The usefulness of PCR amplification of the IS6110 insertion element of M. tuberculosis complex in ascitic fluid of patients with peritoneal tuberculosis

Dimitrios Tzoanopoulos*, Konstantinos Mimidis, Stavros Giaglis, Konstantinos Ritis, Georgios Kartalis

First Division of Internal Medicine, Democritus University of Thrace, Regional Hospital of Alexandroupolis, Alexandroupolis, Greece

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Abstract

Background: The diagnosis of tuberculous peritonitis (TP) may be difficult and elusive. The present study was designed to demonstrate the diagnostic usefulness of a nested polymerase chain reaction (PCR) assay, specific for the IS6110 insertion element of M. tuberculosis complex, in patients with ascites who were suspected of having TP in order to achieve a more timely diagnosis and treatment. Methods: Three HIV-negative patients suffering from fever and ascites were evaluated for suspected TP. Specimens were obtained from ascitic fluid, bone marrow, and peripheral blood and analyzed by both conventional methods and nested PCR for the presence of bacilli. Response to antituberculous treatment was considered as the final criterion for diagnosis of peritoneal tuberculosis. Results: All three patients had an excellent response to antituberculous therapy. Our PCR-based protocol detected M. tuberculosis complex DNA in the ascitic fluid of all patients, whereas conventional methods failed to establish the disease. Furthermore, in one patient, M. tuberculosis was also detected in both bone marrow and peripheral blood. Conclusions: PCR amplification of the IS6110 sequence of M. tuberculosis complex in ascitic fluid is a useful tool when peritoneal tuberculosis is suspected. However, its validity still needs to be established.

Keywords: Tuberculous peritonitis; PCR; IS6110 insertion element

1. Introduction

Tuberculous peritonitis (TP) is a form of abdominal tuberculosis that can involve the omentum, intestinal tract, liver, spleen, or female genital tract in addition to the parietal and visceral peritoneum [1]. It accounts for about 1–2% of all cases of tuberculosis, the incidence having increased after the onset of the HIV epidemic [1].

Clinically, TP is characterized by fever, abdominal pain, anorexia, weight loss, and ascites. However, none of these symptoms is specific for the disease, so it is commonly misdiagnosed, especially as carcinomatous peritonitis in the elderly. Early diagnosis of TP is of major importance in the control of the disease [2,3]. Laparoscopy with direct biopsy is an excellent diagnostic method and must be considered for every patient with unexplained ascites [4]. A definitive diagnosis requires identification of bacilli in ascitic fluid or peritoneum tissue. However, acid-fast staining is usually negative and cultures are positive in 30–40% of cases, making bacteriological confirmation of the disease very difficult [1].

In recent years, advances in molecular techniques have provided a new approach to the rapid diagnosis of tuberculosis by nucleic acid probes and polymerase chain reaction (PCR) [5,6]. The insertion sequence IS6110 has been successfully used as a target for PCR amplification in
clinical samples by many investigators [7,8]. The sensitivity and specificity of IS6110 amplification is variable in different laboratories and depends on the source of the clinical sample, the localization of the tuberculosis, the coexistence of HIV infection, and other technical parameters [9-11]. However, little is known about the value of this approach for patients with TP.

We report here on the molecular detection of M. tuberculosis in peritoneal fluid from three HIV-negative patients with TP using a nested PCR assay, specific for the IS6110 insertion element of M. tuberculosis complex.

2. Materials and methods

2.1. Patients

Three HIV-negative, non-neutropenic patients, admitted to the University Hospital of Alexandroupolis because of ascites and prolonged fever, were investigated for peritoneal bacillus dissemination. Principal clinical features and laboratory findings in all three patients are shown in Table 1.

All three patients underwent extensive investigation for fever and ascites. Two of them had underlying liver disease, one due to chronic hepatitis B and the other due to chronic alcohol abuse. All had a positive tuberculin skin test and were negative for human immunodeficiency virus (HIV) antibodies. Chest X-rays revealed no evidence of active or previous pulmonary tuberculosis. Anemia was found and inflammatory markers (c-reactive protein, fibrinogen, ferritin) were elevated in all of the patients, as well as initial CA-125 levels (>500 U/ml). Viral serology and autoimmunity and malignancy screens were unremarkable. Ascitic fluid, obtained from all patients, was an exudate and contained an abundance of lymphocytes, but microbiology and cytology were negative. Computed tomography (CT) of the abdomen showed ascites and lymph node enlargement but no evidence of malignancy. A broad-spectrum antibiotic trial, administered due to prolonged fever, had no clinical benefit. Peritoneal tuberculosis was suspected and samples from ascitic fluid, as well as peripheral blood and bone marrow aspiration smears, were collected and analyzed by both PCR and conventional methods for the presence of bacilli. Laparoscopy was not performed due to contraindications in two patients (patient nos. 2 and 3, Table 1), whereas the third patient (patient no. 1, Table 1) refused to provide informed consent. Concurrently, a three-drug combination antituberculous regimen (isoniazide 300 mg/day, rifampicin 600 mg/day, and ethambutol 25 mg/kg per day) was initiated [12].

2.2. Acid-fast stain and bacilli culture

All samples were investigated for the presence of acid-fast bacilli by Ziehl-Neelsen and were cultured on Loewenstein-Jensen medium (bioMerieux, France). Liquid culturing systems were not available in the microbiology laboratory of our hospital during that period.

2.3. PCR protocol

A nested PCR protocol was used in order to amplify a 316-bp fragment of the IS6110 sequence of M. tuberculosis complex, as we have previously described [13,14]. Briefly, after DNA isolation by lysing of mononuclear cells (separated by centrifugation of 50 ml of ascitic fluid in 3000 rpm for 10 min) and phenol-chloroform extraction, 0.5-1µg of extracted DNA was used in the 50-µl PCR reaction using the outer primers TJ5, 5'-CCG CAA AGT GTG GCT AAC-3' and TJ3, 5'-ATC CCC TAT CCG TAT GGT G-3' (positions 608 and 998, respectively, and amplified product 409 bp). Nested PCR was carried out in the 50-µl reaction using the inner primers OL15, 5'-AAC GGC TGA TGA CCA AAC-3' and STAN3, 5'-GTC GAG TAC GCC TTC TTG TT-3' (positions 667 and 963, respectively, and amplified product 316 bp). The conditions of primary PCR were: 2 min at 94 °C, followed by 35 cycles (94 °C for 80 s, 57 °C for 60 s, 72 °C for 70 s) and followed by 5 min at 72 °C. The conditions for nested PCR were: 2 min at 94 °C, followed by 35 cycles (94 °C for 80 s, 57 °C for 60 s, 72 °C for 70 s) and followed by 5 min at 72 °C.

<table>
<thead>
<tr>
<th>Pt./Age/Sex</th>
<th>Patient's previous and family history</th>
<th>Clinical presentation</th>
<th>CA125 levels</th>
<th>PPD skin test</th>
<th>Conventional methods</th>
<th>PCR</th>
<th>Response to treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/32/F</td>
<td>Family history of pneumonic tuberculosis</td>
<td>Atypical abdominal pain, small amount of ascites (~1000 ml, ultrasonography finding) and low-grade fever</td>
<td>756 U/ml</td>
<td>10 mm</td>
<td>Negative</td>
<td>Positive in peritoneal fluid</td>
<td>Yes</td>
</tr>
<tr>
<td>2/76/M</td>
<td>Post-viral HBV infection cirrhosis</td>
<td>Prolonged fever, abdominal pain and ascites</td>
<td>1134 U/ml</td>
<td>13 mm</td>
<td>Negative</td>
<td>Positive in peritoneal fluid, peripheral blood, and bone marrow</td>
<td>Yes</td>
</tr>
<tr>
<td>3/71/M</td>
<td>Alcoholic cirrhosis</td>
<td>Prolonged fever, abdominal pain and ascites</td>
<td>668 U/ml</td>
<td>12 mm</td>
<td>Negative</td>
<td>Positive in peritoneal fluid</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Pt. patient; M, male; F, female.
Fig. 1. Agarose gel electrophoresis demonstrating IS6110 sequence amplification. Lane M: 100 bp ladder (Gibco). Lane 6: positive control (commercially available: AMS 19 KIT, Clont, Milano, Italy) Lanes 3–5: nested PCR products (316 bp) originating from patient’s ascitic fluid (patients 1, 2, and 3 according to Table 1, respectively). Lane 2: negative control. Lane 1: blank.

for 60 s, 60 °C for 60 s, 72 °C for 60 s) and followed after the last cycle by 5 min at 72 °C. All PCR amplifications were carried in the PCR-engine apparatus PTC-200 (MJ Research, Watertown, MA, USA). The PCR products were analyzed in 2% agarose gels (Gibco-BRL, Life Technologies, Paisley, UK).

3. Results

Conventional bacteriological methods failed to detect M. tuberculosis in peritoneal fluid after abdominal paracentesis, even in large amounts of ascitic fluid. None of the patients was found to be positive for acid-fast bacilli using the Ziehl-Neelsen staining protocol or using cultures on Loewenstein-Jensen medium. In contrast, PCR amplification readily detected the IS6110 sequence in all three patients in samples originating from the ascitic fluid (Fig. 1). Moreover, in patient 2, M. tuberculosis complex DNA was also detected in both bone marrow and peripheral blood (Table 1).

All three patients were cured after receiving three-drug combination antituberculous therapy, and CA-125 levels returned to normal after the completion of this regimen. Thus, the response to therapy constituted the definitive criterion for the final diagnosis of peritoneal tuberculosis and confirmed the results of our molecular analysis.

4. Discussion

Tuberculous peritonitis (TP) is a relatively rare disease, comprising only 1–2% of all cases of tuberculosis cases, which may be seen in association with the pulmonary or disseminated form of the disease [1]. A rapid and correct diagnosis is of great importance because of the high mortality rate. Unfortunately, clinical presentation as well as laboratory findings, ascitic fluid examination, and serology markers (CA-125) are not specific for the disease, making diagnosis very difficult [2,3]. As a result, TP represents the great mimic and is often misdiagnosed as carcinomatous peritonitis [2,3]. Moreover, conventional bacteriological methods are time-consuming and their sensitivity is low. In most cases, treatment is empirical and physicians are often uncertain as to whether they should continue or discontinue antituberculous therapy.

In our study, TP was demonstrated in three HIV-negative hospitalized patients undergoing investigation for prolonged fever, abdominal pain, and ascites. The diagnosis was based on clinical and laboratory findings, in combination with the response to antituberculous therapy. Acid-fast staining and cultures on Loewenstein-Jensen medium failed to establish the disease when applied to samples originating from ascitic fluid, bone marrow, and peripheral blood, probably due to the fact that the specimens obtained contained bacteria below the detection limit of these conventional and routinely used methods. It is well known that low numbers of bacilli are sufficient to cause tuberculous damage in peritoneum as well as in all other sites involved in extrapulmonary tuberculosis [1]. Furthermore, up to 1 l of ascitic fluid must be obtained, concentrated by centrifugation, and then examined to achieve an 80% rate of sensitivity for mycobacterial culture [15,16]. The combination of small numbers of bacilli and the large amount of ascitic fluid needed to be examined makes bacteriological confirmation of the disease very difficult. In contrast, PCR amplification of the IS6110 sequence was able to detect bacilli in all three patients, and this was in accordance with the response to antituberculous treatment. In addition, the PCR analysis was performed in a small amount of ascitic fluid (50 ml), thus demonstrating the validity of this alternative method for the detection of bacilli in specimens having a number of bacteria undetectable by culture methods.
DNA amplification of *M. tuberculosis* does not always mean viable bacilli, and the PCR result has to be evaluated in combination with other clinical and laboratory findings [17,18]. In our study, the positive PCR result, in combination with the positive tuberculin skin test fever and ascites, was taken as a sign of disease in progress and was treated successfully with an antituberculous regimen. The sensitivity of IS6110 amplification is variable in different laboratories and depends on the source of the clinical sample, the localization of the tuberculosis, the coexistence of HIV infection, and PCR protocols [9–11]. We have previously discussed the sensitivity and specificity of our PCR protocol in diagnosing extrapulmonary tuberculosis in patients presenting with fever of unknown origin [13]. A majority of false-positive results was reported for specimens containing mycobacteria other than tuberculosis (MOTT) [19,20]. The lack of HIV-positive patients in this study, who are more often infected by mycobacteria of other species, as well as the responsiveness to the antituberculous regimen, may explain the absence of false-positive results.

In patient 2 we also detected *M. tuberculosis* complex DNA in both bone marrow and peripheral blood (Table 1). In HIV-positive patients, the sensitivity of IS6110 amplification in peripheral blood is high because of a large amount of mycobacteremia [21]. Condos et al. [22] reported isolation of *M. tuberculosis* in peripheral blood from patients with pulmonary tuberculosis, but this is rare in the extrapulmonary form of the disease. In addition, we have previously shown that bone marrow is a good alternative for molecular detection of *M. tuberculosis* in extrapulmonary disease [13]. This patient was an elderly man who had underlying liver disease due to chronic hepatitis B and who presented with a longstanding disease of 6-month duration. Diagnosis was delayed due to atypical clinical manifestations commonly found in the elderly [23], and that may explain the detection of *M. tuberculosis* in peripheral blood and bone marrow, as a sign of a more disseminated form of the disease.

To conclude, peritoneal tuberculosis should be considered a cause of exudative ascites, particularly in patients with underlying liver disease. Early application of the described PCR protocol in ascitic fluid may be a useful tool for a rapid, safe, and reliable diagnosis, especially when laparoscopy is contraindicated. However, further studies are needed in order to establish the validity of our molecular protocol in diagnosing TP.

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References


