Comparison of Polymerase Chain Reaction with Conventional Techniques for the Detection of *Mycobacterium Tuberculosis* in Pulmonary TB Cases

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**ABSTRACT**

The aim of this study is to evaluate PCR for detection of *Mycobacterium tuberculosis* in sputum for acid-fast bacilli (AFB) comparing with culture and direct smear. This study was carried out between January and October 2006. The sputum specimens were collected at the laboratory of Microbiology from the patient population attending the Alkwafia Hospital and the Center of Endemic and Epidemic Diseases.

This study compares smear, growth in Lowenstein-Jensen medium, and PCR techniques for the detection of *Mycobacterium tuberculosis*. A total of 93 specimens were tested. 18 specimens were as a control group, from individuals with out tuberculosis according to the clinical and x-ray examination. 75 sputum samples were collected from persons suspected of having tuberculosis according to clinical symptoms. They were tested using smear, culture, and PCR techniques.

From these, 25 cases (33.30%) were smear positive and 29 cases (38.70) were positive by culture and 31 cases (58.70) positive by PCR.

The sensitivity and specificity for smear were 86.2 % and 100%, the sensitivity and specificity for culture were 93.54% and 100 %, the sensitivity and specificity for PCR were 100 % and 95.65 respectively. These results confirm that the PCR method PCR is sensitive and specific technique for *M. tuberculosis* detection.

**Keywords:** *Mycobacterium tuberculosis*, PCR, Amplification.

**INTRODUCTION** And Aim Of Study

Among the bacterial disease of the lower respiratory tract are two of the great killer infections at history, pneumonia and tuberculosis. The advent of antibiotic therapy brought the disease under control to considerable extent. Both are making come back to day as a result of spread of HIV and treatment with immunosuppressive drugs for transplant patients and with anti-inflammatory agents for autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis, lowered resistance, overcrowding, chronic disease, and other immunosuppressive factors that also contribute to the severity of problem (Black, 2002).

Tuberculosis remains one of the deadliest diseases in the World. WHO estimates each year more than 8 millions new cases of tuberculosis occur and approximately 3 million persons die yaerly from the disease (WHO, 1996). Ninety five percent of tuberculosis cases occur in developing countries, where few resources are available to ensure proper treatment and where
human immunodeficiency virus (HIV) infection may be common. It's estimated that between 19% and 43% of the world's population is infected with *M. tuberculosis*, the bacterium that causes tuberculosis infection and disease (Suderl et al., 1992). In the united states, an estimated 15 millions people were infected with *M. tuberculosis*, (Stark and Jerb, 1992).

Although the tuberculosis case rate in the united states has declined during the past few years, there remains a huge reservoir of individuals who are infected with *M. tuberculosis*. Without application of effective treatment for latent infections, new cases of tuberculosis can be expected to develop from within this group. Tuberculosis is a social disease with medical implications. It has always occurred disproportionately among disadvantaged populations such as the homeless, malnourished and over crowded (Riley, 1993). Tuberculosis remains one of the major public health problems worldwide (Raviglione et al., 1995; Dye et al., 1999), particularly due to the appearance of drug-resistant *M. tuberculosis* strains (Ramaswamy and Musser, 1998; kremer and Bersa, 2002).

Diagnostic tests devoted to the rapid, sensitive and specific identification of the causative agent are key elements for successful health programs aimed at disease control. Moreover, the accurate determination of mycobacterial burden might be beneficial for fast assessment of patient response to standard therapy, especially in those patients suspected of harboring resistant *M. tuberculosis* strains (Ramaswamy and Musser,1998).

**The present** study is aimed at evaluation of the use of PCR techniqueagnosis of pulmonary tuberculosis in comparison to conventional methods as direct smear examination and culture to achieve an early, rapid and direct detection of tuberculosis by comparing the sensitivity and specificity of PCR to conventional methods.

**PATIENTS AND METHADOLGY**

The present study is conducted between January and October 2006 at the Center of Epidemic and Endemic Diseases and Alkwaefia Hospital of Chest Diseases, in Benghazi, Libya. 75 sputum samples were collected from persons attending to Alkwaefia Hospital and the Center of Epidemic and Endemic diseases suspected of having tuberculosis depending on clinical symptoms. Also 18 sputum samples were included as a control group and they were collected from individuals with out tuberculosis according to the clinical and X-Ray examination. The data of each sample contains information related to sex, age, presence or absence of symptoms and any abnormality of collected samples for bacteriological investigations including direct smear, culture, and PCR.

This study was carried out at the Bacteriology Laboratory in the Hospital of Respiratory Diseases in Benghazi, the Bacteriology Laboratory of the Center of Epidemic and Endemic, Diseases and Department of Microbiology and Immunology, Faculty of Medicine, Assiute University at Egypt. Each sample was divided into two portions, one portion was used for conventional techniques including microscopic examination of acid fast stain and culture, the other portion used for the PCR technique. Microscopic examination and culture were performed by using the following protocols:
Specimens Processing

Examination of Sputum Specimens

Each sputum specimen was digested and decontaminated by treating it with an equal and decontaminated by treating it with an equal volume of N-acetyl-L-cysteine NaOH(5 %) for 15 to 20 min and centrifuged at 3.000 rpm for 15 to 20 min and centrifuged at 3.000 rpm for 15 min. (Kent and Kubica 1985). The excess fluid was poured off, and the sediment was resuspended in 2 ml of phosphate buffer (PH 5.3). The suspension was used to prepare the smears, inoculate Lowenstein-Jensen agar slants and the remainder was stored at -20 C until used for nucleic acid amplification experiments.

Smear Examination

The prepared smears were stained, by Ziehl-Neelsen. The stained smears were examined for the presence of acid fast bacilli.

Culture of Specimens

Lowenstein-Jensen medium (LJ, Becton Dickinson Microbiology System) was inoculated with any of the M. tuberculosis processed specimens, incubated at 37C and examined for growth weekly for 8 weeks. The isolated positive cultures were subjected to biochemical Niacin test.

Identification of by PCR

A 500 µl aliquot of each sample is transferred to a 500 ml centrifuge tube with screw cap and then heated at 80°C for 20 min. to kill the organisms.

DNA Extraction : 2

Samples are sonicated for 3 min. (power level 3) in 1.5 ml centrifuge tubes floating in a water bath, after sonication the samples are boiled for 10 min., and the bacterial debris removed by centrifuge at 13.000 rpm for 20 sec. The supernatant is used directly for PCR.

Purification of DNA :

By addition of BSA (bovine serum albumin) at a concentration of 0.052 %.

Amplification:

The amplification are performed with a (MTB complex 390 \ 750 ic, sacace biotechnologies, Italy). The MTB complex 390 \ 750 ic is an in vitro nucleic acid amplification test for qualitative detection of M. tuberculosis & bovis in sputum and other biological materials.
Principle of Assay:
MTB complex 390/750 ic test is based on three major processes: sample preparation, nucleic acid amplification of DNA using specific M. tuberculosis, bovis primers and detection of the amplified products on agarose gel.

Procedure
1. Prepare required quantity of tubes PCR-mix1.
2. Add for each sample in the new sterile tube 10 μl of 2.5 PCR buffer blue and 0.5μl of hot start polymerase.
3. Add 10 μl of reaction mix into each sample tube. Add 1 drop (15 μl of mineral oil).
4. Add to appropriate tube 10 μl of DNA sample obtained after sample preparation.
5. Add 10 μl of DNA buffer to the tube for negative control of amplification.
6. Add 10 μl of positive control to the tube for positive control of amplification.
7. Add 10 μl of internal control to the tube of internal control tube, serves as an amplification control for each specimen and to identify possible reaction inhibition.
8. Close PCR-mix 1-tubes and transfer them into the thermolcycler only when temperature reaches 95°C.

PCR analysis:
Analysis of results is based on the presence or absence of specific bands of amplified DNA in agarose gel electrophoresis (2%).

The Biochemical Test Used For Identification of M. tuberculosis:
Niacin Test:
Niacin test strips were used (Niacin test strips, TB, Difco)

Procedure:
The Bacto-TB Niacin test strips are prepared with potassium thiocyanate, chloramine T, citric acid and sodium aminosalicylate. The strip when used according to the directions, yields a yellow color. Lowenstein Jensen slopes. The 5 to 6 weeks old cultures were used. The slopes were punctured with a sterile inoculation wire to extract niacin from the medium. One ml of sterile distilled water was added to each slope and kept in a horizontal position for 30 minutes. The test strips were dropped in each L.J.medium tube and stoppered immediately. The tubes were examined after overnight incubation.

Statistical Analysis
Data were analyzed by (SPSS) Statistical package for social science program:
A- Descriptive statistics, tables, figures and measured central tendency (mean, median, mode).
B- Analysis statistic, person chi-square were applied for comparing the different techniques.

RESULTS AND DISCUSSION
A total of ninety three sputum samples were studied 18 as a negative control, the cases were 75 samples, 39 samples from males, 36 from females.

The sensitivity of PCR technique was 100%, while the specificity was 95.65%. We report the results on 75 samples and compare these results with those obtained with direct smear and culture by using Lowenstein Jensen medium.
The sensitivity of smear was 86.2%, with a specificity of 100 %, while the sensitivity of culture was 93.54% with specificity of 100%.

Smear results
Twenty five samples were positive for mycobacteria 33 % and fifty samples 66.70% were negative.

Culture results
Using the Lowenstein culture media twenty nine 38. % samples were positive and forty six 61.3% samples were negative.

PCR results:
Using the PCR technique thirty one samples (41%) samples were positive and sixty four (58%) were negative.

The correlation between PCR and conventional Culture:
The correlation between PCR and conventional culture for all samples was as follows: 29 samples were positive by PCR and positive by culture, and two were positive by PCR and negative by culture for a specificity 95.65 %, a sensitivity 100 %, positive predictive value (PPV) 93.55 % and negative predictive value (NPV) 100 %. Percentage of false negative was zero and percentage of false positive was 4.35 %.

Table 1. Validity of Techniques Used to Examine Sputum with PCR.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity %</th>
<th>specificity %</th>
<th>PPV %</th>
<th>NPV %</th>
<th>P-chi-square</th>
<th>P value</th>
<th>Degree of free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn smear</td>
<td>86.2</td>
<td>100</td>
<td>100</td>
<td>92</td>
<td>95.48</td>
<td>P &gt; .000</td>
<td>1</td>
</tr>
<tr>
<td>culture</td>
<td>93.54</td>
<td>100</td>
<td>100</td>
<td>95.65</td>
<td>67.1</td>
<td>P &gt; .000</td>
<td>1</td>
</tr>
<tr>
<td>PCR</td>
<td>100</td>
<td>95.65</td>
<td>93.55</td>
<td>100</td>
<td>67.1</td>
<td>P &gt; .000</td>
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</table>

Tuberculosis (TB) remains one of the major public health problems worldwide (Raviglione et al., 1995; Dye et al., 1999), particularly due to the appearance of drug-resistant *M. tuberculosis* strains that render TB control programs more cumbersome (Ramaswamy and Musser, 1998; Kremer et al., 2002). Diagnostic tests devoted to the rapid, sensitive, and specific identification of the causative agent are key elements for successful health programs aimed at disease control. Moreover, the accurate determination of mycobacterial burden might be beneficial for fast assessment of patient response to standard therapy; especially in those patients suspected of harboring resistant *M. tuberculosis* strains (Ramaswamy and Musser, 1998). Traditional laboratory techniques (Nolte and Metchock, 1995; van Griethuysen et al., 1996), such as direct microscopy observation and *Mycobacterium* culture on Lowenstein Jensen.
medium, are far from being sensitive and specific or adequate for a fast *M. tuberculosis* identification. Moreover, the harsh decontaminating procedures combined with the lack of homogeneity of the sputum and the tendency of *Mycobacterium* to clump render even quantitative culture systems unreliable.

This combined approach offers three major advantages, namely the following.

1. The assay is extremely fast (within a day), sensitive, and specific, as demonstrated on both AFB-positive and, more importantly, AFB-negative clinical specimens.

2. It allows for the detection of a wide spectrum of Mycobacterium species. In addition, the number of organisms in clinical samples is not always high enough to be detected by microscopic methods, and the generation time of these organisms is long. Not all acid fast – bacilli are *M. tuberculosis* so; species identification must await the results of additional biochemical tests.

**DNA Extraction:**

The majority of methods described for the extraction of DNA from clinical specimens are complex involving multiple enzyme digestion and phenol extraction (8; 25; 15 Brison *et al.*, 1989; Hermans *et al.*, 1990 and Cousins *et al.*, 1992). In the present study a relatively simple system, sonication, (Buck *et al.*, 1992) is used to prepare our samples. Buck *et al.*, 1992 evaluated several simple extraction methods as well and found sonication to be the most effective when working on small samples. Our results confirm that sonication is a simple and efficient in releasing DNA for amplification.

Importantly this processing protocol is considered safe for use in routine laboratory for two reasons: first, dissemination of infectious aerosols is minimized since the tubes remain closed during the processing, and second, infectious organisms are rendered non viable by boiling the sample prior to sonication.

**PCR:**

The PCR has proved to be a valuable tool for the specific detection of microorganisms directly from clinical samples (Dallas *et al.*, 1989; Guatelli *et al.*, 1989 and Carl *et al.*, 1990). An assay of this type for the identification of *M. tuberculosis* would be of great value for several reasons: The number of organisms in a clinical sample is not always high enough to be detected by microscope methods, and the generation time of these organisms is long. In addition not all acid fast bacilli are *M. tuberculosis* so; species identification must await the results of additional biochemical tests.

In our study, we adapted a PCR protocol first developed as a diagnostic test for mycobacteria infecting fish (Talat *et al.*, 1997). The PCR Amplifies a 390bp fragments, which is unique for mycobacteria. The primers showed excellent results, especially with respiratory specimens that may be contaminated with viral and bacterial respiratory pathogens.

In general PCR protocols have two major problems, false positive reactions and false negative reactions; False positive results may be caused by intra and pre laboratory contamination. This hypothesis is supported by the data from Noordhoecck *et al.*, 1994 who described a blind PCR comparison study performed by several laboratories for the detection of *M. tuberculosis* where a high level of false positive PCR (3 to 77%) were found. The authors suspect that this might be due to contamination of buflers and materials used in the pretreatment of the samples or for the extraction of DNA. In our study laboratory associated
contamination of clinical samples is unlikely since we ran negative and positive controls in parallel with the clinical samples during the DNA amplification and detection procedures.

When culture is used as the standard technique in comparison study, specimens containing non-cultivable bacteria, which may lead to a positive PCR result, are initially identified as being a false positive sample. In the absence of an ideal gold standard it is not clear what proportion of the specimens with initial false positive results actually contain non-cultivable bacteria.

Jones et al., 1988, were described 21 specimens that were positive in the gene-assay, but culture negative. Forbes and Hick, 1993, investigated 2 culture negative patients with positive PCR results, in our study there two samples were negative by smear and culture and positive by PCR, these false positive specimens could be attributed to the presence of nonviable organisms in patients with partially treated tuberculosis or may be due to the low number of bacilli which could not be detected by smear or culture. The second problem with PCR protocols is false negative results, the false negative results can be the result of:

1. The presence of inhibitors.
2. Non-homogenous distribution of the bacteria in the specimen so that the fraction tested dose not contain mycobacteria (Brisson et al., 1991).
3. The degradation of mycobacteria DNA during storage at 4°C (Iralu et al., 1993).

Different substances present in clinical samples including phenol and sodium dodecyl sulfate may be potent inhibitors of PCR (Clarrige et al., 1993).

Soini et al., (1992) were suggested that infection due to M. tuberculosis might lead to the introduction of inhibitory substances into sputum as to decreased sputum as well as to decreased sputum production.

The use of internal controls identifies the samples that are inappropriate for PCR or that require processing to remove inhibitors. Although the presence of PCR inhibitors in clinical samples is well recognized, few studies have been performed to specifically address this issue. Purification of DNA prior to amplification has the potential of eliminating inhibitors and concentrating scarce primer targets in the sample and there for has been utilized to increase PCR sensitivity (Boom 1990; Abe et al., 1993). Our findings supported this potential. In an attempt to develop a simple reliable method for treating clinical specimens containing mycobacteria for PCR analysis, Victor et al., 1992 and buck et al., 1992 used a sucrose purification step prior to amplification. Victor, et al 1992 were able to theoretically remove inhibitory substances and provided more reproducible and accurate results, 11 of 169 samples still required re-amplification. Forbes and Hick, used BSA bovine serum albumin and was able to override the presence of interfering substances.

When the performance of a new test system is evaluated, the performance of the standard method is a critical parameter for the detection of M. tuberculosis, culture has always been considered to be the gold standard. The specificity of culture is usually considered to be 100%. The sensitivity, however, though quite satisfactory, is often reported to be <90% (kolk et al. 1992; Schirm et al., 1995).

In the present study, among (75) sputum smears of pulmonary tuberculosis, 25 (33.30 %) were to found to be positive by ZN, 29(38.70 %) positive by culture and 31( 41.30 %) were positive by PCR technique.

The usefulness of the PCR technique for the diagnosis of tuberculous cases is deduced according to the following observation:
1 – It is more advantageous for the rapid diagnosis of suspected cases especially those giving negative microscopic examination.

2 – It can determine whether the acid fast bacilli detected by microscopic examination are MTB or not.

3 – It is also more helpful when large scale screening of mycobacteria is indicated, further more; the PCR will be of great benefit for the follow-up of cases of tuberculosis.

In two samples, PCR results were positive but culture was negative; this could be due to the presence of nonviable mycobacteria in the samples as some of the subjects were receiving anti tubercular treatment.

In our study only two false positive result by PCR test which could be due to the ability of the PCR test to detect very low number and even dead bacteria in a sample which can be present in a symptomatic individual.

To conclude, molecular diagnosis of tuberculosis by PCR has a great potential to improve the clinicians ability to diagnose tuberculosis. This will ensure early treatment to patients and prevent further transmission of disease. However, further work is required for improving sensitivity, specificity and reproducibility of this test and to make it more user friendly and cost effective (Amato et al., 1996; Bechoosh et al., 1997).

This study was conducted to validate a diagnostic protocol that we could use on AFB smear- and culture specimens. The observation of AFB in sputum samples or other biological material should be considered as TB suspected, but smear does not specifically identify M. tuberculosis. Moreover, this procedure is considered the least sensitive methodology for TB diagnosis because the estimated number of bacteria for positive smear is 5,000 / ml (7; 23; 28; 43). This justifies the negative smear result in six samples, in contrast with positive results in culture and PCR, which are more sensitive techniques.

In our study Smear showed negative results in six cases and culture showed negative results in two cases, while positive by PCR, probably due to the fact that the amount of mycobacteria present was not enough to be detected by these techniques, while the number for detection by PCR is in the order of 20 bacterium (Eisenach et al., 1990; Altamirano et al., 1992; Dagmar and Kappe, 2002) and in other studies they could amplify from as low as one single bacterium in specimens.

Furthermore, the contamination of samples before, during, or after amplification could result in PCR positivity, while the conventional tests were negative (Kox et al., 1994), but we have ruled out this possibility with the inclusion of negative controls. Positive samples for smear and culture, with negative PCR, might have been developed by the fact that the protocol was specific to M. tuberculosis as well as the fact that the stain and the culture were not specific to the genus and species. On the other hand, the false-negative PCR result, in a negative smear sample and positive to M. tuberculosis in culture, could be explained by the presence of inhibitors of the amplifying system sample (Clarridge et al., 1993; Nolte et al., 1993 and Burkardt 2000).

Furthermore, these inhibitors substances were common in sputa samples (Clarridge et al., 1993; Pfyffer et al., 1996e). The PCR positive results may not necessarily reflect an active infection by M. tuberculosis because it is not possible to distinguish a previous infection from a minimum quantity of bacteria that does not have clinical significance and does not need treatment (Clarridge et al. 1993).
Therefore, an approach to PCR positive results in asymptomatic patients, prior to considering them false positive, must first discard the possibility of sample contamination, look into the patient’s clinical history or whether the patient is in treatment (Cousins et al., 1992; Kox et al., 1994). Sensitivity of the culture is considered low when biological samples are analyzed with a small number of mycobacteria that must necessarily be viable to yield positive results (Eisenach et al. 1993). On the other hand, in this study, the only criterion for sample selection was TB suspicion in any of its forms, because it is probable that, if more specific clinical criteria are established for a sample for PCR, there will be a significant increase in positive results, mainly when negative smears occur (Kritski et al., 1997).

Therefore, the PCR protocol showed a better sensitivity and specificity to TB diagnosis than the culture. In addition it can be executed in less than 48 h, making it possible to get faster results and minimizing the number of false-positive and -negative results (Cartuyvels et al., 1996).

Finally, when negative PCR results display a discrepancy in relation to other clinical or laboratory criteria, the false-negative results should be discarded due to the presence of inhibitors, to the lack of any basic reaction component, to the use of inappropriate reagent concentrations, or to wrong temperatures (Eisenach et al., 1991, Claridge et al., 1993).

The sensitivity (100%) and specificity (95.65%) of the PCR protocol used in this study is within the limits described by other authors for similar procedures (60% to 100%) (5 ; 37 ; 47), although Lim et al. (2000) found a low sensitivity (44%) and a similar specificity (97%). Padilla et al. (2001) when using the PCR with reverse hybridization line-probe assay to Mycobacterium sp. obtained a sensitivity and specificity of 100%. Similarly, Mehrotra et al., (2002) developed an in-house PCR procedure, a less expensive method, for detection of M. tuberculosis in cytological specimens, and obtained the same results when compared to the other PCR kits.

In conclusion, our study shows that the PCR technique have advantages over AFB smear and culture for predicting diagnosis results in specimens containing a low number of organisms, and this situation could lead to earlier initiation of appropriate therapy and epidemiological intervention. The technique should be used together with the traditional methods of tuberculosis diagnosis until some situation could lead to earlier initiation of appropriate therapy and epidemiological intervention. The technique should be used together with the traditional methods of tuberculosis diagnosis until some of the technical limitations of this technique are solved. Nucleic acid amplification technologies such as PCR are revolutionizing the detection of infectious pathogens such as tuberculosis. Amplification technology offers the potential for the diagnosis of TB in a few hours with a high degree of sensitivity and specificity. However, molecular assays neither replace nor reduce the need for conventional smear and culture, speciation, and antibiotic sensitivity assays.

PCR technique is the method of choice for diagnosis of tuberculosis. This approach not only will provide rapid, clinically significant results but also may detect cases of tuberculosis missed by culture. The test should be performed on all smear –positive specimens and for all specimens from patients at high risk for tuberculosis.

Our data indicate that PCR provides the clinician, investigator and infection control practitioner with very valuable, rapid and clinically relevant information for the diagnosis and control of tuberculosis. The test is easy to perform and requires only one day for results.
The amplification takes 3 hours during which the investigator can perform other tasks. A total of 60 specimens can be analyzed during a single test run the technique will be particularly useful in laboratories serving populations with a high incidence of a typical mycobacterial infections. The assay provides a very sensitive and specific rapid method of detecting *M. tuberculosis*.

**CONCLUSION**

Tuberculosis is on the increase throughout the world. At present, the rapid diagnosis of tuberculosis rests with microscopy, however, this technique is insensitive and many cases of tuberculosis can't be confirmed. Also, culture on solid media even with the Lowenstein media, is too slow for clinical usefulness. We investigated the use of DNA amplification by the polymerase chain reaction (PCR) for detection of *M. tuberculosis* from 75 sputum samples one portion of each sample was processed for smear and culture by standard methods, and the other was submitted for PCR analysis.

The PCR analysis consisted of amplification of a 390-bp segmented within the 16s rRNA gene, and detection by agarose gel electrophoresis. From 75 samples analyzed, 25 samples were positive by smear, 29 samples were positive by culture and 31 were positive by PCR. Two samples were positive by PCR but negative by culture. In comparison with culture, the sensitivity, specificity, positive predictive value and negative predictive value were 100%, 95.65% 93.55% and 100% respectively for PCR. A good correlation was observed between the results obtained with the PCR method that we described and other diagnostic tests currently used.

Thus PCR amplification technique is proposed as a specific, rapid and sensitive test for the diagnosis of infection with *Mycobacterium tuberculosis*.

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