G-50 columns.

Specimen	Dilution	Sephadex G-50	Dilution & Sephadex G-50	Culture*
1	+	+	+	++
2	–	+	+	++++
3	+	–	+	+
4	+	+	+	++
5	–	+	+	+++
6	–	+	+	++
7	–	–	–	+
8	–	–	–	+
9	–	+	+	++
10	–	–	+	+

* Culture + ≤ 5 colonies, ++ > 6-25 colonies, +++ > 26-50 colonies, ++++ > 50 colonies

This study therefore demonstrates that the sensitivity of detection of *M. tuberculosis* by use of the polymerase chain reaction is dependent on the detergents and techniques used to prepare the sample prior to amplification and the amount of target sequence available for amplification.

Based on results of this study NP-40 appears to be the most suitable detergent for processing of clinical samples. Compared to conventional procedures using SDS it is more convenient as an aliquot from the detergent mix can be used directly for PCR without having to include a further purification step involving phenol/chloroform extraction and DNA precipitation. Furthermore during the extraction/precipitation procedure small amounts of DNA may be lost.

However samples that give negative results with NP-40 should be further processed either by sephadex G-50 spun column chromatography and/or a ten fold dilution prior to reamplification. Samples that are further negative would still require culture for a definitive diagnosis as some clinical isolates could either contain a low copy number of target sequence IS 986 or its complete absence.

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