Comparative Diagnostic Methods of Tuberculosis in Blood Samples

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Introduction: Despite the availability of effective preventive measures and chemotherapy, the prevalence of tuberculosis (TB) is increasing in the developing world and in much of the industrialized world as well. Children are among the most vulnerable and the most difficult to diagnose with tuberculosis. Early and precise diagnosis of childhood tuberculosis is necessary in order to prevent mortality and morbidity and unjustified chemotherapy. One of the main objectives of the research in the field of mycobacteriology is the development of new methods that will improve and expedite the diagnosis and treatment of tuberculosis and other mycobacterial infections. HealthTech, in collaboration with DynaGen, Inc., in the United States, developed the MycoDot™ serological assay. Several techniques have been developed to improve the diagnosis of tuberculosis including newer radiometric methods; DNA probes mycolic acid chromatography, polymerase chain reaction. We aimed in our work to diagnose active tuberculosis either pulmonary or extra pulmonary by different new diagnostic methods.

Patients and Methods: In this study included 58 children ranged from 5 to 7 years old and suspected to have tuberculosis, based on the findings of history taking, clinical examination, PPD skin test, chest X-ray, and sputum examination by Ziehl-Nielsen staining for acid fast bacilli. These patients were classified into four classes class 0: no known recent exposure, no infection (PPD-negative), no disease; class I: latent infection as defined by a positive PPD (≥ 10 mm in duration) and no clinical or radiographic evidence of active TB; class II: active TB confirmed by positive clinical pictures and radiographic evidence of active TB with positive sputum staining; Class III: PPD-positive (≥ 5 mm in duration) with evidence of past disease by history or compatible chest radiograph (e.g., upper lobe fibronodular disease) but negative sputum smear (treated patients). All patients sera were subjected to TB identification by rapid test and MycoDot tests and PBMC separated from blood for PCR reaction.

Results: The positive results of the three tests for diagnosis of TB in the four groups of classes were detected as followings: By PCR the four classes were diagnosed as: Class 0 (5.3%), Class I (50%), Class II (70%) and Class III (14.3%). By MycoDot diagnosis of the four groups was as: Class 0 (0%), Class I (4.5%), Class II (50%) and Class III (0%). Diagnosis of the four groups by +ve rapid test was as: Class 0 (0%), Class I (0%), Class II (30%) and Class III (0%).

In conclusion: As a conclusion, PCR is a sensitive and rapid method for detection of latent and active TB within few hours, while in anti-LAM IgG was quite specific detection of active disease. The assay can be performed without sophisticated instrumentation with minimal training, which make the assay for random detection of active TB.

Key words: Active TB, latent TB, PCR and MycoDot.

INTRODUCTION

Despite the availability of effective preventive measures and chemotherapy, the prevalence of tuberculosis (TB) is increasing in the developing world and in much of the industrialized world as well.1-4 According to World Health Organization (WHO) estimates, in 1990 there were 8 million new cases of TB and 3 million deaths due to the disease worldwide; 1.3 million new cases and 450,000 deaths were among children under 15 years of age.5 It is estimated that 1.75 million deaths resulted from TB in 2003. As with cases of disease, the highest number of estimated deaths is in the South-East Asia Region, but the highest mortality per capita is in the Africa region, where HIV has led to rapid increases in the incidence of TB and increases the rate of dying from TB. In developing countries, the risk for TB infection and disease is relatively uniform in the population; annual rates of infection often exceed 2%.5,6 Tuberculosis remains one of the leading causes of death from an infectious disease worldwide. Approximately 1/3rd of the world’s population is infected and about 2 million people die annually from tuberculosis.7

Children are among the most vulnerable and the most difficult to diagnose with tuberculosis. In developing countries,
efforts including Bacille Calmette-Guerin vaccination have failed to control tuberculosis and the disease continues to spread. Basic tools for diagnosis and treatment are often not available in the most affected areas of the world. In developed nations, immigrants from high risk countries have the highest rates of new disease. Better diagnostic techniques, control measures and treatment options are desperately needed; advances will require a worldwide commitment.8

In healthy adults, about 5% to 10% of those infected with tuberculosis will develop active TB at some point in their lifetime if not treated. From data gathered prior to effective treatment for TB, infection with TB is more likely to progress to active disease in younger children with high rates as 45% in the first year of life and around 25% in children of 13 months to 4 years of age.9 Children also have higher rates of extrapulmonary disease as well as TB meningitis. Aggressive disseminated forms such as military and TB meningitis can have a rapid onset within 1 month of infection, usually before the tuberculin skin test has had time to become positive. Unlike adults who usually present with classic clinical findings, children with TB may have no symptoms at all or may have clinical findings that can mimic other more common illnesses.10,11 Tuberculosis in children can present with unusual clinical manifestations and often does not follow classic pulmonary disease seen in adults.12 The diagnosis in children is more difficult and may lead to delay in treatment.13 Pediatric tuberculosis is more aggressive, more often extra pulmonary, and standard diagnostic tests are of little help in over 50% of cases in children.

Early and precise diagnosis of childhood tuberculosis is necessary both in order to prevent mortality and morbidity and unjustified chemotherapy. Clinical manifestations being nonspecific, particularly in early stage and extra-pulmonary forms of tuberculosis, it remains easily misdiagnosed, under-diagnosed or paradoxically over treated. A history of recent exposure to a case of active tuberculosis, tuberculin test, chest radiographs and physical examination are often the only support for the diagnosis. The recovery of tubercle bacilli, which would establish the diagnosis with certainty, is difficult in children. In the past, various workers evaluated the utility of different techniques for early diagnosis of childhood TB. Attempts have been made to improve sensitivity and speed of detection of tubercle bacilli by techniques such as radiometric detection of bacterial growth, BACTEC, fluorescent antibody test, gas chromatography, DNA hybridization, PCR and RIA.

The diagnosis of pulmonary TB is based largely on the microscopic detection of the causative organism in patient sputum, pleural fluid, and bronchoalveolar lavage.15 This method of detection, the acid-fast bacillus (AFB) smear, is error prone. It has a sensitivity of, at best, 20-40% in developing countries and 40-60% in developed countries. Culturing the bacteria on solid media yields significantly better results with slow rate of growth of the bacteria then seldom results are obtained before 4 weeks.15

One of the main objectives of the research in the field of mycobacteriology is the development of new methods that will improve and expedite the diagnosis and treatment of tuberculosis and other mycobacterial infections. HealthTech, in collaboration with DynaGen, Inc., in the United States, developed the MycoDot™ serological assay, which detects anti-mycobacterial antibodies in serum.15,16 The assay procedure, which can be performed using a blood, plasma, or serum sample obtained from a finger prick, takes 20 minutes to perform and requires no special equipment.

Several techniques17-26 have been developed to improve the diagnosis of tuberculosis including newer radiometric methods; DNA probes mycolic acid chromatography polymerase chain reaction, and serologic tests these diagnostic approaches have had A dramatic effect on the ability to diagnose accurately and expeditiously so we aimed in our work to diagnose active tuberculosis either pulmonary or extra pulmonary by different new diagnostic methods.

**PATIENTS AND METHODS**

In this study included 58 children ranged from 5 to 7 years old and suspected to have tuberculosis, based on the findings of
history taking, clinical examination, PPD skin test, chest X-ray, and sputum examination by Zeil-Nielsen staining for acid fast bacilli. They were being seen at the Departments of Pediatrics and Chest disease, El-Minia University Hospital, and from Chest Disease Hospital, El-Minia Directorate of Health and Population during the period from March to August 2005. These patients were classified into four classes class 0: no known recent exposure, no infection (PPD-negative), no disease; class I: latent infection as defined by a positive PPD (≥ 10 mm in duration) and no clinical or radiographic evidence of active TB; class II: active TB confirmed by positive clinical pictures and radiographic evidence of active TB with positive sputum staining; Class III: PPD-positive (≥ 5 mm in duration) with evidence of past disease by history or compatible chest radiograph (e.g., upper lobe fibronodular disease) but negative sputum smear (treated patients). All patients’ sera were subjected to TB identification by rapid and MycoDot tests and PBMC were separated from blood for PCR reaction.

A. Laboratory investigation: Rapid test: Hexagon TB: Immunochromatographic assay for the quantitative determination of M. tuberculosis antibodies. The assay was done as the followings: one drop of serum sample was applied to the antigen in the well, then three drops of antihuman globulin were added and left for three minutes. Finally, positive or negative visualized bands were appeared.

B. The MycoDot test: This test employs lipoarabinomannan (LAM) antigen, bound to plastic combs. When the combs are incubated in diluted serum, specific anti-LAM antibodies from the sample, if present, bind to the antigen. The combs are then washed to remove non-specific antibody, and incubated in a suspension of colored particles, which bind to the bound anti-LAM antibodies. If enough of the specific antibodies are present in the serum sample, a colored spot will form where the antigen is attached to the plastic comb.

C. PCR reactions: Separation of peripheral blood mononuclear cells (PBMCs), which made by collection in heparinized tubes 5-mL blood specimen from all patients. The buffy coat from the heparinized blood was separated using Ficoll Hypaque (density 1.007 gram/ml; Sigma, St. Louis MO) and PBMCs were collected for PCR. This was followed by DNA extraction then DNA precipitation: Thereafter, steps of DNA hydration and PCR amplification were made (optimized specifically for PCR, the Ready-To-Go reaction beads are formulated with high quality buffer, nucleotides (dNTPs), and recombinant puReTaq DNA polymerase only template DNA and template-specific primers need to be added. i- 1 µL forward primer (5-25 pmol). Then 1 µl reverse primer (5-25 pmol), followed by 2 µl of template DNA.

D. Thermal cycling parameters: A standard three-step endpoint PCR cyclizing protocol consists of multiple cycles of denaturation; initial denaturation step at 94 °C for 4 minutes, followed ensure complete denaturation of the template DNA by repeating cycles in the round 35 cycles (each for 30 seconds) at 94 °C. Annealing at 60 °C for 35 cycles (each for 30 seconds). Extension at 72 °C for 35 cycles (each for 60 seconds). The final extension time at 72 °C (each for 8 minutes). Thereafter Gel was prepared with 2% agarose gel. The samples were placed in the gel chambers, then power was supplied on 105 volt for 1 h. Finally, the bands was detected by UV lamp.

Statistical analysis
For MycoDot and PCR techniques, specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for all patient cases of different classes.

RESULTS
Table 1 shows the demographic data of the different patients groups. The table also shows Class 0 as a negative control group, Class I is the only positive immuno response (latent infection), Class II express the patients suffered from active TB and Class III showed patients who have cured (received treatment). Table 2 showed the number and percentage of children with positive or negative results of PCR, MycoDot, and rapid test in children of each of the four Classes of TB. Table 3 shows the number of patients and their percentages with only positive results of the three tests for diagnosis of TB in the four groups of classes.
By PCR the four classes were diagnosed as: Class 0 (5.3%), Class I (50%), Class II (70%) and Class III (14.3%). By MycoDot diagnosis of the four groups was as: Class 0 (0%), Class I (4.5%), Class II (50%) and Class III (0%). Diagnosis of the four groups by +ve rapid test was as: Class 0 (0%), Class I (0%), Class II (30%) and Class III (0%). In Table 4, Class I (latent infection) has a specificity of MycoDot and rapid test (100%) reactions higher than that with PCR (94.7%), but the sensitivity of PCR (50%) is higher than those of MycoDot (4.5%). The positive predictive value (PPV) of MycoDot (100%) is higher than that of PCR (91.7%). The negative predictive value (NPV) of PCR (62%) is higher than that of MycoDot test (47.5%). In Class II (active pulmonary disease) specificity of MycoDot reactions of 100%, which is higher than that with PCR (94.7%), but the sensitivity of PCR (70%), which is higher than those of MycoDot (50%). The positive predictive value (PPV) of MycoDot (100%) is higher than that with PCR which is 87.5%. The NPV of PCR (85.2%) is higher than those with MycoDot test (79%). In Class III (postinfection), whereas the specificity of MycoDot reactions is 100%, and it is higher than that of PCR (94.7%). However, the sensitivity with PCR is 14.3%, which is higher than those with MycoDot. Additionally, the PPV with PCR is 50%, while those of MycoDot which is of 0%. Moreover, the NPV with PCR is 75%, which is higher than those of MycoDot test, and equals 73%. Figure 1 shows positive samples MycoDot test in Class I and one of positive case of Class II, while Figure 2 shows the rest of the obtained of positive samples in Class II. Figures 3-5 show the positive bands obtained by PCR reaction in different classes of patients.

Table 1: Patients demographic data.

<table>
<thead>
<tr>
<th>Patient Classification</th>
<th>Clinical Picture</th>
<th>PPD skin test</th>
<th>Chest X-ray</th>
<th>Sputum Examination</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 0</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>19</td>
</tr>
<tr>
<td>Class I</td>
<td>negative</td>
<td>positive ≤ 10 ml</td>
<td>negative</td>
<td>negative</td>
<td>22</td>
</tr>
<tr>
<td>Class II</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>10</td>
</tr>
<tr>
<td>Class III</td>
<td>negative</td>
<td>positive ≤ 5 ml</td>
<td>positive</td>
<td>negative</td>
<td>7</td>
</tr>
</tbody>
</table>

Table (2): Results of total patients of the research.

<table>
<thead>
<tr>
<th>Patients Classification</th>
<th>Number of Patients</th>
<th>PCR</th>
<th>MycoDot</th>
<th>Rapid Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Class 0</td>
<td>19</td>
<td>1</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Class I</td>
<td>22</td>
<td>11</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Class II</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Class III</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

PPD: Posterior Probable Diagnosis, PCR: Polymerase Chain Reaction, MycoDot, Rapid Test
Table (3): Number (percentage) of children had +ve results in each of the 3 tests in the 4 Classes of patients of the study.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Class 0 (No=19)</th>
<th>Class I (No=22)</th>
<th>Class II (No=10)</th>
<th>Class III (No=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve PCR</td>
<td>1 (5.3%)</td>
<td>1 (50%)</td>
<td>7 (70%)</td>
<td>1 (14.3%)</td>
</tr>
<tr>
<td>+ve MycoDot</td>
<td>0 (0%)</td>
<td>1 (4.5%)</td>
<td>5 (50%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>+ve Rapid Test</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (30%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Table 4. Specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) in children of Classes I-III.

<table>
<thead>
<tr>
<th>Class</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>PCR</td>
<td>94.7%</td>
<td>50%</td>
<td>91.7%</td>
</tr>
<tr>
<td></td>
<td>MycoDot</td>
<td>100%</td>
<td>4.5%</td>
<td>100%</td>
</tr>
<tr>
<td>Class II</td>
<td>PCR</td>
<td>94.7%</td>
<td>70%</td>
<td>87.5%</td>
</tr>
<tr>
<td></td>
<td>MycoDot</td>
<td>100%</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>Class III</td>
<td>PCR</td>
<td>94.7%</td>
<td>14.3%</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>MycoDot</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Fig. 1. MycoDot test: Lane 1 is +ve control. Lane 2 is –ve control. The +ve samples are in lanes 7 and 8 (Class I and II).

Fig. 2. The +Ve samples are in lanes 3, 5, 7 and 8 (Class II).
**Fig. 3.** Lane 1 is 100 pb ladder. Lane 2 is the –ve control. Some positive samples employ lanes 3-7 which are in Class I in addition to lane 8 in Class 0.

**Fig. 4.** Lane 1 is 100 pb ladder. Lane 2 is the –ve control. Positive samples employ lanes 3-9 which are in Class II.

**Fig. 5.** The positive PCR sample in class III (lane 11).

**DISCUSSION**

It is estimated that one-third of the world's population is infected with *M. tuberculosis* with approximately 8 million new cases of active TB and 3 million deaths per year. Such extensive infection of the human population requires a diagnostic test that is capable of discrimination an active from a latent infection. The gold standard for diagnosis of TB is demonstration of mycobacteria from various body fluids. This is often not possible in children due to pauci-bacillary nature of illness. In pediatric age group, sputum is difficult to obtain. In our study we investigated different classes of patients as cleared in the results sections. These results were obtained during applying of three techniques including rapid test and MycoDot in patients sera. The third one was PCR in peripheral blood mononuclear cells (PBMCs). In the rapid test in the sera of different classes of patients, we found low percentage (30%) only in case of active groups (Class II), which makes this technique of low importance in diagnosis of different classes of TB. In our study MycoDot anti-LAM IgG was evaluated in patients sera with only positive cases in Classes I and II. In Class I, the sensitivity was of low value (4.5%), while in Class II this sensitivity was 50%, which expresses the importance of the test for detection active TB. The detection of anti-LAM antibodies has been previously evaluated in various developing countries (Thailand, Ghana, Tanzania) and in Europe (Italy and Spain). The specificity of the test was excellent, ranging from 84 to 100%.
In HIV-negative patients, the sensitivity of the MycoDot anti-LAM IgG test in Tanzania, Ghana, and Thailand was 33%, 56%, and 63.2%, respectively. The formal results agreed with our results as the sensitivity related to the detection of TB by MycoDot is 50%, whereas its specificity is 100%. In contrast, the sensitivity was 89% in Italy and in Spain, overall sensitivity was only 21.5%, although in relapse cases. These differences in the sensitivity of anti-LAM IgG between the developing and European countries. The formal differences of sensitivity are related to the immune and nutritional status. Chain et al reported on anti-LAM IgG is a sensitive marker of active TB in a U.S. population. It is known that humoral or cellular immune response of foreign antigens can vary among individuals and therefore some patients may not develop a detectable antibody titer to LAM. Furthermore, confounding variables such as an underlying illness (e.g., malnutrition), medication use (e.g., corticosteroids), or even TB itself may impair the humoral and/or cellular immune response. Seropositivity against LAM was observed in both (S. Nochur, DynaGen Inc., Cambridge, MA, personal communication). Gupta and colleagues, who showed that antibody levels to antigen 60 were lower in patients who were receiving treatment for TB versus those measured before treatment, also observed a similar phenomenon. Nevertheless, the overall detection of active TB based on the presence of anti-LAM IgG was substantially more sensitive than results usually obtained from sputum smears.

On the other side, our PCR results from PBMCs indicated only one case from the 19 patients of the negative group (Class 0). In Class I, it was found that 50% (11 from 22 patients) of cases were positive by PCR detection in PBLs, which gave the diagnostic test as its importance in early detection of TB with early treatment and better prognosis and also in latent infection of TB. The PCR test in active group from PBMCs (Class II) showed sensitivity of 70% and specificity of 94.7%, which agreed by the results by reported by Mirza, S. of PCR test of sensitivity of 65.2% from PBMCs, he suggested that the PBMCs is highly sensitive when compared with conventional other techniques. In our results and from the cured class (Class III, it was found that only one case gave positive result by PCR with a sensitivity 14.3%, which could be non-living TB Bacilli. It is clear from our study that PCR technique is a good method used for early detection of TB within a few hours compared with traditional bacterial culture technique that normally take few weeks. In addition, PCR technique is an effective method for active TB diagnosis with a sensitivity 70% and specificity 94.7%. The PCR has gained its importance in the clinical diagnosis of M. tuberculosis, and is now important support in the clinical diagnosis of TB in developed countries (Brown TJ, 1999). Laifer, G. showed that the sensitivity of PCR for TB testing was higher than that of acid fast staining in overall specimens 64% vs. 20%; in addition, the specificity of PCR tests was found as 96.2%. The working hypothesis for the PBMC-PCR is that peripheral macrophages have scavenged M. tuberculosis genomic material from the infected sites, or even whole bacilli. The detection of M. tuberculosis DNA, but our inability to obtain culture-positive blood from the same individuals, suggested low-level bacteremia undetectable by culture, or most likely, amplification of DNA remnants from non-viable mycobacteria, presumably inside phagocytic vacuoles from peripheral macrophages. As the PCR technology improves, new advances allowing real time assays, high sensitivity and specificity.

**Conclusion:** As a conclusion PCR is a sensitive and rapid method for detection of latent and active TB within few hours, while in anti-LAM IgG was quite specific detection of active disease. The assay can be performed without sophisticated instrumentation with minimal training, which makes the assay for random detection of active TB.

**REFERENCES**


