The predictive value of Gen-Probe’s amplified *Mycobacterium tuberculosis* direct test compared with culturing in paraffin-embedded lymph node tissue exhibiting granulomatous inflammation and negative acid fast stain

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**KEYWORDS**
Tuberculosis; Molecular testing; Granuloma; *Mycobacterium tuberculosis* direct (MTD) test; Gen-Probe

**Summary**

*Background and objectives:* The diagnosis of granulomatous inflammation with possible tuberculosis (TB) infection in histopathology is often difficult. There is a need for a rapid and reliable diagnostic test. Thus, we evaluated the performance of the *Mycobacterium tuberculosis* direct (MTD) test in specimens with granulomatous lymphadenitis and negative acid fast stains.

*Methods:* The *M. tuberculosis* direct (MTD) test by Gen-Probe was performed on 45 formalin-fixed paraffin-embedded tissue samples including 34 lymph nodes. We measured the predictive values of the MTD test in specimens with granulomatous lymphadenitis and negative acid fast stains.

*Results:* The overall test sensitivity was 73.9%, and specificity was 95.4%. The MTD test sensitivity and specificity for lymph node tissue were 72.7% and 91.67%, respectively. In the presence of granulomatous inflammation, the MTD test sensitivity and specificity were higher than those for all tissue samples, at 75% and 100%, respectively.
Introduction

The diagnosis of granulomatous inflammation with possible tuberculosis (TB) infection in histopathology is often frustrating for the treating physician and pathologist. The pathologist cannot ascertain that granulomas with caseous necrosis are caused by TB infection unless they demonstrate acid fast bacilli (AFB). As a result, clinicians often have to wait for another six to eight weeks before they can receive confirmatory culture results. The treatment decision is often made based upon clinical suspicion and the knowledge that such a diagnosis cannot be ignored. The challenge is that most patients with extrapulmonary TB have little or no symptoms and are considered a low-suspicion group. There is a real need for a rapid and reliable diagnostic test to allow the pathologist to give an evidence-based diagnosis to the treating physician when granulomas are encountered in asymptomatic patients.

Several authors have demonstrated a consistent ability to identify Mycobacterium tuberculosis complex (MTC) in formalin-fixed paraffin-embedded (FFPE) tissue, with variable sensitivity [1–3]. However, none of the published reports measured the predictive value of these tests in light of a low clinical suspicion level and negative special histochemical stains.

This study simulates the frequently encountered scenario of granulomatous inflammation with low clinical suspicion for TB infection and negative acid fast stain (AFS). Most of the tissue selected was from lymph node biopsies because it represents the most frequently encountered specimen from those patients in our population [4].

This study measures the predictive value of applying molecular methods such as the M. tuberculosis direct (MTD) test by Gen-Probe to detect MTC in extracted nucleic acids from FFPE tissue. In addition to lymph nodes, the sensitivity and specificity of this technique were also measured for different tissue types against the results of fresh tissue culture as the gold standard. The patient’s clinical probability level was evaluated as low clinical suspicion by a qualified infection disease specialist.

Materials and methods

The files of Saudi Aramco Medical Services Organization were searched for cases that had TB culture performed on the same fresh biopsy tissue from patients with low clinical suspicion for TB. Low clinical suspicion was defined as no systemic symptoms of fever, night sweats, anorexia or weight loss. Patient symptoms included cervical lymph adenopathy, abnormal chest X-ray, one case of brain lesion and one case of osteomyelitis. A total of 45 cases were selected, and 23 cases had granulomatous inflammatory reaction and a negative Zeel-Nelson histochemical special stain. Seventy-five percent of the cases were from lymph node biopsy specimens (34/45 cases). The reference culture methods used included the liquid-based mycobacteria growth indicator tube (MGIT) culture system, conventional solid Löwenstein–Jensen (LJ) medium and Middlebrook medium.

The removal of the tissue from the paraffin was performed as described by Wright et al. with modifications [5]. Three 35-μm-thick sections of FFPE tissue were placed in 1.5-ml Eppendorf tubes. To avoid cross-contamination, a new microtome blade was changed after sectioning each sample. Deparaffinization of the sample was performed by adding 1 ml of xylene, followed by vortexing for 1 min at room temperature and centrifuging at 16,000 × g for 10 min; the xylene was then removed. This step was repeated at least twice or until the wax was completely dissolved. The remaining xylene was washed off by adding 1 ml of absolute alcohol with vortexing for 20 s and centrifugation at 16,000 × g for 10 min. The ethanol was then removed. This last step was repeated twice, and the tubes were left open to dry.

The nucleic acid was extracted following QIAamp Mini DNA kit instructions (Qiagen GmbH, Hilden, Germany). We followed the manufacturer’s instructions but used double volumes for all reagents to match the high amount of tissue used. In addition, the elution volume was increased to 300 μl using PCR-grade water for 10 min or more. The DNA concentration was then measured using an Eppendorf
biophotometer. The lowest DNA concentration level achieved in positive cases was 13 μg/ml.

The detection of MTC was performed according to instructions of the amplified *M. tuberculosis* direct (MDT) (Gen-Probe, San Diego, CA) manufacturer’s package insert, as follows. All reaction volumes were brought to a total level of 450 μl for the test sample and the controls using NALC/NaOH/phosphate buffer solution. To achieve that, a total of 200 μl of the eluted nucleic acid sample was transferred to 250 μl of the NALC/NaOH/phosphate buffer solution. To monitor any inhibitory effect, 100 μl of the eluted sample was transferred to a new tube containing 300 μl of NALC/NaOH/phosphate buffer solution and was spiked with 50 μl of *M. tuberculosis* positive control. The remaining detection and reporting steps followed the Gen-Probe manufacturer’s instructions.

**Results**

The distribution of the 45 tissue samples was as follows: 34 lymph nodes, six biopsies from the lung and pleura, and five cases from soft tissue, bone and brain and meninges (see Table 1). Granulomatous inflammation was detected in 23 of the 45 cases. Twenty-two of the 34 lymph nodes showed granulomatous lymphadenitis (64.7%). The total number of positive cases by the MTD test was 18 out of 45 cases (40%). One of the 18 cases showed equivocal results twice and was considered positive according to the manufacturer recommendation. This was a lymph node tissue with no granulomas (Table 2). Because the culture was negative, this case was considered a false-positive case. This was the only false-positive case out of the total 45 cases (2.2%). Thus, 17 of the 45 cases were positive by both the culture and MTD test (37.7%). The cases that were negative by both MTD and culture were considered as the negative control group. This included 21 samples (46.6%). Three out of the 21 negative control cases showed granulomatous inflammation (Table 2). Two of the three cases were lymph node samples and the patients were considered as a possible sarcoidosis or atypical TB infection and improved with anti-TB treatment. The third case was that of a 73-year-old male smoker who was diagnosed with right upper lobe pneumonia and improved with antibiotics treatment. The false negative group included six out of 45 cases that were positive by culture and negative by MTD test (13.3%) (Table 1).

Considering the culture results as a gold standard and with no regard to the presence of granulomas, the Gen-Probe method on all the selected cases showed a detection sensitivity of 73.9% and a specificity of 95.4%. Accordingly, the positive predictive value was 94.4%, and the negative predictive value was 77.7% (see Table 2).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Distribution of tissue samples with Gen-Probe (MDT) and culture results.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>34</td>
</tr>
<tr>
<td>Lung and pleura</td>
<td>6</td>
</tr>
<tr>
<td>Other tissue</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>N = 45 (100%)</td>
</tr>
</tbody>
</table>

*MTD, Mycobacterium tuberculosis* direct test by Gen-Probe; POS., positive; Neg., negative.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Predictive values of MTD test in the presence or absence of granulomas against tissue culture.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue type</td>
<td>Culture Pos.</td>
</tr>
<tr>
<td>Granulomas</td>
<td>23</td>
</tr>
<tr>
<td>No granulomas</td>
<td>22</td>
</tr>
<tr>
<td>All tissue</td>
<td>45</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mycobacterium tuberculosis direct test.  
<sup>b</sup> Sensitivity.  
<sup>c</sup> Specificity.  
<sup>d</sup> Positive predictive value.  
<sup>e</sup> Negative predictive value.
Table 3  Predictive values of MTD test and culture results in lymph node tissue with and without granulomas.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Culture Pos. MTD</th>
<th>Culture Neg. MTD</th>
<th>Culture Pos. MTD</th>
<th>Culture Neg. MTD</th>
<th>Sen^b</th>
<th>Spe^c</th>
<th>PPV^e</th>
<th>NPV^f</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN^d with granuloma</td>
<td>22</td>
<td>14</td>
<td>5</td>
<td>2</td>
<td>73.68</td>
<td>66.67</td>
<td>93.3</td>
<td>28.57</td>
<td></td>
</tr>
<tr>
<td>LN^d with no granuloma</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Total LN^d cases</td>
<td>34</td>
<td>16</td>
<td>5</td>
<td>2</td>
<td>72.73</td>
<td>91.67</td>
<td>94.12</td>
<td>64.71</td>
<td></td>
</tr>
</tbody>
</table>

^a Mycobacterium tuberculosis direct test.  
^b Sensitivity.  
^c Specificity.  
^d Lymph node.  
^e Positive predictive value.  
^f Negative predictive value.

When a sub-analysis was performed to compare the MTD test predictive values between cases with granulomas and all cases, the group with granulomas showed a slightly increased sensitivity and specificity and positive predictive value; however, the negative predictive value became remarkably lower (37.5%). This is caused by the low number of cases that had granulomatous inflammation and negative culture (Table 2).

The test predictive values for all lymph node tissue showed high sensitivity and specificity of 72.73% and 91.67%, respectively. However, the specificity of the test decreased to 66.67% when we limited the analysis to only lymph node cases with granulomatous inflammation (see Table 3).

Discussion

The low suspicion of tuberculosis continues to be a major challenge, with the diagnosis of granulomatous inflammation often being the first medical evidence for TB infection. At least 15% of TB infection occurs in extrapulmonary sites [6]. It has been reported by Small et al. in a large retrospective study that 30% of the HIV population has only extrapulmonary TB infection [7]. In a previous study from our institution, extrapulmonary tuberculosis constituted 51% of all culture-positive tuberculosis, and most cases presented with lymphadenopathy [4]. Unfortunately, histopathologists continue to insist on visualizing the TB organism in situ with the help of special immunological or histochemical stains to confirm TB infection, despite the low sensitivity of these methods. Consequently, many false negative results are issued, leading to delayed diagnosis and treatment. The reported contributing factors to this low sensitivity include a number of organisms in tissue sections below the required $10^4$–$10^6$ organisms per milliliter and the anti-TB treatment effect on the bacterial cell wall [8,9].

Under such circumstances, the laboratory fails to deliver the needed conclusive evidence at a very critical time in patient management. This laboratory evidence is needed to treat patients promptly or to prevent the administration of inappropriate treatment, such as steroids to patients with mediastinal lymphadenopathy. Due to the high negative predictive value of this test, clinicians will have the option in low-suspicion cases to safely delay empiric anti-TB treatment during the eight-week period until the culture results are finalized. This will lead to significant treatment cost savings and will prevent the harm of unnecessary anti-TB medication. In addition, early and adequate treatment will reduce the patient’s risk from progressing to generalized disease or developing multi-drug resistant TB infection. There is a clinical need for a diagnostic test that is faster than culturing and has strong predictive values. Such a test is needed to support early treatment decisions when granulomas are unexpectedly encountered in low-suspicion cases with negative AFS. The cases selected for this study simulated this frequently encountered, difficult scenario and focused on cases with granulomatous lymphadenitis.

Although rapid molecular detection methods for TB are now highly standardized and automated, they are often limited to a specific specimen type and collection techniques to avoid PCR inhibition or to overcome the low number of TB organisms [10–12]. However, many authors have demonstrated the reproducibility of applying these techniques on FFPE tissue, with variable sensitivity [1–3]. Manzano et al. compared the sensitivity
and specificity of the Gen-Probe MTD test and LCx MTB test by Abbott [2]. They reported sensitivities of 52% and 67%, respectively, and the specificity for both tests was 100%. This is in line with our overall sensitivity of 73.9% and specificity of 95.4%. Despite the reported difficulties in applying molecular techniques on FFPE tissue, recent nucleic acid extraction methods, such as that described by Qiagen, have significantly improved the quality of the extracted nucleic acid material almost to the level of fresh frozen tissue [13].

Lymph nodes represented most of the specimens in this study (75%), which reflected the nature of the clinical presentation of lymphadenopathy in our patient population of extra pulmonary tuberculosis cases [4]. Cases from other tissues were included to explore the possible effect of tissue type on the predictive values in comparison to lymph nodes.

As expected, the predictive values of the MTD test on lymph nodes with granulomatous inflammation were of equal sensitivity (73.68%) to overall tissue samples (73.9%) because most of the positive cases were from lymph nodes. However, the specificity of the MTD test on granulomatous lymphadenitis was lower than the total number of cases due to the low number of cases with negative culture in this group (see Table 3).

The predictive values of non-lymph node tissue were not reliable due to the low number of cases with positive results. This is expected because lymph nodes act as bacterial filters, which results in a higher number of bacterial loads in the specimen. The Gen-Probe MTD test was chosen for this study because of its higher sensitivity, whereby more than 2000 copies of ribosomal RNA are produced per cell rather than per target sequence [2,14,15]. Because of its increased sensitivity, this test method was among the first tests approved by the Food and Drug Administration (FDA) for smear-negative sputum testing, with a calculated sensitivity of 41–48%. The test sensitivity and specificity in our study were found to be higher than those reported by Manzano et al. when using the same MTD molecular test on FFPE tissue [2]. The possible contributing factors, in addition to a high percentage of lymph node tissue samples, include using three 35-μm-thick sections compared to two 10-U-thick sections. In addition, we used double volumes from all extraction reagents, which resulted in higher nucleic acid concentration. Our result confirmed the conclusion of Marchetti et al., who listed DNA concentration among the factors that must be controlled in detecting TB in FFPE. In addition, these authors listed adequate numbers of the MTB organisms and an adequate target DNA size [1]. By using culture results as a gold standard, we also demonstrated that the granulomatous reaction did increase the MTD test sensitivity. However, when the sub-analysis was limited to the granuloma-positive group, the specificity and the negative predictive value decreased. This was caused by the low number of cases that had negative culture in the presence of granulomatous inflammation.

Our findings are also consistent with those reported by Vlaspolder et al. for fresh sputum samples and Lo et al. for FFPE tissue—culturing is a better test than MTD in ruling out MTC infection in extrapulmonary disease [15,16]. A feasible explanation is related to the rare false-positive results for the MTD test due to its ability to detect non-viable organisms that cannot be cultured. However, in our study, we came across this scenario only in one case where the MTD test was positive and the culture was negative. Aggarwal et al. reported the advantage of using the MTD test in addition to tissue culture and conventional tests as a rapid method to evaluate synovial fluid and rule out TB arthritis prior to surgery [17].

In addition, under these conditions, the 73.9% sensitivity of the MTD test is higher than the sensitivities reported for histochemical or immunohistological stains, 47.09% and 59.35%, respectively. This is statistically significant because the margin of error in our study and those reported by Grang et al. and Whily et al. are less than 0.05% [8,9].

As a result of this investigation, we recommend that conventional histochemical or immunological staining methods should be supplemented by the MTD test when evaluating lymph node tissue with a granulomatous inflammation to deliver stronger evidence to support the clinical decision. Prior to accepting a case for analysis, a minimum DNA concentration level of 13 μm/ml should be achieved, and the pathologist should ensure the presence of granulomatosus inflammation. In addition, the results should be reported with a note highlighting the test predictive values for the tissue type under these conditions. More comparative studies between molecular techniques on FFPE tissue and culture methods are needed to identify the most predictive test. These studies should take into consideration the effect of tissue type on the test sensitivity due to the variability in bacterial load.

In conclusion, the Gen-Probe test method for FFPE lymph node tissue is a reliable and reproducible one. It has stronger predictive values to detect MTB organisms than those reported for histochemical and immunohistochemical staining methods. This test should be used in addition to immunoperoxidase and histochemical staining to provide strong evidence to the treating physician at an earlier time when dealing with unexpected
granulomatous lymphadenitis in low-suspicion TB patients.

Conflict of interest

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