Occurrence of Overlooked Zoonotic Tuberculosis: Detection of *Mycobacterium bovis* in Human Cerebrospinal Fluid

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Tuberculosis (TB) is a chronic, systemic infectious disease caused by *Mycobacterium tuberculosis*. The most common clinical manifestation is pulmonary TB. The inhaled bacilli can localize in alternate sites, leading to extrapulmonary TB (EPTB). Among the different manifestations of EPTB, tuberculosis meningitis (TBM) has been considered to be a fatal form (41). Fatality rates in developing countries have been reported to range from 44 to 69% (15, 18, 30). In fact, delayed identification to the species level of mycobacterial pathogens can be made. The present report describes the utility of a nested PCR (N-PCR) assay (A. Mishra, A. Singh, D. S. Chauhan, V. M. Katoch, K. Srivastava, S. S. Thakral, S. S. Bharadwaj, V. Sreenivas, and H. K. Prasad, J. Clin. Microbiol. 43:5670–5678, 2005) in detecting *M. tuberculosis* and *M. bovis* in human CSF. In 2.8% (6/212) of the samples, *M. tuberculosis* was detected, and in 17% (36/212), *M. bovis* was detected. Mixed infection was observed in 22 samples. Comparative analysis of clinical diagnosis, smear microscopy, and N-PCR in 69 patients (TBM, 25; non-TBM, 44) showed that the sensitivity of N-PCR (61.5%) was greater than that of smear microscopy (38.4%). Determination to the species level is important from the viewpoint of determining the prevalence of these mycobacteria in a community and would influence strategies currently adopted for the prevention of tuberculosis.

### MATERIALS AND METHODS

**Patients.** CSF samples from 212 patients were investigated. The patient distribution was as follows. For the first part of the study, 112 patients admitted to the neurology ward of the All India Institute of Medical Sciences (AIIMS), New Delhi, were investigated. Subsequently, for the second part of the study, CSF samples from 100 children (≤12 years old) admitted to the pediatric ward of Safdarjung Hospital, New Delhi, India, were investigated. The institutional ethical committee approved the study. At the time of data analysis, the AIIMS hospital records of the 69 patients were obtained. These 69 cases were separated into TBM and NTBM (nontuberculous meningitis) groups on the basis of the criteria described by Ahuja et al. (1) (Table 1). CSF was collected under aseptic conditions by lumbar puncture. Five hundred microliters to 2.0 ml of CSF was available for the study. Samples were stored at −20°C, prior to processing for target DNA for N-PCR and smear microscopy for acid-fast bacilli (AFB; auramine O stain).

**Mycobacterial strains.** *M. tuberculosis* H37Rv DNA was obtained from TB research material, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. Lowenstein-Jensen slant cultures of *M. tuberculosis* H37Rv and *M. bovis* ANS were obtained from the Central JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India.

**Preparation of mycobacterial cell lysates containing target DNA.** (i) **Mycobacterial cultures.** One milliliter of a logarithmic-phase culture of *M. tuberculosis* or *M. bovis* grown in 7H9 Middlebrook broth was pelleted and resuspended in 100 μl of 0.1% of Triton X-100. The suspension was boiled (90°C for 40 min) and centrifuged (10,000 × g for 10 min). The supernatant was stored at −20°C and used as template DNA in a PCR.
N-PCR products were cloned into the pGEMT vector with a TA cloning kit. The 116- and 89-bp products obtained in this case were generated from a mixture of the target DNAs that showed an 89-bp product of M. tuberculosis and M. bovis with the CLUSTALW software (http://www.ebi.ac.uk/clustalw/).

The sequences obtained were aligned with the sequences of M. tuberculosis and M. bovis by N-PCR in CSF samples derived from patients clinically categorized was determined by the trend chi-square test.

RESULTS

Two hundred twelve CSF samples were processed for the detection of M. tuberculosis and M. bovis by the N-PCR assay. Determination to the species level of the mycobacterial pathogens, namely, M. tuberculosis and M. bovis, present in the human CSF samples was established by molecular size analysis of the N-PCR products electrophoresed on a 10% polyacrylamide gel with appropriate controls.

Detection and identification of M. tuberculosis and M. bovis in CSF. The detection and differentiation of M. tuberculosis and M. bovis in representative CSF samples are depicted in Fig. 1. Figure 1B illustrates the single-band N-PCR products obtained in four of the five CSF samples (lanes 2, 3, 7, and 8). The PCR products in these samples were found to align with the PCR product obtained in the case of the standard M. bovis (Fig. 1B, lane 6) and was distinctly different from the PCR product obtained in the case of standard M. tuberculosis (Fig. 1B, lane 4).

Figure 1C shows the dual amplified bands occasionally detected on polyacrylamide gel electrophoresis. Two bands of

<table>
<thead>
<tr>
<th>TABLE 1. Criteria for categorizing TBM patients</th>
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<tbody>
<tr>
<td><strong>Criterion or category</strong></td>
</tr>
<tr>
<td>Clinical (A)</td>
</tr>
<tr>
<td>CSF (B)</td>
</tr>
<tr>
<td>Radiological (C)</td>
</tr>
<tr>
<td>Extraneural (D)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Categories</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definite TBM</td>
<td>A + isolation of bacteria or AFB positive</td>
</tr>
<tr>
<td>Highly probable TBM</td>
<td>A + all 3 of B, C, and D</td>
</tr>
<tr>
<td>Probable TBM</td>
<td>A + any 2 of B, C, and D</td>
</tr>
<tr>
<td>Possible TBM</td>
<td>A + any 1 of B, C, or D</td>
</tr>
</tbody>
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DETECTION OF M. TUBERCULOSIS AND M. BOVIS IN CSF

The detection and differentiation of M. tuberculosis and M. bovis in CSF samples. (A) Positions of the primers in the hupB gene (Rv2986c in M. tuberculosis, Mb301kc in M. bovis) sequence are depicted. Primers N and S (S-PCR) are specific for the hupB gene, and internal primers F and R (N-PCR) are specific for the C-terminal part of the gene. (B) The ethidium bromide-stained amplification products of M. tuberculosis and M. bovis generated by using primers F and R were electrophoresed on nondenaturating 10% polyacrylamide gels. The 116- and 89-bp products obtained in M. tuberculosis and M. bovis, respectively, are indicated. Samples showing dual infection are shown in lanes 11 and 17. Lanes: 1, CSF 53; 2, CSF 55; 3, CSF 80; 4 and 15, M. tuberculosis; 5 and 14, 100-bp molecular size marker; 6 and 13, M. bovis; 7, CSF 38; 8, CSF 40; 9 and 10, negative control; 11,CSF 83; 12, CSF 81; 16, dual positive control; 17, CSF 71; 18, CSF 67.

DNA sequencing, Primer F- and R-amplified N-PCR products from 10 samples that showed an 89-bp product of M. bovis were eluted from agarose gel with a mini Elute Gel Extraction kit (QIAGEN). The extracted DNA was biotyped and sent for sequencing to the DNA Sequencing Resource Center at the Rockefeller University.

Alternatively, with CSF samples showing mixed infection, both of the bands (116 and 89 bp) were cut from a 10% polyacrylamide gel and DNA was extracted by the crush-and-soak method as previously described (37). These extracted N-PCR products were cloned into the pGEMT vector with a TA cloning kit (Promega) according to the manufacturer’s instructions. The clones were sequenced at the DNA sequencing facility, South Campus Delhi University, New Delhi, India.

The trend in detection of M. tuberculosis and M. bovis by N-PCR in CSF samples derived from patients clinically categorized was determined by the trend chi-square test.

FIG. 1. N-PCR for detecting and differentiating M. tuberculosis and M. bovis in CSF samples. (A) Positions of the primers in the hupB gene (Rv2986c in M. tuberculosis, Mb301kc in M. bovis) sequence are depicted. Primers N and S (S-PCR) are specific for the hupB gene, and internal primers F and R (N-PCR) are specific for the C-terminal part of the gene. (B and C) The ethidium bromide-stained amplification products of M. tuberculosis and M. bovis generated by using primers F and R were electrophoresed on nondenaturating 10% polyacrylamide gels. The 116- and 89-bp products obtained in M. tuberculosis and M. bovis, respectively, are indicated. Samples showing dual infection are shown in lanes 11 and 17. Lanes: 1, CSF 53; 2, CSF 55; 3, CSF 80; 4 and 15, M. tuberculosis; 5 and 14, 100-bp molecular size marker; 6 and 13, M. bovis; 7, CSF 38; 8, CSF 40; 9 and 10, negative control; 11, CSF 83; 12, CSF 81; 16, dual positive control; 17, CSF 71; 18, CSF 67.

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116 and 89 bp were seen in samples 71 and 83 (Fig. 1C, lanes 11 and 17), which were identical in molecular size to those generated in the dual positive control (Fig. 1C, lane 16). The higher-molecular-weight PCR product in lanes 11 and 17 (Fig. 1C) corresponded to and aligned with the PCR product obtained for \( M. \) tuberculosis (Fig. 1C, lane 15). The lower band corresponded to and aligned with the standard \( M. \) bovis strain (Fig. 1C, lane 13). Single bands matching the standard \( M. \) bovis and \( M. \) tuberculosis strains were seen in lanes 12 and 18, respectively (Fig. 1C). These assorted patterns obtained for the 112 CSF samples from the AIIMS hospital investigated have been summarized in Table 2.

Of the 112 samples (neurology ward, AIIMS hospital) investigated, 37 (33%) were positive for \( M. \) tuberculosis and \( M. \) bovis by N-PCR (Table 2). A mixed pattern of PCR products was seen in the CSF samples (Table 2). In 17% (19/112) of the samples, \( M. \) bovis was detected. Infection with \( M. \) tuberculosis alone was detected in 2.7% (3/112) of the samples investigated. Simultaneous infection with both pathogens was established in 15/112 samples (13.4%). In 12.5% (14/112) of the samples, AFB were detected microscopically (Table 2).

In all samples positive for AFB, \( M. \) tuberculosis and/or \( M. \) bovis were detected by N-PCR. However, a limited number of the smear-negative samples (23/112) were positive by N-PCR.

Twenty-seven of the 100 CSF samples from the pediatric ward of Safdarjung Hospital were found to be positive for \( M. \) tuberculosis and/or \( M. \) bovis by N-PCR assay. The positive-case distribution was as follows: 3 samples were positive for \( M. \) tuberculosis, 17 were positive for \( M. \) bovis, and mixed infection was detected in 7 samples.

**Clinical categorization of patients.** The 69 patients from the AIIMS hospital were categorized into definite (\( n = 9 \)), highly probable (\( n = 3 \)), probable (\( n = 5 \)), possible (\( n = 8 \)) TBM and NTBM (\( n = 44 \)) (Table 3) on the basis of the criteria described in Table 1 (1).

### TABLE 3. Comparative analysis of N-PCR results and microscopy of 69 patients

<table>
<thead>
<tr>
<th>Patient category</th>
<th>Total no.</th>
<th>N-PCR results</th>
<th>Microscopy results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Definite TBM</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Highly probable TBM</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Probable TBM</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Possible TBM</td>
<td>8</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>NTBM</td>
<td>44</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>20</td>
<td>49</td>
</tr>
</tbody>
</table>

* Patients were categorized on the basis of the criteria described in Table 1.

Correlation of clinical categorization of 69 patients with smear microscopy and N-PCR results. The results of the N-PCR were matched with the clinical categorization and smear microscopy results (Table 3). Positivity in the case of smear microscopy was limited to the nine cases grouped as definite TBM. The trend in detection of \( M. \) tuberculosis and \( M. \) bovis in CSF corresponded to the clinical classification of the patients (Fig. 2). All patients classified as definite TBM were positive by N-PCR. However, the N-PCR positivity for \( M. \) tuberculosis and \( M. \) bovis decreased with the decreased probability of clinical assessment of TBM (Table 3). Five of eight (62.5%), 1/8 (12.5%), and 5/44 (11.4%) cases classified as highly probable-to-probable TBM, possible TBM, and NTBM, respectively, were N-PCR positive. Comparative scrutiny of three parameters, namely, clinical diagnosis, smear microscopy, and N-PCR, showed that the sensitivity of the N-PCR (60.0%) was greater than that of smear microscopy (36.0%, Table 4). The specificity was 88.6% compared to that of smear microscopy (Table 4).

### TABLE 4. Comparative analysis of N-PCR and smear microscopy for AFB with clinical diagnosis of 69 patients investigated

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
<th>No. of patients with clinical diagnosis* of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TBM ((n = 25))</td>
</tr>
<tr>
<td>N-PCR</td>
<td>Positive</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>10</td>
</tr>
<tr>
<td>Microscopy</td>
<td>Positive</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>16</td>
</tr>
</tbody>
</table>

* Clinical diagnosis based on criteria described in Table 1.

b TP/(TP + FN) \times 100, where T is true, F is false, P is positive, and N is negative.

c TN/(TN + FP) \times 100.
Sequence analysis of single or dual N-PCR products in a representative CSF sample. The dual bands obtained from CSF samples were extracted and cloned individually into the pGEMT vector as described in Materials and Methods. The sequence analyses of the cloned inserts is represented in Fig. 3B to E. Each insert was compared with the sequence of the C-terminal end of the \( hupB \) gene of \( M.\) \( \text{tuberculosis} \) (Rv2986c, tb-116, accession no. NC_000962) and \( M.\) \( \text{bovis} \) (Mb3010c, bo-89, accession no. Y18421) showing the 27-bp difference between the two. Panels B and C depict a sequence alignment of csf-clone7 with tb-116 and bo-89. Panels D and E depict a sequence alignment of csf-clone6 with tb-116 and bo-89. The start and end of each sequence are indicated by arrows.

**FIG. 3.** Sequence alignment of the clones obtained from a CSF sample which showed mixed infection. The 116- and 89-bp amplicons were cloned separately into vector pGEMT and sequenced. Panel A depicts a comparative alignment of the sequences of N-PCR products of the \( hupB \) gene of \( M.\) \( \text{tuberculosis} \) (Rv2986c, tb-116, accession no. NC_000962) and \( M.\) \( \text{bovis} \) (Mb3010c, bo-89, accession no. Y18421) showing the 27-bp difference between the two. Panels B and C depict a sequence alignment of csf-clone7 with tb-116 and bo-89. Panels D and E depict a sequence alignment of csf-clone6 with tb-116 and bo-89. The start and end of each sequence are indicated by arrows.
M. tuberculosis (Fig. 3C). Comparison with the sequence of M. bovis (Fig. 3B) revealed an additional 27 bp in the sequence of the cloned insert. Hence, it was inferred that the higher-molecular-weight N-PCR product was derived from M. tuberculosis.

Figure 3D and E show a comparative sequence analysis of the clone containing the lower-molecular-weight N-PCR product (89 bp) as an insert. Complete identity was seen with that of M. bovis (bo-89, Fig. 3D). However, comparison of the insert sequence with that of M. tuberculosis (tb-116, Fig. 3E) revealed a discrepancy in the alignment; this was due to the additional 27 bp in the larger hupB gene of M. tuberculosis. This additional stretch of 27 bp was absent in the insert sequence. Therefore, it was concluded that the lower-molecular-weight N-PCR product was derived from M. bovis.

Direct DNA sequencing of N-PCR products of 10 samples that gave an amplified product equivalent to that of M. bovis on a polyacrylamide gel was done. All sequences of these samples matched the hupB gene sequence of M. bovis (bo-89). Hence, it was concluded that the N-PCR products of these samples were derived from M. bovis.

**DISCUSSION**

Though human TB is caused mainly by M. tuberculosis, an unknown proportion of cases is due to M. bovis (10). Human TB due to M. bovis is rare in developed nations. This is mainly due to the practice of animal TB control and elimination programs, together with milk pasteurization (15). In developing countries, animal TB is widespread and therefore constitutes a potential infectious source for humans (zoonotic TB). In Africa, approximately 85% of cattle and 82% of the human population live in areas where the disease is prevalent (10). Human disease caused by M. bovis has been confirmed in several African countries (28), France (35), Australia (11), and England (14). Infection of humans with M. bovis occurs mainly by consumption of contaminated milk or other dairy or meat products. The oral route has been the most common route of infection. Besides the oral route, inhalation of aerosolized infectious pathogens from infected animals has been considered to be a potential port of entry into susceptible hosts (3). Due to differences in the routes of infection, M. bovis is more likely to cause nonpulmonary disease (15). There are limited reports in India (27, 39, 40) and in underdeveloped countries (10, 16) relating to the prevalence of infection of cattle with M. tuberculosis and/or M. bovis. In India, there are no surveys to date to assess the public health problems posed by zoonotic TB. Human infection with M. bovis in immunocompetent, as well as in immunocompromised, individuals has been reported (3, 9, 16, 21, 25, 33). The epidemic of human immunodeficiency virus in developing countries, particularly where M. bovis infection prevails in animals and conditions favor zoonotic transmission, constitutes a serious public health threat (2).

The present study focused on the diagnosis of TBM. The tests currently used for rapid diagnosis of TBM are limited to the detection of AFB in smears, followed by culture and biochemical tests. However, both of these approaches are limited in the ability to identify the mycobacterial pathogen to the species level. Moreover, the commonly used Lowenstein-Jensen medium for primary isolation of mycobacteria from clinical samples is not conducive to M. bovis isolation (17). Hence, the isolation of M. bovis from clinical samples has been scanty. Further, TB caused by M. tuberculosis in humans is clinically, radiologically, and histopathologically indistinguishable from TB caused by M. bovis (44).

The problems associated with the sensitivity of TBM diagnosis by smear microscopy and the prolonged time taken by culture have been overcome by molecular techniques (4, 7, 9, 20, 22, 42). However, most of these techniques have been limited to detection of pathogenic mycobacteria as belonging to the MTC. Therefore, they are incapable of differential identification of the members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC. Unlike these targets, the hupB gene target used in the N-PCR assay has the potential advantage of detecting and identifying the closely related members of the MTC.
injected cattle to humans (zoonosis) and from infected humans to cattle (reverse zoonosis) (13).

In summary, this study shows that infection by members of the MTC can be determined with appropriate molecular techniques and defined gene targets. This would help in constituting appropriate strategies for prevention of human TB by mycobacterial pathogens other than the usual M. tuberculosis.

ACKNOWLEDGMENTS

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fulness of the GenoType MTBC assay for differentiating species of the Mycobacterium tuberculosis complex in cultures obtained from clinical spec-


