Evaluation of the detection of Mycobacterium tuberculosis with metabolic activity in culture-negative human clinical samples

N. Cubero¹,², J. Esteban³, E. Palenque⁴, A. Rosell² and M. J. Garcia¹

¹) Departamento de Medicina Preventiva, Facultad de Medicina, Universidad Autonoma de Madrid, Madrid, ²) Servicio de Neumología, Hospital Universitario de Bellvitge, L'Hospitalet de Llobregat, Barcelona, ³) Servicio de Microbiología, IIS-Fundacion Jimenez Diaz, Avenida Reyes Catolicos, Madrid and ⁴) Servicio de Microbiología, Hospital General Universitaria Doce de Octubre, Universidad Complutense, Madrid, Spain

Abstract

Mycobacterium tuberculosis is assumed to remain in a quiescent state during latent infection, being unable to grow in culture. The aim of this study was to evaluate the detection of viable but non-cultivable bacilli with metabolic activity in human clinical samples using a procedure that is independent of the immunological status of the patient. The study was performed on 66 human clinical samples, from patients subjected to routine diagnosis to rule out a mycobacterial infection. Specimens from pulmonary and extra-pulmonary origins were verified to contain human DNA before testing for M. tuberculosis DNA, rRNA and transient RNA by real-time quantitative PCR. Clinical records of 55 patients were also reviewed. We were able to detect viable but non-cultivable bacilli with a metabolic activity in both pulmonary and extra-pulmonary samples. Mycobacterium tuberculosis RNA was detected in the majority of culture-positive samples whereas it was detected in one-third of culture-negative samples, 20% of them showed metabolic activity. Amplifications of the ftsZ gene and particularly of the main promoter of the ribosomal operon rrnA, namely PCL1, seem to be good targets to detect active bacilli putatively involved in latent infection. Moreover, this last target would provide information on the basal metabolic activity of the bacilli detected.

Keywords: Latent tuberculosis, quantitative polymerase chain reaction, ribosomal operon rrn-PCL1 promoter, tuberculosis, viable but non-cultivable bacilli

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Introduction

Tuberculosis (TB) is a granulomatous disease caused by the infection by Mycobacterium tuberculosis. The resurgence of TB and its importance as a worldwide cause of morbidity and mortality have focused attention on the need for more rapid and reliable diagnostic procedures [1].

Following infection by the tubercle bacilli progression to disease depends on the ability of the immune system of the patient to resist the pathogen [2]. TB is a disease with complex pathogenic mechanisms, the lung being the main tissue targeted by the infection. However, albeit at a lower frequency, the disease can also affect extra-pulmonary tissues where TB is usually more difficult to diagnose, mainly because of unspecific symptoms and technical difficulties in isolating the bacteria as well as a lower bacillary load [3].

The bacilli, once inside the human host, can cause either a latent disease (about 90% of cases) or an active disease (about 10% of cases), a condition that may also result from the progression of the endogenous reactivation of a latent infection.

Latent TB infection (LTBI) is a state characterized by the presence of a persistent immune response, with absence of symptoms or signs of the disease [4]. Although the clinical definition of LTBI appears clear, the state of the bacteria in
such a condition is poorly characterized [5–7]. The metabolic state of the *M. tuberculosis* during latent infection remains to be characterized and, to date, it is not possible to determine if the bacilli are alive in these circumstances [4]. Traditionally, latent TB is supposed to be caused by viable but non-cultivable bacilli [8] that remain quiescent in the lung granulomas. However, new data indicate that *M. tuberculosis* DNA can be detected in tissues without granulomas [9,10], and the recent dynamic hypothesis of LTBI suggests that the quiescent bacilli may be spread out from the granulomas during latency [11]. This is in accordance with the recent consideration of *M. tuberculosis* infection as a continuous spectrum extending from sterilizing immunity to clinical disease [12].

An LTBI is usually diagnosed by a tuberculin skin test (TST), which has been used for more than a century. However, bacillus Calmette–Guérin vaccinations [13], repeated TST, exposure to other mycobacteria and immunodeficiencies limit the efficacy of TST. New diagnostic tools were developed for latent infections, including in vitro tests to detect specific cell-mediated immune responses against *M. tuberculosis* (interferon-γ release assays), that overcome some of the limitations of TST [14,15]. However, the differentiation between latent and active TB remains difficult [4].

Diagnostic assays based on nucleic acid amplification methods, including PCR, are currently used for a rapid TB diagnosis. However, their usefulness for LTBI diagnosis remains uncertain [16].

The purpose of this study was to evaluate the detection of viable but non-cultivable bacilli with metabolic activity through detection of bacterial transient RNA in clinical samples that were negative for *M. tuberculosis* by using standard microbiological diagnostic procedures. Furthermore, we aimed to develop a procedure of identification of LTBI that was independent of the immunological status of the patient. To this end, we performed quantitative reverse transcription PCR (qRT-PCR) to detect the presence of bacterial RNA in both pulmonary and extra-pulmonary clinical samples.

**Materials and Methods**

**Design of the study**

From February 2006 to June 2008, a total of 103 samples from 90 patients were collected in the laboratories of Hospital Doce de Octubre and Fundacion Jimenez Diaz in Madrid (Spain). Samples were collected from individuals suspected of having mycobacterial disease. Most of the biopsies were sent for mycobacterial culture following the Hospital protocols, regardless of the suspicion of mycobacteriosis.

Clinical records were studied retrospectively. Data considered as putatively related to a mycobacterial infection were collected that included immune status; previous or actual cancer; fever; previous history of TB or contact with TB patients; TST results; and symptoms and signs related to TB.

The study was approved by the Ethical Committees for Clinical Research of the hospitals where the samples were processed.

**Microbiological analysis**

After standard procedures of decontamination with N-acetylcyesteine-NaOH, the samples were inoculated in liquid media (BacT-Alert (BioMérieux, Marcy l’Etoile, France) or MGIT (Becton Dickinson, Franklin Lakes, NJ, USA) according to the Hospital) and solid media (Löwestein–Jensen and Coletos; BioMérieux). Cultures were considered negative when no growth was detected after 45 days at 37°C in liquid media or after 8 weeks in solid media. Positive cultures were confirmed by acid-fast fluorescent stain. Mycobacteria were identified using standard tests including specific molecular probes (AccuProbe; GenProbe, San Diego, CA, USA).

**Total nucleic acid isolation**

Aliquots were collected directly from decontaminated samples, after reception in both clinical microbiology laboratories, in Eppendorf tubes containing guanidium chloride (Merck, Whitehouse Station, NJ, USA) and glass beads (150–212 mm; Sigma, St Louis, MO, USA) and maintained frozen at −70°C until required.

Samples were defrosted and mechanically lysed with Fast Prep (Bio101 Savant), using seven pulses at maximum speed, with 2 min on ice after each pulse. Lysates were extracted once with phenol–chloroform–isoamyl alcohol (25:24:1), followed by three extractions with chloroform–isoamyl alcohol (24:1) [17]. The supernatant was ethanol precipitated overnight at −70°C. After high-speed centrifugation, sediments were resuspended in 20 μL H2O dyethyl pyrocarbonate and 10 μL were used for direct detection of human and bacterial DNA. The remaining 10 μL was reverse-transcribed to 40 μL of cDNA using random primers (Promega, Madison, WI, USA) and Superscript III (Promega). DNAse treatment was not performed to avoid the loss of the material through further purifications.

**Quantitative PCR**

Quantitative PCR was performed using a capillary Light Cycler real-time amplifier and SYBR Green as fluorescent label (Roche, Indianapolis, IN, USA) using primers described in Table I. LightCycler Fast Start DNA master SYBR Green I
TABLE 1. Targets, primers and conditions used in real-time quantitative PCR

<table>
<thead>
<tr>
<th>Targets</th>
<th>Primer name and sequence (5’–3’)</th>
<th>Ann. temp. (°C)</th>
<th>Sizeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>GH20–CATGAGCCAGCACGAGGTAC3</td>
<td>60</td>
<td>268</td>
</tr>
<tr>
<td>IS6110</td>
<td>PC04–CCACATTCACAGAGTTCACC3</td>
<td>68</td>
<td>123</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>165-F–ATAGGCGGCTGGGGTGTGA3</td>
<td>66</td>
<td>120</td>
</tr>
<tr>
<td>mA-PCLI</td>
<td>PCLITB-F–CTGTTTGGTGTTCACTATT3</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>fsz</td>
<td>PCLTB-R–GGGAAAGGGGACCAAAGTGA3</td>
<td>62</td>
<td>71</td>
</tr>
<tr>
<td>hspX</td>
<td>268FL– AGCAGAAAGGCACCGGTCA3</td>
<td>64</td>
<td>60</td>
</tr>
</tbody>
</table>

aAnnealing temperature for each primer set.

bThe size of the amplicons is indicated in base pairs.

reagent (1 μL) was supplemented with 3.5 mM MgCl2 (final concentration) and 0.5 μM of each primer in a 7 μL volume. Sample (3 μL) was added to the mix. PCR cycling programme: denaturation, one cycle of 95°C for 10 min with a transition rate of 20°C/s; amplification, 45 cycles at 95°C for 0 s, the annealing temperature (Table 1) of each primer for 5 s and an extension step at 72°C for 10 s with a single fluorescence acquisition. Specificity of the reaction was verified by analysis of the melting curve and by agarose gel electrophoresis of the final amplified product. Cross-contamination was ruled out using standard manipulation procedures of control.

An arbitrary threshold was set at position one of the log of fluorescence level versus cycle number plot. The Ct value is defined as the cycle number at which the fluorescent level crosses this threshold. Only those samples with Ct value in the upper limit of detection in the Light Cycler system (100 copies of M. tuberculosis H37Rv genome equivalent corresponding to 0.5 pg) were considered positive.

Targets analysed by quantitative PCR

Only the samples that amplified the β-globin eukaryotic gene [18] were tested for the presence of bacterial nucleic acids. Presence of M. tuberculosis DNA in the samples was determined by detection of IS6110 [19] a multi-copy target of this bacterium. These values were used to evaluate the potential bacterial DNA contamination in the cDNA. To do so, ten copies of the IS6110 were considered as a mean value that was subtracted from the amount of cDNA obtained in the corresponding sample.

Four transcripts were amplified in the cDNA: 16S rRNA [20]; mA-PCLI [21]; fsz gene [22]; and hspX gene [22]. Targets and primers (Roche) used for the amplifications are summarized in Table 1. All primers are specific for M. tuberculosis, except for 16S rRNA which are common for all mycobacteria. Each of the cDNA amplifications was performed in duplicate.

Results

Detection performances in the samples using quantitative PCR

In all, 103 specimens were collected including a higher number of samples from extra-pulmonary (58) than from pulmonary (45) origins. All samples were tested for the presence of eukaryotic DNA by qPCR. Thirty-seven samples did not amplify that target, probably because of the presence of PCR inhibitors. Other unknown problems may have occurred as a result of the unplanned collection or during the manipulation of the samples. The 66 samples positive for human DNA were then studied to detect the presence of M. tuberculosis DNA and RNA (Table 2). Complete data are summarized in Supplementary material, Table S1.

The bacterial IS6110 was detected in 19 samples and mycobacterial cDNA was amplified from 35 samples (Table 2). As expected, cDNA was detected in a higher number of samples than DNA (Table 2).

Table 3 shows the PCR results according to the anatomico-cultural origin of the samples and culture results. The M. tuberculosis nucleic acids were detected in over 75% of culture positive samples and over 17% of culture negative samples. Mycobacterial nucleic acids were found in culture negative samples from both pulmonary and extra-pulmonary samples.

Mycobacterium tuberculosis cDNA was amplified in 30.2% of the culture negative samples (16/53), including 20.8% of samples carrying bacilli with evidence of transient RNA activity (11/53) (Table 3).

TABLE 2. Detection of mycobacterial nucleic acids in β-globin-positive clinical samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary</td>
<td>Culture-positive</td>
<td>8</td>
<td>5 (62.5)</td>
</tr>
<tr>
<td></td>
<td>Culture-negative</td>
<td>20</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Extra-pulmonary</td>
<td>Culture-positive</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td></td>
<td>Culture-negative</td>
<td>33</td>
<td>3 (9.1)</td>
</tr>
<tr>
<td>Total</td>
<td>Culture-positive</td>
<td>13</td>
<td>10 (76.9)</td>
</tr>
<tr>
<td></td>
<td>Culture-negative</td>
<td>53</td>
<td>9 (17)</td>
</tr>
</tbody>
</table>

Number of PCR-positive samples. Percentages corresponding to total values of each (culture-positive, culture-negative and total samples) are indicated in parentheses (%).

Seven extra-pulmonary culture-negative samples that were PCR-negative for mycobacterial DNA and amplified 16SrRNA as single RNA target were considered to represent detection of non-tuberculous mycobacteria and were labelled as [NTM].

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The 16S rRNA was detected as the single mycobacterial target only in culture-negative extra-pulmonary samples (Table 3). The seven culture-negative samples amplifying 16S rRNA as the unique target were considered as putative non-tuberculous mycobacteria carriers (indicated as [NTM] in Table 3) and were not included in the viable but non-cultivable analysis presented in the rightmost column of Table 3. Non-tuberculous mycobacteria were confirmed by sequencing of the 16SrRNA in three of these samples (data not shown). That detection in extra-pulmonary samples agreed with previous publications [23,24].

The comparison of the detection of cDNA according to the transcripts amplified showed, as expected, that all but one culture-positive sample was amplified whereas only one-third of the culture-negative samples were amplified (Table 4). Transcripts involved in the active growth of the bacteria (PCL1 and ftz) were more frequently amplified in culture-positive samples, indicating the presence of actively dividing bacilli. The 16S rRNA was most frequently detected in culture-negative samples (Table 4).

**Discussion**

We studied the presence of *M. tuberculosis* with metabolic activity in culture-negative samples from those received for routine diagnosis from two different hospitals. No selection criteria of the samples were established, allowing the collection of pulmonary and extra-pulmonary specimens. Only the

### TABLE 3. Detection of mycobacterial nucleic acids in β-globin-positive clinical samples according to the anatomical origin

<table>
<thead>
<tr>
<th>Samples</th>
<th>Culture+a +/-</th>
<th>IS6110</th>
<th>16SrRNA only</th>
<th>Any target</th>
<th>Mtb VBNCb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum (n = 9)</td>
<td>8/1</td>
<td>5/1</td>
<td>0/0</td>
<td>7/1</td>
<td>1</td>
</tr>
<tr>
<td>Bronchial (n = 17)</td>
<td>0/17</td>
<td>0/5</td>
<td>0/5</td>
<td>0/1</td>
<td>6</td>
</tr>
<tr>
<td>Other (n = 2)</td>
<td>0/2</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0</td>
</tr>
<tr>
<td>Total pulmonary (n = 28)c</td>
<td>8/20</td>
<td>5 (62.5)/6 (30)</td>
<td>0/5 (25)</td>
<td>7 (87.5)/2 (10)</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Adenopathy (n = 6)</td>
<td>3/3</td>
<td>3/0</td>
<td>1/1</td>
<td>1/1</td>
<td>1</td>
</tr>
<tr>
<td>Liquids (n = 6)d</td>
<td>0/6</td>
<td>0/0</td>
<td>0/1 [NTM]</td>
<td>0/3</td>
<td>3</td>
</tr>
<tr>
<td>Pleura (n = 8)</td>
<td>1/7</td>
<td>1/1</td>
<td>1/0</td>
<td>1/0</td>
<td>0</td>
</tr>
<tr>
<td>Synovia (n = 5)</td>
<td>0/5</td>
<td>0/0</td>
<td>0/2 [NTM]</td>
<td>0/1</td>
<td>1</td>
</tr>
<tr>
<td>Othere (n = 13)</td>
<td>1/12</td>
<td>1/2</td>
<td>0/3 [NTM]</td>
<td>1/4</td>
<td>4</td>
</tr>
<tr>
<td>Total extra-pulmonary (n = 38)f</td>
<td>5/33</td>
<td>5 (100)/3 (9.1)</td>
<td>1 (20)/7 [NTM] (21.2)</td>
<td>4 (80)/9 (27.3)</td>
<td>9 (27.3)</td>
</tr>
<tr>
<td>Total samples (n = 66)</td>
<td>13/53</td>
<td>10 (77)/9 (17)</td>
<td>1 (7.7)/12 (22.6)</td>
<td>11 (84.6)/11 (20.8)</td>
<td>16 (30.2)g</td>
</tr>
</tbody>
</table>

Number of samples that were culture-positive (+) or culture-negative (–) are indicated as +/-.

1Other pulmonary samples: one biopsy; one abscess.

2Total results globally and for pulmonary and extra-pulmonary samples are indicated. Percentages corresponding to total values of each (culture-positive or culture-negative) are indicated in parentheses (%).

3Samples negative for IS6110 and positive for 16S rRNA as single target were considered to represent non-tuberculous mycobacteria [NTM].

4Liquid extra-pulmonary samples: three urine; three cerebrospinal fluid.

5Other extra-pulmonary samples: nine biopsies; two abscesses.

6This value includes five pulmonary culture-negative samples that amplified 16S rRNA as single RNA mycobacterial target and also amplified *M. tuberculosis* DNA, therefore they were not considered to carry non-tuberculous mycobacteria. The value corresponds to 22.7% of the total setting.

### TABLE 4. Detection of mycobacterial RNA in β-globin-positive clinical samples according to the targets studied

<table>
<thead>
<tr>
<th>Culture</th>
<th>16SrRNA</th>
<th>mAPCL1</th>
<th>ftz</th>
<th>hspX</th>
<th>PCR+ samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (n = 13)</td>
<td>7 (53.8)</td>
<td>11 (84.6)</td>
<td>10 (77)</td>
<td>6 (46.1)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td>Negative (n = 53)</td>
<td>11 (20.8) [NTM] (13.2)</td>
<td>9 (17)</td>
<td>5 (9.4)</td>
<td>5 (9.4)</td>
<td>16 (30.2) 7 [NTM] (13.2)</td>
</tr>
<tr>
<td>Total (n = 66)</td>
<td>25 (37.9)</td>
<td>20 (30.3)</td>
<td>15 (22.7)</td>
<td>11 (16.7)</td>
<td>28 (42.4)</td>
</tr>
</tbody>
</table>

Number of samples qRT-PCR positive. Percentages corresponding to total values of each (culture-positive or culture-negative) are indicated in parentheses (%).

[NTM], non-tuberculous mycobacteria.
The main limitation of our study was the number of samples available. However, this limitation may reinforce the hypothesis that the number of samples containing viable but non-cultivable bacilli may be increased in a controlled study.

Another limitation was that the level of DNA contaminating the cDNAs was unknown, and could only be approximated through the level of IS6110 detected (see Materials and methods). We have no clear solution to solve this problem because it is recommended to avoid DNase treatment to minimize the loss of this scarce material during further purifications.

Finally even though this is a rare event except for some South Asian countries [29], the possibility of viable but non-cultivable M. tuberculosis bacilli with a low or zero copy number of IS6110 in their genomes should also be considered. Other IS multi-copy elements could be targeted instead for DNA detection when these circumstances are suspected.

The detection of mycobacterial cDNA in pulmonary and extra-pulmonary culture-negative samples agreed with previous data suggesting that M. tuberculosis may establish a latent infection in both types of anatomical locations with or without granulomas [8,9,30,31].

Concluding Remarks

According to our results, we suggest that the PCL1 promoter of the rRNA operon of M. tuberculosis may be a target of interest to detect viable and metabolically active bacilli in culture-negative samples.

The advantages of this detection procedure to test for LTBI would be to avoid the secondary effects that may be observed with TST. Moreover, it would not be influenced by the immune system state of the patient as observed with TST and interferon-γ release assays can be used advantageously in children and can be performed on every type of sample, of either pulmonary or extra-pulmonary origin.

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Transparency Declarations

All authors declare no potential conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical data of patients and microbiological data of corresponding samples qPCR positive for the eukaryotic gene β-globin.

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References