Evaluation of conventional molecular diagnosis of *Mycobacterium tuberculosis* in clinical specimens from Morocco

Fathiah Zakham¹,²,³, Halima Bazoui¹, Mohammed Akrim¹, Sanœe Lamrabet¹, Ouafae Lahlou¹, Mohamed Elmzibri⁴, Abdelaziz Benjouad⁵, My Mustapha Ennaji²*, Rajae Elaouad¹*

¹ My M. Ennaji and Rajae Elaouad share senior authorship in this study (equal contribution).

1 Institut National d’Hygiène, Rabat, Morocco
2 Laboratoire de Virologie et Hygiène et Microbiologie, Faculté des Sciences et Techniques, Mohammedia, Morocco
3 Laboratoire de Biochimie et Immunologie, Faculté des Sciences, Université Mohammed V- Agdal, Rabat, Morocco
4 Unité de Biologie et Recherches Médicales, Centre National de l’Energie, des Sciences et Techniques Nucléaires, Rabat, Morocco

Abstract

Introduction: Tuberculosis is a major public health threat, annually affecting new individuals worldwide, especially those in developing countries. Rapid detection of the agent and effective treatment are two important factors in controlling this disease.

Methodology: The present study aimed to evaluate polymerase chain reaction (PCR) as a rapid and direct molecular method for the diagnosis of *Mycobacterium tuberculosis* (MTB) in 70 clinical specimens (62 sputum samples, six cerebrospinal fluids, and two biopsies) using heat shock protein (*hsp65*) as the gene target. Automated sequencing of the same gene was used for the identification of MTB to the species level.

Results: The sensitivity of PCR was 81.13%, with specificity of 88.24%; the positive and negative predictive values were 95.56% and 60%, respectively.

Conclusion: Based on these results, the *hsp65* gene sequence can be used to differentiate the members of MTB complex from non-tuberculosis mycobacteria (NTM).

Key words: *Mycobacterium tuberculosis*; polymerase chain reaction; PCR; *hsp65* target gene; diagnosis


(Received 16 January 2011 – Accepted 17 May 2011)

Copyright © 2012 Zakham et al. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

The World Health Organization (WHO) reports that, tuberculosis (TB) remains a major public health problem as a first-line infectious disease [1].

It is estimated that one-third of the global population is infected with *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis, and that approximately between 8.9 and 9.9 million new cases of tuberculosis arise annually and cause over 1.3 million deaths among HIV-negative people and 0.38 million deaths among HIV-positive people [1].

In Morocco, TB remains a major public health issue, even though the results of the antituberculosis program were positive, as demonstrated by the decrease in new cases, and 25,562 cases were recorded between 2000 and 2007 [2]. However, effective care for MTB-infected individuals requires an effective diagnosis.

The conventional laboratory diagnosis of tuberculosis is based on the method of Ziehl-Neelsen acid–fast bacilli stain and culture of MTB [3-5]. The Ziehl-Neelsen stain is a rapid and cheap method, but it lacks sensitivity. The culture requires time and viable microorganisms, which are difficult to obtain, especially in treated patients. Several rapid methods for MTB diagnosis, such as DNA probes that require sophisticated equipment [6], have been developed. The polymerase chain reaction (PCR) is an alternative method that can amplify a small fragment of DNA with high specificity for the diagnosis of infectious diseases [7]. PCR has recently been used to detect MTB in respiratory samples [8-10] and other clinical specimens [11-14].

In this study, conventional PCR was used as a rapid and direct molecular method for the detection of MTB in the clinical specimens. For this purpose the *hsp65* gene was targeted; because this gene is
highly conserved among mycobacterial species and present in all mycobacteria, it is more variable than the 16S rRNA gene sequence and for this reason it is a potential tool for the identification of genetically related species of mycobacteria [15,18].

Automated sequencing of the same gene was used for the identification of MTB to species level. The results were then compared with the results of the conventional methods to evaluate the direct PCR diagnosis of MTB in clinical samples.

Methodology

Sampling

A total of 70 (62 sputum samples, six cerebrospinal fluid samples and two biopsies) specimens were collected and sent to the laboratory of Medical Microbiology at the National Institute of Hygiene (NIH) in Rabat, Morocco. The specimens were examined using the Ziehl-Neelsen method; bacterial culturing was performed according to the Petroff method, which includes decontamination of sputa, then neutralization and inoculation onto Lowenstein Jensen (LJ) medium [19]. Species identification included a macroscopic analysis of colonies on LJ medium and a microscopic analysis. In addition, complementary niacin, nitrate reductase, and catalase tests at room temperature and at 68°C were performed to confirm the MTB species [19].

For PCR, sputum samples were first liquefied and then decontaminated by adding 2 ml of NaOH 40g/L (4%), after which they were vortexed and incubated at 37°C for 20 minutes and 18 ml of neutral buffer (3.40 g KH₂PO₄ + 3.55g Na₂HPO₄/ L, [pH 6.8]) were added. After centrifugation at 3500g for 20 minutes, the supernatant was discarded. Finally, 1 ml of the same neutral buffer was added and after shaking, the specimens were stored at -20°C until testing.

Bacterial lysis

MTB bacteria were lysed using the heat shock treatment. The specimen was first thawed and centrifuged at 6,000g for one minute. The supernatant was discarded and the pellet was resuspended in 200 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The mixture was then vortexed and placed in a boiling water bath for 15 minutes to inactivate bacteria and release DNA. After centrifugation at 16,000 g for five minutes, an aliquot of 100 μl of the supernatant was transferred to a sterile tube and stored at -20°C for PCR testing [20].

PCR for amplification of hsp65 gene

The forward primer Tb11 (5’ ACCAACGATGGTGTGG TCCAT-3’) and the reverse primer Tb12 (5’ CTTGTCGAACCCGCATACCCT-3’) were used to amplify a 441-bp portion of the hsp65 gene [17]. A final volume of 100 μl of the Master Mix (Roche Diagnostic, Manheim, Germany) for the PCR reaction mixture contained 1x PCR buffer, 25 mM MgCl₂, 2.5 mM each of deoxynucleoside triphosphates (i.e., deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate), 0.5 mM each, primer Tb11 and primer Tb12, and five units of Taq DNA polymerase enzyme (Roche Diagnostic, Manheim, Germany). The DNA from the clinical samples was added, and the volume increased with autoclaved distilled water. Each set of PCR reactions contained a positive control containing DNA from the H37Rv strain of MTB and a negative control containing the same amount of autoclaved water. The thermal cycler (Gene Amp, PCR system 9700, Applied Biosystems, Foster City, US) was programmed for 35 cycles with initial denaturation at 94°C for four minutes. Each cycle was performed with denaturation at 94°C for one minute, annealing at 64°C for one minute with an extension at 72°C for two minutes. At the end of the last cycle, the mixtures were incubated at 72°C for 10 minutes.

Visualisation of the PCR products

The amplified product was submitted to electrophoresis on a 1.5% agarose gel in 1X Tris-borate-EDTA buffer at pH of 8.6. The gel was stained with ethidium bromide of 10 mg/ml 2 μl in 100 ml of 1X Tris-borate-EDTA, and the 441-bp amplified band was visualized on an ultraviolet transilluminator to check for DNA amplification.

Sequencing reaction

Direct sequencing of amplified PCR products was performed on an ABI PRISM sequencing apparatus (ABPRISM 310 Genetic Analyser, Applied Biosystems) using Big Dye Terminator kit (Applied Biosystems) that includes didesoxynucleotides marked with four fluorochromes of different colours. For each PCR product, both strands were sequenced, in independent reactions, using Tb11 or Tb12 primers.
Analysis of the sequences

Data analysis was performed by sequencing analysis software. The sequence consensus of the gene hsp65 was entered into the database of the Mycobacterium available on the Basic Local Alignment and Research Tool (BLAST) program to compare the obtained sequences with those of other mycobacterial species in GenBank. The analysis of the sequences on the site provides information about the sub-type of Mycobacterium spp.

The resulting electrophoregrams were manually edited to ensure sequence accuracy and added to the alignment component of MEGA software version 4.

Results

Amplification of mycobacterial DNA

This study was performed on 70 clinical specimens. As shown in Table 1, conventional analyses, including Ziehl-Neelsen staining and bacterial culture, showed the presence of MTB infection in 53 samples (75.71%). Molecular MTB infection was based on the presence of a 441 bp band corresponding to the hsp65 gene amplification after electrophoresis. Using the direct PCR approach, 45 specimens, including 44 sputum samples and one cerebrospinal fluid, were positive (64.29%) (Table 1).

A comparison of MTB detection in the 70 specimens using conventional and molecular techniques is reported in Table 2.

Concordance of results was found in 58 clinical specimens: 43 were specimens MTB positive and 15 were MTB negative. A total of 10 samples were positive in culture and negative in PCR. The two samples that were positive by PCR and negative in culture were subject to further analyses for confirmation. Sequence analysis of the two strains showed that both were affiliated with the MTB complex, and thus considered true positives. PCR technique had good sensitivity (81.13%) and good specificity (88.24%) with the positive and negative predictive values of 95.56 % and 60%, respectively.

We may suggest that those results are comparable to those of the culture method which is accepted as the gold standard.

Automated partial DNA sequencing of the hsp65 gene and sequence alignment

The PCR products of the hsp65 gene from 10 isolated strains were sequenced. The sequences were compared with available DNA sequence databases using the BLAST program. Results showed that the 10 strains present a high percentage of similarities to both M. tuberculosis and M. bovis (Table 3).

The hsp65 gene sequences from the 10 isolates were aligned with the sequence from the MTB H37 reference strain (Figure 1) and analysed by MEGA software version 4. Results showed high similarity, confirming the BLAST analyses. Those sequences are available in GenBank at NCBI and their accession numbers are reported in Table 3.

Discussion

Tuberculosis is a major public health problem, annually affecting new individuals, especially in developing countries. According to the last report of the Ministry of Health, tuberculosis remains one of the first line of infectious diseases in Morocco, with an incidence of 82.1 new cases per 100,000 inhabitants in 2007 [2]. Tuberculosis especially affects young adults and thus has a high impact on the socio-economic status of the country. In fact, 65.9% of total TB cases are between 15 and 45 years of age, with a significant male predilection (59.3%).

Worldwide, the main problems in TB management remain the early diagnosis of MTB and drug-resistance testing. Rapid diagnosis and appropriate chemotherapy become the first priorities and a serious challenge in the improvement of TB treatment and the reduction of the dissemination of MTB strains. Currently, TB screening is done by conventional techniques, including the method of Ziehl-Neelsen staining and MTB culture.
Table 1. Detection of MTB by direct examination (ZN staining), culture, and PCR in different kinds of specimens

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>Total number of specimens</th>
<th>ZN staining</th>
<th>Culture</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Sputum</td>
<td>62</td>
<td>52</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>Biopsy</td>
<td>2</td>
<td>Nil</td>
<td>2</td>
<td>Nil</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

MTB: *Mycobacterium tuberculosis*, PCR: Polymerase Chain Reaction

Table 2. Comparison of MTB detection by conventional and molecular techniques

<table>
<thead>
<tr>
<th>Techniques of detection of MTB</th>
<th>Conventional technique (culture)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Molecular technique (Direct PCR)</td>
<td>43</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>17</td>
</tr>
</tbody>
</table>

MTB: *Mycobacterium tuberculosis*, PCR: Polymerase Chain Reaction

Table 3. The BLAST results of identification of 10 PCR products by automated sequencing of the gene *hsp65*.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Sequenced product</th>
<th>NCBI GenBank Accession number</th>
<th>Percentage of similarity to <em>M. Tuberculosis</em></th>
<th>Percentage of similarity to <em>M. bovis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 1</td>
<td>408</td>
<td>JF921162</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Strain 2</td>
<td>378</td>
<td>JF921154</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Strain 3</td>
<td>397</td>
<td>JF921155</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Strain 4</td>
<td>381</td>
<td>JF921153</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Strain 5</td>
<td>369</td>
<td>JF921156</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Strain 6</td>
<td>377</td>
<td>JF921160</td>
<td>98%</td>
<td>98%</td>
</tr>
<tr>
<td>Strain 7</td>
<td>381</td>
<td>JF921157</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Strain 8</td>
<td>399</td>
<td>JF921158</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Strain 9</td>
<td>310</td>
<td>JF921159</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Strain 10</td>
<td>376</td>
<td>JF921161</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>

BLAST: Basic Local Alignment and Research Tool, PCR: Polymerase Chain Reaction, gene *hsp65*: gene of heat shock protein 65, NCBI: National Center for Biotechnology Information
Unfortunately, the Ziehl-Neelsen staining lacks sensitivity, and bacteria culture is time-consuming [6]. There is a clear need to implement reliable molecular techniques for the detection of all forms of TB strains to improve the management of TB. Molecular techniques are rapid and prevent crucial delays. In the last decade, advances in molecular biology have made it possible to use rapid and specific techniques to detect MTB DNA in samples. The current study was planned to evaluate the use of the PCR technique to detect MTB DNA directly from clinical specimens.

It is widely accepted that PCR is a new and rapid technique for the diagnosis of bacterial DNA with high specificity, even in patients who have undergone antibiotic treatment because the DNA is still detectable in the absence of a viable microorganism [7].

The sensitivity of PCR is largely dependent on the efficiency of the DNA extraction procedure [21].

Successful amplification of mycobacterial DNA targets is a challenge when biological specimens such as sputum contain several inhibitors of the PCR reaction. In this study, the PCR technique was evaluated for the detection of limited mycobacteria cells, from clinical specimens, by using the target gene hsp65. Among the 70 tested cases, 45 strains were detected by PCR, providing good sensitivity and specificity, as compared to the conventional technique. The specificity (88.24%) and sensitivity (81.13%) of the detection of MTB from clinical specimens by PCR are in concordance with the overall specificity and sensitivity of the PCR technique as reported by Zamirian et al. [14]. Moreover, the sensitivity of the detection of MTB by PCR from clinical specimens was reported to range between 55% and 90% [3]. Furthermore, many studies, using different commercial amplification systems to detect the mycobacterial DNA, have used cultured bacteria as the gold standard [22-24].

We were not able to cultivate two strains which were PCR positive, and this may possibly be related to improper decontamination procedures or inhibition of growth due to antimicrobial treatment.

The main limitations of the PCR technique are the false-negative results (10 strains in this study), which can be attributed to either the paucibacillary nature of the specimen, the inefficient extraction of the DNA, or the presence of PCR inhibitors. The presence of PCR inhibitors has been reported in sputum, pus samples and tissue biopsies [25,26].

The molecular approach based on amplification of MTB DNA by PCR in clinical specimens is a valuable screening method, especially when the limitations of the conventional techniques have a high impact on the patient health; for example, when there is potential for disease relapse or therapeutic failure and in HIV-seropositive patients.

The target gene hsp65 is present in all mycobacteria. It is more variable than the 16S rRNA gene and is the most conserved gene among the mycobacterial species. In this study, sequence analyses showed that DNA extracted from clinical specimens are related to the MTB complex. Moreover, hsp65 gene sequencing is a potential tool for differentiating between mycobacteria at the species level, even in the genetically related species, based on PRA and DNA sequencing studies [15-18]. Therefore, the hsp65 gene could be used for differentiating between the MTB complex and other non-tuberculosis mycobacteria.

The results of this study demonstrate that all the sequenced products share the consensus sequence, and that there is a high similarity between them with the species M. tuberculosis and M. bovis, which belong to the MTB complex and share the same ancestor in their evolutionary events as previously reported [27,28].

These results suggest that the automated sequencing of the hsp65 gene PCR product is one of the most reliable techniques for confirming the laboratory diagnosis of MTB complex. It could be a potential tool in differentiating between the members of MTBc from other non-tuberculosis mycobacteria [16,18]. However, discrimination between members of MTB complex, especially between MTB and M. bovis, must combine hsp65 and pncA gene sequencing [29].

In conclusion, a molecular approach, based on the amplification of hsp65 gene by PCR, is a reliable and rapid method that could be used to detect MTB strains in clinical specimens.

Acknowledgments
The authors thank H. Eloudiyi, M. Melloul, M. Terta and the staff of the Department of Medical Microbiology in the Institute of Hygiene in Rabat, Morocco.

References
Conflict of interests: No conflict of interests is declared.

Corresponding author: Zakham F, Department of Virology and Microbiology, Faculty of Medicine, University of Casablanca, Morocco. Email: zakham@unicas.ac.ma