

# Identification of *Mycobacterium tuberculosis* in the cerebrospinal fluid of patients with meningitis using nested PCR

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**Abstract.** Tuberculous meningitis (TBM) is the most severe form of tuberculosis. It is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*; MT) and it is very difficult to diagnose. The symptoms are similar to other infectious neurological diseases, such as neurocysticercosis, neuroborreliosis, or herpes viral infection. The aim of this study was to identify tuberculosis (TB) in cases of meningitis with clinical and laboratory evidence suggestive of TBM, and to confirm our findings with molecular tests for TB infection. We recruited patients with neurological symptoms who were examined at the neurology services of Hospitals of Instituto Mexicano del Seguro Social (IMSS) in Mexico City. A total of 144 consecutive patients with suggestive infectious meningitis were initially included; 94 cases of meningitis with clinical and laboratory evidence suggestive of TBM were included, but only 50 of these cases fulfilled the criteria for probable TBM. As the controls, we included 50 cases of meningitis with clinical and laboratory evidence suggestive of non-TBM. Cerebrospinal fluid (CSF) was collected from all 100 patients (cases and controls) and tested for TB by multiplex and nested PCR analyses. Nested PCR detected 0.1 fg of *M. tuberculosis* DNA. TB infection was confirmed with molecular tests in 49 patients from the 50 cases suggestive of TBM and in 1 of the 50 non-TBM cases. The analysis exhibited a sensitivity of 98.0%, a specificity of 92.0%, a positive predictive value of 88.0% and a negative predictive value of 98.0%. The use of CSF for the analyses proved to be effective for the rapid diagnosis

of TBM using a developed system of multiplex and nested PCR analyses in patients presenting neurological symptoms.

## Introduction

Tuberculosis (TB) remains among the leading infectious causes of death in developing nations, with *Mycobacterium tuberculosis* (*M. tuberculosis*; MT) as the species responsible for most of these deaths (1,2). According to the World Health Organization (WHO), in 2014, the estimated number of tuberculosis cases was 9 million, including 3.5 million new cases and 1.1 million human immunodeficiency virus (HIV)-positive cases. WHO reported a prevalence of 370 tuberculosis cases per 100,000 individuals in the United States (3). In Mexico and other developing nations, tuberculosis is ranked 17th among the leading causes of all deaths in the working-aged general population (4).

The two main presentations of tuberculosis are pulmonary and extra pulmonary (5). Extra-pulmonary tuberculosis results from bacterial spread to other sites in the body, and occurs in approximately 10% of patients hospitalized with pulmonary tuberculosis (6). Tuberculous meningitis (TBM) is the most severe form of the infection, which is usually caused by *M. tuberculosis* and poses a serious threat to human health worldwide (7). If left untreated, the mortality associated with TBM is almost 100% and delayed treatment may lead to permanent neurological damage (3,8). The conventional 'gold standard' bacteriological methods, namely direct smear and culture isolation, are hardly able to detect *M. tuberculosis* in the cerebrospinal fluid (CSF) of patients with TBM. The clinical severity of TBM demands a rapid diagnosis and appropriate treatment in order to improve the clinical outcome. In infections of the central nervous system, such as TBM, the bacterial load is extremely low, thus making diagnoses based on the culture or staining of CSF difficult, as the results are usually negative. Furthermore, the clinical manifestations of TBM are often non-specific, similar to those of other forms of meningitis, such as neurocysticercosis, neuroborreliosis, or viral infections; thus, its diagnosis is currently based on

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clinical characteristics, radiological tests, responses to treatment and in the characteristics of CFS (9-14).

Molecular tests, in particular those based on the PCR for amplification of genes specific for the infecting organism may identify a wide variety of microorganisms, including mycobacteria (15-18). The analysis of the genomic sequence of *M. tuberculosis* (19) and studies on comparative genomics (20,21) have identified major deletions that specifically characterize different species of the *M. tuberculosis* complex, and are useful in differentiating these from non-tuberculous mycobacteria (22-25). Some studies have reported the use of PCR and quantitative PCR to identify *M. tuberculosis* in the CSF of patients with TBM, with varying sensitivity and specificity (25,26). However, the reported methods are based primarily on primers that bind IS6110, which allow the identification of the genus of the tuberculosis complex, but does not discern between species. Thus, the aim of this study was to identify TB in cases of meningitis with clinical and laboratory evidence suggestive of TBM, and to confirm our findings with molecular tests for TB infection.

### Patients and methods

**Bacterial strains and DNA isolation.** The mycobacterial strains used as controls were *M. tuberculosis* H37Rv (donated by The Pasteur Institute, Paris, France), *M. bovis* AN5 (35734; ATCC, Rockville MD, USA), *M. avium*, *M. intracellulare* and *M. habana* (clinical isolates previously identified by rRNA 16S sequencing), *M. celatum* (51130; ATCC) and *M. marinum* (clinical isolate given by Dr Ruth Parra of the National Medical Center, Mexico City, Mexico). The strains were grown in Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ, USA) and DNA was isolated using the phenol-chloroform method, as previously described (27,28). DNA was precipitated with isopropanol and resuspended in 50  $\mu$ l of distilled water, and a 10  $\mu$ l aliquot was used for PCR amplification (100 ng of DNA for the test), as previously described (15).

**Ethics statement.** The study protocol was approved by the Ethics Committee of the Mexican Social Security Institute (IMSS).

**Patient selection.** We recruited 144 consecutive patients with suspected meningitis and neurological symptoms who were examined at the neurology services of 4 Hospitals of IMSS in Mexico City between June 2008 and February 2013. The patients were both male and female, and included children and adults. Written informed consent was obtained from all the patients or the parents/legal guardians prior to recruitment. A total of 94 cases of meningitis with clinical and laboratory evidence suggestive of TBM were included, but only 50 of these cases fulfilled the criteria for probable TBM, the other 44 cases were eliminated. As the controls, we included 50 cases with neurological disease other than TBM, such as neuropathy, neurocysticercosis, neuroborreliosis, or viral infections. CSF was extracted from these patients as part of the protocol for diagnosis; a fraction of the sample was processed immediately for DNA isolation and was stored at -70°C until analysis.

**Definition of case and control.** As the bacterial load in the CSF is extremely low and culture is difficult using these samples, we diagnosed the cases of TBM based on clinical findings, CSF

Table I. Baseline characteristics of tuberculous meningitis (TBM) cases.

| Characteristics        | TBM cases, n (%) |
|------------------------|------------------|
| Age (years)            |                  |
| <18                    | 21 (42)          |
| >18                    | 29 (58)          |
| Gender                 |                  |
| Male                   | 27 (54)          |
| Female                 | 23 (46)          |
| Clinical manifestation |                  |
| Headache               | 32 (64)          |
| Fever                  | 19 (32)          |
| Neck stiffness         | 18 (36)          |
| Vomiting               | 18 (36)          |
| Abnormal behavior      | 26 (52)          |
| Unconsciousness        | 3 (6)            |
| Drowsiness             | 11 (22)          |
| Seizures               | 24 (48)          |
| Nausea                 | 10 (20)          |
| Blurred vision         | 10 (20)          |

TBM (cases), n=50; average age, <18 [10.8 (0.5-18)] and >18 [42.3 (19-63) years].

Table II. The cerebrospinal fluid (CSF) characteristics of the tuberculous meningitis (TBM) cases and non-TBM controls.

| CSF characteristics                      | TBM cases     | Non-TBM controls |
|--|---------------|------------------|
| Total cell count (cell/mm <sup>3</sup> ) | 96.5 (17-176) | 59 (12-106)      |
| Sugar (mg/dl)                            | 49 (15-83)    | 65 (9-121)       |
| Protein (mg/dl)                          | 151 (98-204)  | 74 (53-95)       |

criteria or both. The clinical criteria of meningitis included headache, fever and neck stiffness, with or without an altered consciousness. The CSF criteria were a cell count >10 cells/mm<sup>3</sup>, a protein concentration >45 mg/dl and a glucose concentration <40 mg/dl (9), either alone or in combination. TBM was defined as meningitis with typical CSF findings in conjunction with either suggestive chest X-ray abnormalities or suggestive TB lymphadenitis. We chose to use clinical and laboratory data to define the cases and the controls, as previously described (9,13,14). A TBM case was defined as a patient suffering from fever, headaches and meningismus (stiff neck), along with focal neurological deficits, behavioural changes and alterations in consciousness, with CSF characteristics of moderate lymphocytic pleocytosis, moderately elevated protein levels and hypoglycorrhachia (Tables I and II) (12). In addition, all the TBM cases should respond clinically and radiologically to specific anti-tuberculosis treatment. A history of a positive tuberculin skin test, or exposure to tuberculosis, was also considered as risk for TBM. A non-TBM case was defined as a patient with a confirmed diagnosis of neuropathy other

Table III. Primers used in the study.

| Gene fragment                     | Primer target (description) | Primer sequences 5'→3' (forward/reverse) | Amplicon size (bp) | Authors/year/(Refs.)  |
|-----------------------------------|-----------------------------|--|--------------------|---|
| <i>p32</i>                        | MT1                         | TTC CTG ACC AGC GAG CTG CCG              | 506                | Del Portillo <i>et al</i> , 1991, 1996 (30,31)<br>Nava <i>et al</i> , 2005 (33) |
|                                   | MT2                         | CCC CAG TAC TCC CAG CTG TGC              |                    |   |
| IS6110                            | IS 5                        | CGG AGA CGG TGC GTA AGT GG               | 984                | Del Portillo <i>et al</i> , 1991, 1996 (30,31)<br>Nava <i>et al</i> , 2005 (33) |
|                                   | IS 6                        | GAT GGA CCG CCA GGG CTT GC               |                    |   |
| <i>mtp40</i>                      | PT1                         | CGG CAA CGC GCC GTC GGT GG               | 396                | Del Portillo <i>et al</i> , 1991 (30,31)<br>Herrera and Segovia, 1996 (32)      |
|                                   | PT2                         | CCC CCC ACG GCA CCG CCG GG               |                    |   |
| Internal fragment of <i>mtp40</i> | PT3                         | CAC CAC GTT AGG GAT GCA CTG C            | 223                | Gori <i>et al</i> , 1996 (35)   |
|                                   | PT4                         | CTG ATG GTC TCC GAC ACG TTC G            |                    |   |

than TBM. From our examination, there were 50 patients who fulfilled the criteria for TBM (the cases) and 50 patients who fulfilled the criteria for the controls (non-TBM).

**Sample size calculation.** The sample size was estimated with the EPI info 3.5.1 (2008) programme, aiming to achieve 90% sensitivity for our multiplex-nested PCR analysis of the CSF of patients with neurological manifestations suggestive of TBM (26,29). The sample size calculation was 20 cases and 20 controls. We recruited 50 cases and 50 controls for this study.

**Multiplex and nested PCR.** We used 100 ng of DNA from the *M. tuberculosis* strain H37Rv to standardize the test. The sequences of the primers are shown in Table III. In the initial amplification, primers MT1 and MT2 amplified the gene encoding the 32-kDa  $\alpha$  antigen present in all described mycobacteria, whereas primers IS5 and IS6 amplified the IS6110 insertion element (30,31), and PT1 and PT2 were used to amplify the species-specific gene *mtp40*. Nested PCR further amplified an internal region of the *mtp40* gene of *M. tuberculosis* (32). All reactions were performed in a final volume of 25  $\mu$ l, with 10X reaction buffer, 1.25 U of Taq DNA polymerase, 0.2 mM of each deoxynucleotide, 2.5 mM MgCl<sub>2</sub>, 10 pmol of MT1 and MT2, 15 pmol of IS5 and IS6, and 20 pmol of PT1 and PT2. The cycling parameters for the initial PCR were 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 71°C for 2 min and extension at 72°C for 3 min, followed by a final extension at 72°C for 10 min. Following amplification, the PCR products were analysed by horizontal electrophoresis on 2.0% agarose gels (33) using DNA molecular marker 1 kb (Invitrogen Life Technologies, Carlsbad, CA, USA). The multiplex PCR products were subjected to nested PCR under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 74°C for 2 min, and 72°C for 2 min, followed by a final extension at 72°C for 7 min.

**Laboratory sensitivity and specificity.** Before testing the clinical samples, the specificity of the assay was tested using DNA from the following *Mycobacterium* species: *M. tuberculosis* H37Rv, *M. bovis* AN5, *M. intracellulare*, *M. avium*, *M. celatum*, *M. habana* and *M. marinum*, and DNA from *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) (clinical isolate from the clinical laboratory of Pediatric

Hospital of the National Medical Center, Mexico City, Mexico). The sensitivity of the assay was estimated using 10-fold dilutions of *M. tuberculosis* DNA, ranging from 100 ng to 0.01 fg.

**Processing of the clinical samples.** All CSF samples from the patients with meningeal infection were analyzed by multiplex and nested PCR, and the study was blinded. An aliquot of 500  $\mu$ l of each CSF was subjected to thermal shock, and centrifuged at 17,000 x g for 15 min. The DNA was isolated from the pellet with guanidine isothiocyanate according to the procedure previously described by Chomczynski and Sacchi (27) and Chomczynski (28), and stored at -70°C until use. A 100 ng sample of DNA from each CSF sample was used for the multiplex and nested PCR. The results of PCR for the cases and controls were used to estimate the clinical specificity and sensitivity of the test.

**DNA sequences and analysis.** Four of the amplified nested PCR products from the clinical samples were purified using the QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany), and sequenced with the CEQ 8800 Genetic Analysis system (Beckman Coulter, Inc., Brea, CA, USA), according to the manufacturer's instructions. The sequences generated were analyzed with the BLAST tool (available online at [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) and aligned with the available *M. tuberculosis* H37Rv genome sequence database, accessible at <http://genolist.pasteur.fr/Tuberculist>, to estimate the degree of homology.

**Statistical analysis.** Measures of central tendency were the descriptive statistics used to analyze the quantitative variables. A 2x2 contingency table was used to determine the sensitivity (S), specificity (E), positive predictive value (PPV) and negative predictive value (NPV) of the diagnostic test using clinical samples (27).

## Results

**Multiplex and nested PCR.** The sizes of the amplified fragments were sufficiently different to be distinguishable on agarose gels; the primers had no potential matches with sequences at non-specific target sites, and the optimal DNA-primer annealing temperatures were almost the same for all template-primer combinations (15). Fig. 1A shows the fragments amplified when each individual primer set was used

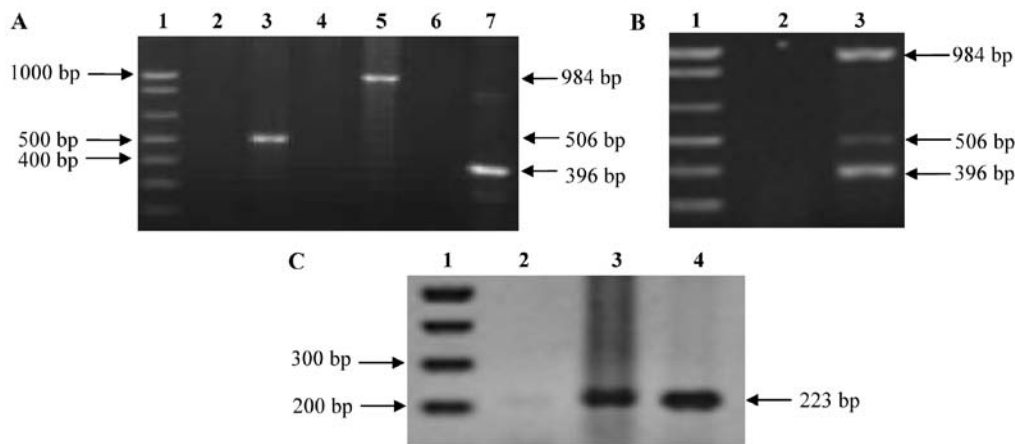


Figure 1. Electrophoresis of PCR and nested PCR products of *Mycobacterium tuberculosis* (*M. tuberculosis*) H37Rv DNA. (A) Amplification of *p32*, *IS6110* and *mtp40* fragments by conventional PCR. Lane 1, DNA molecular marker 1 kb; lane 2, negative control of MT1 and MT2 primers; lane 3, *p32* amplification fragment; lane 4, negative control of IS5 and IS6 primers; lane 5, *IS6110* amplification fragment; lane 6, negative control of PT1 and PT2 primers; and lane 7, *mtp40* amplification fragment. (B) Amplification of *p32*, *IS6110* and *mtp40* fragments by multiplex PCR lane 1, DNA molecular marker 1 kb; lane 2, negative control; and lane 3, amplification products of multiplex PCR of *M. tuberculosis* H37Rv DNA. (C) Amplification of internal fragment of *mtp40* by nested PCR. Lane 1, DNA molecular marker 1 kb; lane 2, negative control; lanes 3 and 4, *M. tuberculosis* H37Rv DNA.

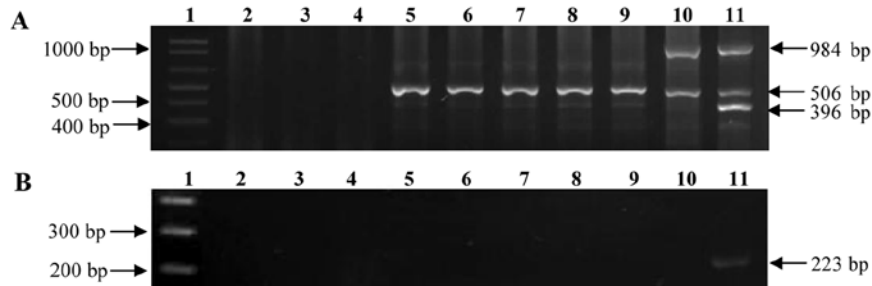


Figure 2. Amplification of DNA from other bacteria. (A) Multiplex PCR products. (B) Nested PCR products. Lane 1, DNA molecular marker 1 kb; lane 2, negative control; lane 3, *Escherichia coli* (*E. coli*) DNA; lane 4, *Staphylococcus aureus* (*S. aureus*) DNA; lane 5, *M. habana* DNA; lane 6, *M. celatum* DNA; lane 7, *M. marinum* DNA; lane 8, *M. avium* DNA; lane 9, *M. intracellulare* DNA; lane 10, *M. bovis* AN5 DNA; and lane 11, *Mycobacterium tuberculosis* (*M. tuberculosis*) H37Rv DNA.

separately. Fig. 1B, lane 3, shows the simultaneous amplification of the 3 fragments. A nested PCR was used to increase the sensitivity of the system, with a pair of primers that amplifies a fragment of 223 bp, corresponding to the internal region of the species-specific sequence of the *mtp40* gene (Fig. 1C).

**Laboratory specificity and sensitivity of the multiplex and nested PCR system.** The specificity of the assay was evaluated with DNA from different *Mycobacterium* strains (*M. tuberculosis*, *M. bovis* AN5, *M. intracellulare*, *M. avium*, *M. celatum*, *M. habana* and *M. marinum*) and DNA from *S. aureus* and *E. coli*. Following multiplex PCR amplification, *M. tuberculosis* DNA amplify the 3 expected bands of genus (506 bp), complex (984 bp) and species (396 bp), and *M. bovis* DNA amplify the 2 expected bands of 506 and 984 bp. We also tested DNA from other *Mycobacterium* species (*M. intracellulare*, *M. avium*, *M. celatum*, *M. habana* and *M. marinum*), and in all these strains, only the fragment corresponding to the *p32* gene of the *Mycobacterium* genus was amplified. Finally, no amplification was observed with any of the primers when DNA from *S. aureus* and *E. coli* was tested (Fig. 2A).

These results confirm that multiplex PCR is specific for *M. tuberculosis*. Moreover, the products obtained from the

multiplex PCR were reamplified in the nested PCR, and the internal fragment of the *mtp40* gene only amplified with the *M. tuberculosis* DNA, confirming that this system specifically identifies *M. tuberculosis* (Fig. 2B).

The sensitivity of the multiplex PCR assay was 10 ng of *M. tuberculosis* H37Rv DNA where it amplified the 3 expected products (Fig. 3A). However, the sensitivity of the nested PCR was 0.1 fg of DNA for the fragment corresponding to an internal region of the *mtp40* gene (Fig. 3B).

**CSF sample analysis.** Following the standardization of the multiplex and nested PCR system with the *Mycobacterium* strain, we assessed its usefulness in the CSF samples obtained from clinical cases suggestive of TBM. We analyzed 100 CSF samples from patients with diverse neurological symptoms, from 50 patients with a clinical diagnosis of TBM (cases) and 50 patients with a confirmed neurological aetiology other than TBM (controls). In the initial multiplex PCR, only one (patient 3) of the 100 CSF samples amplified a fragment of 396 bp corresponding to the *mtp40* gene (Fig. 4A). In the nested PCR, the 223-bp amplification product of the *mtp40* gene specific for *M. tuberculosis* was amplified from 53 samples (Fig. 4B). Forty-nine of these samples corre-

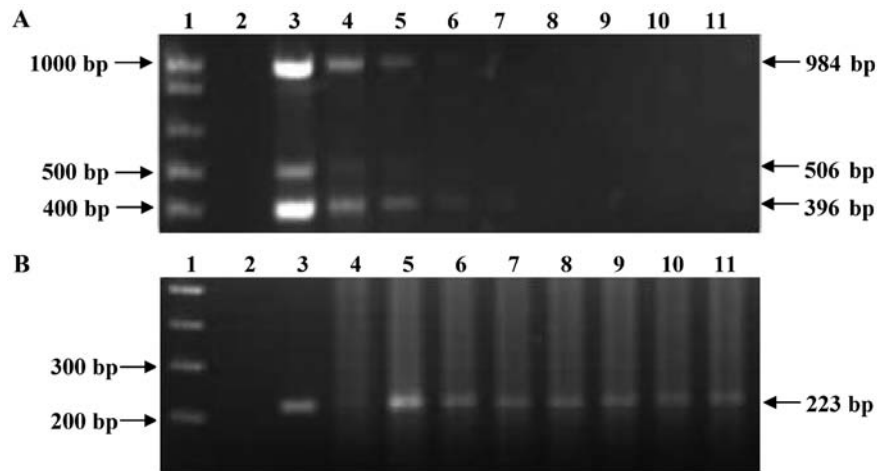


Figure 3. Sensitivity test of the multiplex and nested PCR for detect *Mycobacterium tuberculosis* (*M. tuberculosis*) DNA. (A) Multiplex PCR products of different concentrations of *M. tuberculosis* H37Rv DNA. (B) Nested PCR products from multiplex PCR of different concentrations of *M. tuberculosis* H37Rv DNA. Lane 1, DNA molecular marker 1 kb; lane 2, negative control; lane 3, 100 ng; lane 4, 10 ng; lane 5, 1 ng; lane 6, 100 pg; lane 7, 10 pg; lane 8, 1 pg; lane 9, 100 fg; lane 10, 10 fg; and lane 11, 1 fg.

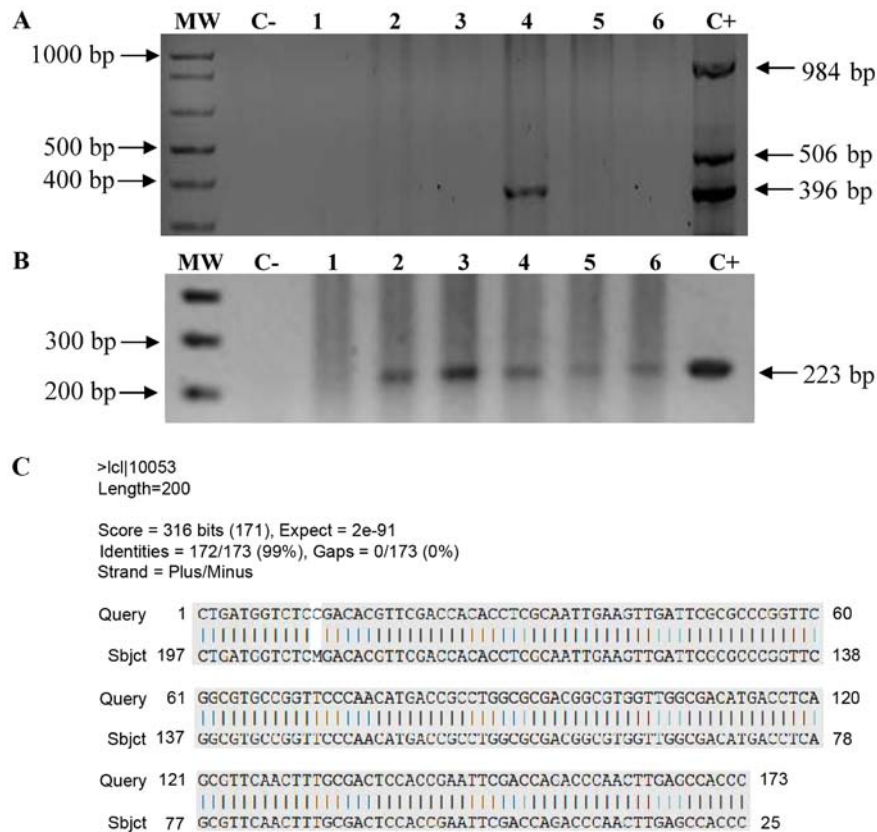


Figure 4. Multiplex and nested PCR for detect *Mycobacterium tuberculosis* (*M. tuberculosis*) in cerebrospinal fluid (CSF). (A) Multiple-PCR test applied to CSF samples from patients with neurologic diseases. (B) Nested PCR products from multiple-PCR products. Lane 1, patient 16 (true negative); lane 2, patient 11; lane 3, patient 8; lane 4, patient 3; lane 5, patient 12; and lane 6, patient 17 (patient 11, 8, 3, 12 and 17 were true positive). MM, molecular marker 1 kb; C-, negative control; C+, positive control DNA from *M. tuberculosis* H37Rv strain. (C) Alignment of the *mtp40* internal fragment with sequence of nested PCR product using PT3 and PT4 primers. The highlighted positions indicate the sequence of the *mtp40* internal fragment (Query) from 2630676 to 2630827 positions, and sequence of nested PCR product (Sbjct).

sponded to the group of cases and responded to specific treatment against *M. tuberculosis*, thus confirming the presence of *M. tuberculosis* DNA in the CSF. Moreover, we had one false-negative sample in the nested PCR. The negative control run with each set of test samples produced no visible product on ethidium bromide-stained agarose gel.

**DNA sequence of PCR products.** We determined the DNA sequence of the amplified 223-bp fragment corresponding to the *mtp40* gene from 4 positive samples to confirm that the product was specific for *M. tuberculosis*. The sequence alignment showed 99% homology between the nested PCR products and the corresponding region of the *mtp40* gene (Fig. 4C).

Table IV. Sensitivity and specificity as a diagnostic test for tuberculous meningitis (TBM).

| Nested PCR  | %  |
|-------------|----|
| Sensitivity | 98 |
| Specificity | 92 |
| PPV         | 88 |
| NPV         | 98 |

PPV, positive predictive value; NPV, negative predictive value.

*Sensitivity and specificity of the test.* The clinical sensitivity of this method was 98.0% and its specificity was 88.0%; the positive predictive value (PPV) was 91.0% and the negative predictive value (NPV) was 98.0% (Table IV).

## Discussion

The suboptimal and often delayed results of traditional microbiological techniques used in the diagnosis of TBM underscore the need for a more rapid and more accurate diagnostic method to facilitate early treatment. Several molecular-based methods used for the diagnosis of tuberculosis in respiratory specimens have been evaluated for their applicability to the diagnosis of TBM (23). Many of these methods have failed with CSF samples, mainly due to the fact that the number of bacilli typically present in TBM is low and that amplification inhibitors are present in the CSF. Commercially available methods of nucleic acid amplification (NAA) for the direct detection of the *M. tuberculosis* complex have been approved in the United States for testing respiratory specimens; Ling *et al*, performed an extensive literature search and identified a total of 125 separate studies from 105 articles that reported NAAT results from respiratory specimens. The results showed that sensitivity and specificity estimates for commercial NAATs in respiratory specimens were highly variable, with sensitivity lower and more inconsistent than specificity. Thus, summary measures of diagnostic accuracy are not clinically meaningful. The use of different cut-off values and the use of specimens other than sputum could explain some of the observed heterogeneity (34). However, there are few NAA methods that have been approved for testing the CSF and several studies have evaluated their performance in patients with TBM (31,32).

The PCR system also has been used as an epidemiological tool in strain classification (23,24). The most widely used target sequence for the diagnosis of tuberculosis has been the IS6110 insertion element, present in a different number of copies in the genome of species of the *M. tuberculosis* complex (12,35) and PCR techniques based on this sequence have shown to be useful for diagnosis. However, studies have demonstrated that some *M. tuberculosis* strains do not carry the IS6110 sequence (36,37). Therefore, the use of a PCR method based on the detection of IS6110 for the diagnosis of *M. tuberculosis* may in some cases lead to false-negative results. Additionally, PCR-based diagnoses based exclusively on the IS6110 sequence would also fail to distinguish *M. tuberculosis* from other mycobacteria of the *M. tuberculosis* complex.

In the present study, we used the multiplex PCR method described by Del Portillo *et al* (30,31) to detect *M. tuberculosis* in pulmonary-type tuberculosis, with some modifications to identify *M. tuberculosis* in CSF samples. Additionally, we used the nested PCR described by Herrera and Segovia (32). In this study, we demonstrated that using a multiplex and nested PCR system, it is possible to simultaneously amplify the  $\alpha$  antigen gene (*p32*), which identifies the *Mycobacterium* genus, the IS6110 insertion element and the species-specific *mtp40* gene. The nested amplification of a 396-bp fragment corresponding to the *mtp40* gene ensures that the results are specific for *M. tuberculosis*. In addition, the nested PCR allowed us to reach a limit of detection to 0.1 fg of mycobacterial DNA, which theoretically would allow the detection of one bacterium in the sample (16). The PCR amplification of the *mtp40* sequence provides a sensitive and specific method for the diagnosis of tuberculosis (35,38) and it may be useful for the identification of *M. tuberculosis*, as 98.5% of strains possess this gene (39). We used a multiplex-PCR method followed by a nested PCR, reaching high specificity and sensitivity.

With the multiplex PCR we were able to detect only the *mtp40* gene in a single CSF sample; this may be due to the extremely low number of bacteria in the CSF. However, with the nested PCR, we detected the presence of *M. tuberculosis* in 53 patients with the amplification of the species-specific *mtp40* gene. The products of the nested PCR were sequenced to further confirm that the amplified fragment corresponded to the *mtp40* *M. tuberculosis* gene, and demonstrated that the sequence had a 99% homology with the *mtp40* gene. Thus, we were able to detect the presence of *M. tuberculosis* in the CSF samples of patients with suggested TBM using a species-specific multiplex and nested PCR test.

Our test showed a sensitivity of 98.0% and a specificity of 88.0% for the diagnosis of TBM in CSF samples, which are better than those reported previously (40,41). In relation to the false-negative result, it is possible that a fraction of *mtp40* gene was excised by IS6110 recombination as previously described Vera-Cabrera *et al* (42), which could explain the lack of genes in some *M. tuberculosis* strains described by Weil *et al* (43). Concerning the false-positive results, we suggest that they may represent cases of co-infection, where clinical diagnosis could have masked the *M. tuberculosis* symptoms, focusing on the diagnosis of the other disease. The test presented a reasonably good PPV (91.0%) and NPV (98.0%), which contrast with the findings reported by Lima *et al* (37), who applied a nested PCR to the peripheral blood samples from patients with extra pulmonary tuberculosis, with a rather low PPV (55.6%) and an acceptable NPV (92.7%).

In conclusion, the present study demonstrates that the identification of *M. tuberculosis* in the CSF of patients with meningitis is possible using a developed system of multiplex and nested PCR. This study supports the implementation of two molecular tests as a sensitive and specific diagnostic tool, which offers an improved alternative for the diagnosis of TBM. The multiplex-nested PCR test is a rapid, sensitive and specific tool which may have a beneficial impact on the management of patients with suspected TBM, allowing a more timely anti-tuberculosis treatment to reduce sequelae and mortality.

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