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 which 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	hose based on amplification of mycobacterial DNA
have been compared only in relatively few studies.
The present study was therefore conducted in order to
have been compared only in relatively few studies.
The present study was therefore conducted in order to
increase the sensitivity of detection of Mycobacterium
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 dif-
ferent sample preparation techniques with a variety of
detergents to optimize DNA extraction from Mycobacterium
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 Mycobacterium
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 solu-
tion 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 resus-
pended 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 am-
plification.

Treatment of sputum for PCR

To five 50 µl aliquots of the processed sample were
added freshly prepared lysozyme (1 mg/ml) in TE pH
8.0, (TE: 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0)
and incubated for 1 hour at 37°C. Following lyso-
zyme 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 deter-
gents used were 0.5% tween 20, 1.0% sodium dodecyl
sulphate (SDS), 3.5% N-lauryl sarcosine, 0.45% non-
iden P-40 (NP-40) and 1.0% triton X-100 in final
concentrations. Subsequently proteinase K was in-
activated 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 ali-
quots of the remaining processed sample was subjected
to conventional phenol/chloroform DNA extraction
and ethanol precipitation (Manniat is 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) 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris HCl
(pH 8.3), 0.01% (wt/vol) gelatin, 0.2 mM 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 (Manniat is 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 (Mannitiis 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.

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 inhibit of Taq polymerase. Such methods therefore require careful purification 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.

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<th>Specimen Dilution</th>
<th>Sephadex G-50 Dilution &amp; Culture* Sephadex G-50</th>
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* 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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REFERENCES


