THE DETECTION OF TUBERCULOSIS IN PAUCIBACILLARY SPUTUM SAMPLES IN GHANA USING PCR


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SUMMARY
In Ghana, the detection of tuberculosis (TB) is traditionally done by microscopy. However, the low sensitivity and poor specificity of microscopy do not allow detection of Mycobacterium tuberculosis (M. tb) among certain types of patients. Polymerase chain reaction (PCR) is a rapid, sensitive and specific technique, which has been applied to the diagnosis of tuberculosis. In the present study, we report on the use of PCR for the detection of tuberculosis among patients who are negative by microscopy. This PCR is based on the amplification of 249bp fragment of the multi-copy insertion sequence IS6110 present in the genome of the M. tuberculosis and detection by agarose gel electrophoresis. The sensitivity of PCR as a diagnostic tool for smear-negative samples was 59.1% and the specificity was 48.8% as compared to culture. Significantly, 34.9% of ZN-negative samples were found to be positive by PCR. We conclude, therefore, that the PCR technique would be very useful for the diagnosis M. Tb in atypical presentations.

INTRODUCTION
Tuberculosis remains one of the world’s most serious and infectious diseases killing as many as 3 million people annually. In addition, the emergence of drug-resistant tuberculosis has worsened the situation. The incidence of tuberculosis is increasing worldwide and is a significant public health problem in sub-Saharan Africa. The HIV epidemic has also contributed to the high mortality arising from infection due to M. tuberculosis. In Ghana, the overall adult prevalence rate in 2001 was 0.2%, with a case notification rate of 62 per 100,000 and a treatment failure rate of 1.6%.

After Ziehl-Neelsen (ZN) staining for acid-fast bacilli (AFB). ZN staining is cheap, relatively simple to perform and available at district hospitals equipped with a microscope. Since ZN staining will be positive for any mycobacteria it is not specific for M. tuberculosis. Furthermore, it has a detection limit of (5-10) x 103 bacilli/ml and its sensitivity is between 40 and 60%.

In the hands of overworked and poorly resourced laboratory staff this sensitivity might decrease even further. For this reason the International Union Against Tuberculosis and Lung Diseases, (IUATLD), recommends a maximum of 20-30 slides per technician per working day.

One of the main objectives national tuberculosis control programmes is to reduce or eliminate tuberculosis transmitted by coughing. Patients who produce paucibacillary sputa pose a dilemma for tuberculosis control strategies. Whilst they may not transmit the disease through coughing, they may act as a reservoir for the bacteria that may be reactivated when the patient’s immunity is compromised. This is typically the case in patients with HIV AIDS. It is therefore important to diagnose patients whose bacterial load is below the detection limit of microscopy.

In order to overcome the limitations of microscopy such as poor sensitivity and specificity, various other techniques have been tried. These include gas chromatography-mass spectrometry to identify tuberculosis, a fatty acid component of the mycobacterial cell wall. Although excellent results have been reported for this technique, it is not available in the average diagnostic laboratory. Enzyme-linked immunosorbent assay (ELISA) techniques, based on the detection of antibodies to the mycobacteria have been suggested but are not

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in regular use because of poor specificity\(^9\,10\). ELISA tests based on antigen detection are under development and not readily available for diagnosis at the present time\(^9\). Culture is probably the most reliable technique for unambiguous identification of *M. tuberculosis*. The disadvantage of culture as a diagnostic tool is that, it may take up to eight weeks and is generally unavailable even at regional hospitals in Ghana. Nucleic acid amplification techniques, specifically PCR, have been used elsewhere\(^11\,12\,13\,14\) for the diagnosis of tuberculosis. The PCR technique is rapid, sensitive and specific and may be used to analyze many samples simultaneously. Usually results are obtainable within 24 hours after receipt of the sample and the detection limit is theoretically one bacterium. Because of this high sensitivity, the technique has been used to detect extra pulmonary tuberculosis. The aim of this work was to investigate the diagnostic utility of the PCR technique for the detection of *M. Tb* in samples that would have been passed negative by microscopy.

In this communication we report on PCR studies carried out on smear-negative sputum samples obtained from patients suspected of having tuberculosis. The PCR used in this study is based on the amplification of a 249-bp DNA fragment of a multicopy insertion sequence IS6110 that is present in the genome of *M. tuberculosis* complex\(^11\) and it is therefore specific for its identification. In addition, we report of culture studies carried out on the same sputum samples for comparison with the polymerase chain reaction.

**MATERIALS AND METHODS**

**Sample Collection**

New tuberculosis suspects presenting at the Chest Clinic of the Korle Bu Teaching Hospital (KBTH) with smear-negative sputum samples were enrolled into the study. The inclusion criteria for the patients were: weight loss, persistent cough for more than three weeks and night sweat. Two samples were obtained from each patient: one at midnight and the other at 5am the following morning. The chemotherapeutic status of patients was not known at the time of sampling. The samples were collected into sterile containers and were each processed for Ziehl-Neelsen microscopy, PCR and culture.

**Ziehl-Neelsen (ZN) microscopy**

Microscopy was carried using carbol-fuschin staining procedure for mycobacteria\(^16\) as follows: Sputum samples were smeared onto glass slides using disposable spatulae in a sink enclosed in a P2 hood. Each slide was flooded with carbol-fuschin stain ([obtained by dissolving 0.3g basic fuschin in 10.0ml 95% ethanol and then mixing this with a solution obtained by mixing 5.0ml of melted phenol in 95.0ml distilled water and allowing the resultant solution to stand for three days]) with slow intermittent heating for about 5 minutes. The slide was then washed briefly but carefully with tap water and then decolourised with acid-alcohol mixture (97.0ml of 95% ethanol mixed with 3.0ml of concentrated hydrochloric acid) until no more stain was observed in the running water. Counterstaining with methylene blue ([0.3g methylene blue in 100.0ml distilled water]) was carried out after a further rinse with tap water. Upon microscopic examination, Ziehl-Neelsen negative sample were indicated by a blue background without red spots.

**Decontamination and culture of sputum samples**

The sputum samples were decontaminated according to standard procedure\(^17\) by initially mixing with an equal volume of a solution containing 1M NaOH, 0.1M sodium citrate and 0.5% N-acetyl-L-cysteine (NAC) in a 15ml screw-capped polypropylene tube. The mixture was vortexed for about 20 seconds with regular inversion for about 20 minutes and then neutralized with 8ml of 67mM phosphate buffer (pH 6.8) and centrifuged at 3000g for 15 minutes. The pellet was resuspended in 400μL of the phosphate buffer. Part of the suspension was used for culture\(^16\) by inoculating Lowenstein-Jensen slants. The slants were incubated at 37°C, initially in a slanting position, for a week thereafter vertically for five to seven weeks. They were observed weekly. They were observed weekly for growth.

**DNA extraction**

The extraction of *M. tuberculosis* DNA was carried out on the remaining part of decontaminated sputum sample using a slightly modified Wilson method\(^15\). Briefly the digested sputum samples were incubated at 80°C for 20 minutes and washed three times with 0.1M Tris-HCl buffer, pH 6.4. About 1mL of Tris-HCl buffer, pH 6.4, was added to each sample in a tube, centrifuged at 12,800g for 10 minutes and the supernatant discarded. This procedure was repeated twice. After the final wash the pellets were re-suspended in 1mL of TE (10mM Tris-1mM EDTA), pH 8.3, centrifuged at 12,800g for 10 minutes and the supernatant discarded. Chloroform (100μL) was added to each
pellet followed by vigorous shaking for 1 minute to disrupt the cells. Tris-EDTA (100μL) was added to each tube, the mixture vortexed for 1 minute and centrifuged at 12,800g for 10 minutes. The aqueous phase containing the DNA was transferred into fresh tubes.

DNA amplification and analysis
Ten microfilters (10μL) of the solution containing DNA template was added to 35μL of amplification mixture which contained: 1.5mM MgCl₂, 10mM Tris-HCl buffer, pH 9.0, 50mM KCl, 0.01% (w/v) gelatine, 0.1% Triton X-100, 0.2mM of each of four dTPPs, pH 8.3 (Boehringer Mannheim), 0.2μM of each oligonucleotide primer, 1.0 Unit of Taq DNA polymerase (HT Biotechnology) and 2 units of uracil-N-glycosylase, UNG, (GibcoBRL). The set of primers used were P1-18F GAAACCGTAGGCGACATCGAG and INS2R06GC GCGTACGCGTACGACAAA (Isogen Biosciences BV). This set of primers amplifies a 249-bp portion of the IS6110 element of M. tuberculosis and a 301bp control fragment from M. smegmatis DNA. PCR reactions were carried out in a final volume of 50μL in a MJ Research (PTC-100) thermal cycler fitted with a hot bonnet. The following cycling parameters were used for amplification: 10 minutes at 40°C for optimal activity of UNG, then 40 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute 30 seconds at 65°C and extension for 2 minutes at 72°C; the reaction mixture was held at 72°C until analysis. The PCR products were analysed by electrophoresis on 2% agarose gel stained with ethidium bromide (0.5μg/mL) and visualized on a UV transilluminator (Vilber Lourmat, France).

Controls for PCR
Each batch of PCR analysis was performed with five positive controls, four of which were different concentrations of M. tuberculosis DNA and another containing a known concentration of M. smegmatis 1008 DNA (kindly supplied by Dr. AHJ Kolk of the Royal Tropical Institute, Amsterdam). The target in the M. smegmatis control DNA is identical to that in M. tuberculosis except that the former incorporates a 56 base pair cloned fragment that is not present in the latter. There were three negative controls, two of which contained TE in place of template DNA and the other being a sample obtained from a non-tuberculous patient from Amsterdam (kindly supplied by Dr. AHK Kolk of KIT, Amsterdam). The negative controls were processed from the decontamination stage to the point of inoculating into the PCR mix alongside the samples. The two samples from each patient were run in duplicate, one neat and the other spiked with 5 x 10⁷ g/ml M. smegmatis 1008 DNA (5μl). The spiked sample served as an internal control for Taq polymerase inhibitors. Deoxyuridine triphosphate (dUTP) was used in place of dTTP to control for amplification contamination; the inclusion of uracil-N-glycosylase in the reaction mix ensured to destruction of dUTP-containing ampiclons from previous reactions.

RESULTS
Sixty-three (63) samples were obtained from the patients enrolled in the study. Of these, 34 or 54% were male (mean age was 41.5 years, median age was 42.5 years and the age range was 13-74 years) and 29 or 46% were female (mean age was 42.0 years, median age was 40.0 years and the age range was 6-89 years) giving a male/female ratio of 1:2:1.

![Figure 1](image1.png)

Figure 1 Ethidium bromide stained agarose gel electrophoresis analysis of PCR products.

Lanes 1-4: 1pg, 1000pg, and 5 respectively of M. tuberculosis DNA (positive controls); Lanes 5: 250pg M. smegmatis 1008 DNA (control for internal inhibitors); Lane 6: TE (negative control); Lane 7: distilled water (negative control); Lane 8-9: sample 77B; Lane 10-11: sample 84A; Lanes 12-13: sputum sample from non-tuberculous patient (negative control); Lanes 14-15: sample 94A; Lanes 16-17: sample 77A; Lane 18-19: sample 33B; Lanes 20 and 39: molecular weight marker (100bp ladder); Lane 21-22: sample Cab Lane 23-24: sample 96A; Lane 25-26: sample 86A; Lane 27-28: sample 68A; Lane 29-30: sample 63A; Lane 31-32: sample 58B; lane 33-34: sample 71B; Lane 35-36: sample 69B; Lane 37-38: sample 71B; The first of each duplicate sample was spiked with 250pg of M. smegmatis, 1008 DNA while the other was not.

Figure 1 shows the PCR results of 15 representative specimens. The remaining 58 specimens were analysed similarly (not shown). Using this method we were able to detect or exclude the presence on M. tuberculosis DNA in sputum samples from smear negative TB suspects within two days.
Table 1 Results of polymerase chain reaction (PCR) compared with culture

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<th>Culture +ve</th>
<th>Culture -ve</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR +ve</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>PCR -ve</td>
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<td>20</td>
</tr>
<tr>
<td>Totals</td>
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<td>41</td>
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Table 2 PCR results indicating male-female distribution

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<th>Female</th>
<th>Totals</th>
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<tbody>
<tr>
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<td>15</td>
</tr>
<tr>
<td>PCR -ve</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Totals</td>
<td>34</td>
<td>29</td>
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Table 3 Culture results indicating male-female distribution

<table>
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<tr>
<th>Male</th>
<th>Female</th>
<th>Totals</th>
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</thead>
<tbody>
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<td>7</td>
</tr>
<tr>
<td>Culture -ve</td>
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<td>22</td>
</tr>
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DISCUSSION
Table 1 shows the results of polymerase chain reaction (PCR) conducted on the 63 specimens compared with culture while tables 2 and 3 shows the male-female distribution in the PCR and culture results respectively. From the table 1 the sensitivity and specificity were calculated to be 59.1% and 48.8% respectively. The results also indicate that about 41% of the culture-positive samples were negative by PCR. Although PCR is very sensitive it is important to observe that the samples used in this study were obtained from patients whose samples were negative by microscopy and therefore probably had low bacterial loads. All the samples that were assayed by PCR had been decontaminated for culture. The decontamination time of 20 minutes is optimized for culture but not long enough for the efficient extraction of DNA for PCR. It is possible that under these conditions, the DNA of the mycobacteria would not be extracted. Such samples would erroneously be classified as PCR negative samples but might be culture positive. The results also suggest that about one-third (34.9%) of ZN-negative samples are indeed samples form patients with infection due to M. tuberculosis.

The importance of this work lies in its ability to detect tuberculosis in ZN-negative samples. The polymerase chain reaction could have a significant impact on the management of tuberculosis suspects and hence improve the efficiency of the national tuberculosis control programme. In the absence of the final clinical diagnosis, PCR seems to perform better than culture in diagnosing tuberculosis among patients with low bacterial load and is therefore a valuable asset in the diagnostic facilities available to a tuberculosis laboratory. The authors are unaware of the existence of PCR facilities for the detection of tuberculosis in any hospital in the country. The technique would be too expensive for routine use at most hospitals but might be useful in the Teaching Hospitals and some selected Regional Hospitals for the resolution of difficult cases.

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