Original Article

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

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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: *Mycobacterim tuberculosis*; polymerase chain reaction; PCR; *hsp65* target gene; diagnosis

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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 identificaiton 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 KH_2PO_4 + 3.55g Na_2HPO_4/ 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 6,000*g* 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' ACCAACGATGGTGTG TCCAT-3') and the reverse primer

Tb12 (5' CTTGTCGAACCGCATACCCT-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 1× PCR buffer, 25 mm MgCl, 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.

Figure 1. Consensus sequence of the *hsp65* gene obtained by MEGA software version 4

1	ATCGGCGCCGAGCTGGTCAAAGAGGTAGCCAAGAAGACCGATGACGTCGC	50
51	CGGTGACGGCACCACGACGGCCACCGTGCTGGCCCAGGCGTTGGTTCGCG	100
101	AGGGCCTGCGCAACGTCGCGGCCGGCGCCAACCCGCTCGGTCTCAAACGC	150
151	GGCATCGAAAAGGCCGTGGAGAAGGTCACCGAGACCCTGCTCAAGGGCGC	200
201	CAAGGAGGTCGAGACCAAGGAGCAGATTGCGGCCACCGCAGCGATTTCGG	250
251	CGGGTGACCAGTCCATCGGTGACCTGATCGCCGAGGCGATGGACAAGGTG	300
300	GGCAACGAGGGCGTCATCACCGTCGAGGAGTCCAACACCTTTGGGCTGCA	350
351	GCTCGAGCTCACCGAGGG	368

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 *Mycobacterim* 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

Type of specimen	Total number of specimens	ZN staining		Culture		PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
Sputum	62	52	10	52	10	44	18
Biopsy	2	Nil	2	Nil	2	Nil	2
Cerebrospinal fluid	6	1	5	1	5	1	5

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

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

Techniques of detection of MTB		Conventional technique (culture)		Total
		Positive	Negative	
Molecular technique (Direct PCR)	Positive	43	2	45
	Negative	10	15	25
Total		53	17	70

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*.

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

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 *hsp 65*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.

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References

1. WHO Report (2010) Global Tuberculosis Control: epidemiology, strategy, financing. WHO/HTM/TB/2010. 300. www.who.int/tb/publications/global-report/2010.
2. DELM « Direction de l'épidémiologie et de lutte contre les maladies » (2007) Epidemiologic bulletin. Kingdom of Morocco. Ministry of Health N°:69-70-71-72. P: 32-33.

3. Brooks, GF, Butel, JS, and Morse, SA (1998) Jawetz, Melnick, Adelberg's Medical Microbiology. In Brooks GF, Butel JS and Morse SA, editors. Mycobacteria. 21st edition, United States of America: Lange Press. 279-283.
4. Gupte, S (1999) Short Textbook of medical microbiology, In Gupte, S, editor. Mycobacteria. 7th edition. New Delhi: Jaypee Press, 264-283.
5. Cheesbrough, M (2000) District laboratory practice in tropical countries. In Cheesbrough, M, editor. Mycobacterium tuberculosis. 2nd part. Cambridge: Cambridge University Press. 207-211.
6. Nagesh BS, Sehgal S, Jindal SK, and Arora, SK (2001) Evaluation of Polymerase Chain Reaction for Detection of *M. tuberculosis* in Pleural Fluid. Chest 119: 1737-1741.
7. Rebollo MJ, Garrido RS, Folgueir D, Palenque E, Lumbreras C, Aguado JM (2006) Blood and urine samples as useful sources for the direct detection of tuberculosis by polymerase chain reaction. Diagn Microbiol Infect Dis 56: 141-146.
8. Jonas V, Alden MJ, Curry JI, Kamisango K, Knot CA, Lankford R, Wolfe JM, and Moore DF (1993) Detection and Identification of *M. tuberculosis* Directly from Sputum Sediments by Amplification of rRNA. J Clin Microbiol 31: 2410-2416.
9. Kim K, Lee H, Lee MK, Lee SA, Shim TS, Lim SY, Koh WJ, Yim JJ, Munkhtsetseg B, Kim W, Chung SI, Kook YH, Kim BJ (2010) Development and application of multiprobe real-time PCR method targeting the hsp 65 gene for differentiation of *Mycobacterium* species from isolates and sputum specimens. J Clin Microbiol 48: 3073-3080.
10. Soo PC, Horng YT , Hsueh PR, Shen BJ, Wang JY, Tu HH, Wei JR, Hsieh SC , Huang CC, Lai HC (2006) Direct and Simultaneous Identification of *M. tuberculosis* complex (MTBc) and M. tuberculosis (MTB) by Rapid Multiplex nested PCR-ICT assay. J Microbiol Methods 66: 440-448.
11. Yam WC, Cheng VC., Hui WT, Wang LN, Seto WH, Yuen KY (2004) Direct detection of *M. tuberculosis* in clinical specimens using single-tube biotinylated nested polymerase chain reaction-enzyme linked immunoassay (PCR-ELISA). Diagn Microbiol Infect 48: 271-275.
12. Restrepo BI, Gomez I, Shipley G, McCormick JB, Fisher-Hoch SP (2006) Selective enrichment and detection of mycobacterial DNA in paucibacillary specimens. J Microbiol Methods 67: 220-229.
13. Takahashi T, Tamura M, Takahashi SN, Matsumoto K, Sawada S, Yokoyama, E, Nakayama T, Mizutani T, Takasu T, Nagase H (2007) Quantitative nested real-time PCR assay for assessing the clinical course of tuberculous meningitis. J Neuro Sci 255: 69-76.
14. Zamirian M, Mokhtarian M, Motazedian MH, Monabati A, Rezaian GR (2007) Constrictive pericarditis: Detection of *M. tuberculosis* in paraffin-embedded pericardial tissues by polymerase chain reaction. Clin Biochem 40: 355-358.
15. Telenti A, Marchesi F, Balz M, Bally F, Bottger E, Bodmer T (1993) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 31: 175-178.
16. Douglas S, Swanson D, Pan XI , Musser JM (1996) Identification and Subspecific Differentiation of *M. scrofulaceum* by Automated Sequencing of a Region of the Gene (hsp65) Encoding a 65-Kilodalton Heat Shock Protein. J Clin Microbiol 34: 3151-3159.
17. Taylor TB, Patterson C, Hale Y, and, Safranek WW (1997) Routine Use of PCR-Restriction Fragment Length Polymorphism Analysis for Identification of Mycobacteria Growing in Liquid Media. J Clin Microbiol 35: 79-85.
18. Ringuet H, Akoua-Koffi C, Honore S, Varnrot A, Vincent V, Berche P, Gaillard JL, and Pierre AC (1999) Hsp65 Sequencing for Identification of Rapidly Growing Mycobacteria. J Clin Microbiol 37: 852-857.
19. David H, Levy Frebault V, Thorel MF (1987). Methodes de Laboratoire pour Mycobactériologie Clinique. Unité de tuberculose et des mycobacteries, Institut Pasteur Paris: Institut Pasteur Press 87 p.
20. Aldous WK, Pounder JI, Cloud JL, Woods GL (2005) Comparison of Six Methods of Extracting *M. tuberculosis* DNA from Processed Sputum for Testing by Quantitative Real-Time PCR. J Clin Microbiol 43: 2471-2473.
21. Amita J, Vandana T, Guleria RS, Verma RK (2002) Qualitative Evaluation of Mycobacterial DNA Extraction Protocols for Polymerase Chain Reaction. Molecular Biology Today 3: 43-50.
22. Carpentier E, Droullard B, Dailloux M, Moinard D, Vallee E, Dutilh B, Maugen J, Bergogne E, Caronnelle B (1995) Diagnosis of Tuberculosis by Amplicor *M. tuberculosis* Test: a Multicenter Study. J Clin Microbiol 33: 3106-3110.
23. Piersimoni C, Callegaro A, Nista D, Bornigia S, De Conti F, Santini G, De Sio G (1997) Comparative Evaluation of Two Commercial Amplification Assays for Direct Detection of *M. tuberculosis* Complex in Respiratory Specimens. J Clin Microbiol 35: 193-196.
24. Scarparo C, Piccoli P, Rigon A, Ruggiero G, Scangelli M, Piersimoni C (2000) Comparison of Enhanced *M. tuberculosis* Amplified Direct Test with COBAS AMPLICOR Mycobacterium tuberculosis Assay for Direct Detection of *M. tuberculosis* Complex in Respiratory and Extrapulmonary Specimens. J Clin Microbiol 38: 1559-1562.
25. Amicosante M, Richeldi L, Trenti G, Paone G, Campa M, Bisetti A, Saltini C (1995) Inactivation of polymerase inhibitors for *M. tuberculosis* DNA amplification in sputum by using capture resin. J Clin Microbiol 33: 629-630.
26. Kesarwani RC, Pandey A, Misra A, Singh AK (2004) Polymerase chain reaction (PCR): Its comparison with conventional techniques for diagnosis of extra-pulmonary tubercular diseases. Indian Journal of Surgery 66: 84-88.
27. Zakham F, Belayachi L, Ussery D, Akrim M, Benjouad A, El Aouad R, Ennaji M (2011) Mycobacterial species as Case study of comparative genome analysis. Cell. Mol. Biol. 57: 1462-1469.
28. Cole, ST, Comparative and functional genomics of the *M. tuberculosis* complex. Microbiology. 148: 2919-2928.
29. Bannalikar AS and Verma R (2006) Detection of *Mycobacterium avium & M. tuberculosis* from human sputum cultures by PCR-RFLP analysis of *hsp65* gene & *pncA* PCR. Indian J Med Res 123: 165-172.

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