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

N. P. Shah,[1]† A. Singhal,[1]† A. Jain,[1]‡ P. Kumar,[1] S. S. Uppal,[2] M. V. P. Srivatsava,[3] and H. K. Prasad[1]*

Departments of Biotechnology[1] & Neurology,[3] All India Institute of Medical Sciences, and Department of Pediatrics, Safdarjung Hospital,[2] New Delhi 110029, India

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The paucibacillary nature of the cerebrospinal fluid (CSF) has been a major obstacle in the diagnosis of human tuberculous meningitis (TBM). This study shows that with molecular techniques direct precise determination 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. Singhal, 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.

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, tuberculous 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 or erroneous diagnosis often results in serious long-term debilitating complications. Central nervous system involvement has frequently been found secondary to TB elsewhere in the body, particularly the lungs. The presence of TB elsewhere in the body favors the diagnosis, although its absence does not exclude it. The great majority of patients with neuro-TB are diagnosed on the basis of clinical criteria, imaging, and laboratory investigation of the cerebrospinal fluid (CSF). The clinical response to antituberculosis therapy in all forms of neuro-TB is excellent, provided the diagnosis is made early, before an irreversible neurological defect occurs.

Hence, precise and rapid clinical diagnosis of TBM is a critical component of the management of TBM patients (12, 24, 25). Culture and Ziehl-Neelsen staining of the CSF are specific but insensitive due to the paucity of bacilli. Nucleic acid amplification techniques have shown promising results in overcoming this drawback (20, 22, 30, 42).

M. tuberculosis is a member of the *M. tuberculosis* complex (MTC), which includes *M. bovis*, "*M. canetti*," *M. microti*, and *M. africanum*. *M. bovis* infection of the central nervous system may lead to EPTB, similar to *M. tuberculosis* infection, with identical symptomatologies (10, 16). *M. bovis* infection has been reported in immunocompetent, as well as in immunosuppressed, individuals (9, 21, 25, 33). Hence, the present study was undertaken to detect *M. tuberculosis* and *M. bovis* in human CSF by using an in-house nested PCR (N-PCR) assay (26). The assay targets the 27-bp difference in the C terminus of the *hupB* gene in *M. tuberculosis* (*Rv2986c*) and *M. bovis* (*Mb3010c*) to differentiate *M. tuberculosis* from *M. bovis* (32). The utility of the assay for direct detection of *M. tuberculosis* and *M. bovis* in bovine samples was confirmed in comparison with standard culture techniques (26).

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 (nontubercular 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* AN5 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.

* Corresponding author. Mailing address: All India Institute of Medical Sciences, Department of Biotechnology, Ansari Nagar, New Delhi 110029, India. Phone: 91 11 26594994. Fax: 91 11 26852286. E-mail: hk_prasad@hotmail.com.

† N.P.S. and A.S. contributed equally to this report.

‡ Present address: Department of Biochemistry, Lady Hardinge Medical College, New Delhi 110001, India.

TABLE 1. Criteria for categorizing TBM patients[a]

Criterion or category	Findings
Criteria	
Clinical (A)	Fever and headache for 14 days, vomiting, altered sensorium
CSF (B)	Sugar, <60 mg%; protein, >100 mg%; negative cytology for malignant cells; pleocytosis with >20 cells
Radiological[b] (C)	Exudate in basal cistern, hydrocephalus, infarcts, gyral enhancement
Extraneural (D)	Active TB of lungs, GIT,[c] UTI,[d] lymph node, bone, skin
Categories	
Definite TBM	A + isolation of bacteria or AFB positive
Highly probable TBM	A + all 3 of B, C, and D
Probable TBM	A + any 2 of B, C, and D
Possible TBM	A + any 1 of B, C, or D

[a] Data from reference 1.
[b] Findings based on computerized tomography of the head.
[c] GIT, gastrointestinal tract.
[d] UTI, urinary tract infection.

(ii) CSF. Target DNA was prepared from CSF by a modification of the method of Chakravorty and Tyagi (6). CSF was filtered (0.22-μm pore size; Millipore). The CSF container was rinsed with 2 ml of inhibitory removing solution (25 M guanidinium isothiocyanate, 0.025 M EDTA, 0.05 M Tris, 0.5% Sarkosyl, 0.186 M β-mercaptoethanol, pH 7.5) and filtered. The membrane was transferred to a 1.5-ml Eppendorf tube containing 500 μl of inhibitory removing solution, incubated for 15 min at room temperature, and centrifuged (10,000 × *g* for 10 min). The supernatant was discarded, and the membrane was washed with sterile water. Two hundred microliters of 0.1% Triton X-100 was added to the membrane and vortexed vigorously. The suspension was used to make smears and subsequently boiled in a dry bath (90°C for 40 min), followed by centrifugation (10,000 × *g* for 10 min). The supernatant was stored at −20°C and used as template DNA.

N-PCR assay for *hupB* DNA target (international patent application PCT/IN03/00302, 1 July 2004, L. S. Davar and Co., Calcutta, India). Primers N (5′-GGAGGGTTGGGATGAACAAAGCAG-3′) and S (5′-GTATCCGTGTG TCTTGACCTATTTG-3′) were used to amplify the *hupB* gene as previously described (N-S PCR, Fig. 1A) (32). The N-PCR assay for the C-terminal portion of the *hupB* gene was carried out with primers F (5′-CCAAGAAGGCGA CA AAGG-3′) and R (5′-GACAGCTTTCTTGGCGGG-3′) (Fig. 1A) (26). Four microliters of the amplified product of a PCR with primers N and S was used as the template DNA for the N-PCR. Each reaction mixture (40 μl) of the N-PCR contained 1.25 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 0.5 μM primers F and R, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P-40, and 1.0 U of *Taq* DNA polymerase. The reaction mixture was subjected to initial denaturation at 94°C for 10 min and 35 cycles each of 1.0 min at 94°C and annealing and extension at 59°C for 0.30 min, followed by a final extension at 72°C for 7 min. The products were analyzed on a 10% polyacrylamide gel and stained with ethidium bromide. The expected sizes of amplicons for *M. tuberculosis* and *M. bovis* were 116 and 89 bp, respectively. The limit of detection was 50 fg, which is equivalent to five tubercle bacilli (26). Further, in an earlier report, the *hupB*-based PCR assay was shown to be specific for *M. tuberculosis* and *M. bovis* when tested with members of the MTC, as well as with other mycobacterial species (32). N-PCR products generated from standard strains of *M. tuberculosis* and *M. bovis* were included (positive control). A dual positive control was also included to discern mixed infection with *M. tuberculosis* and *M. bovis*. The N-PCR products in this case were generated from a mixture of the target DNAs of *M. tuberculosis* H37Rv and *M. bovis* AN5.

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 lyophilized 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 sequences obtained were aligned with the sequences of *M. tuberculosis* and *M. bovis* with the CLUSTALW software (http://www.ebi.ac.uk/clustalw/).

Statistical analysis. 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.

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

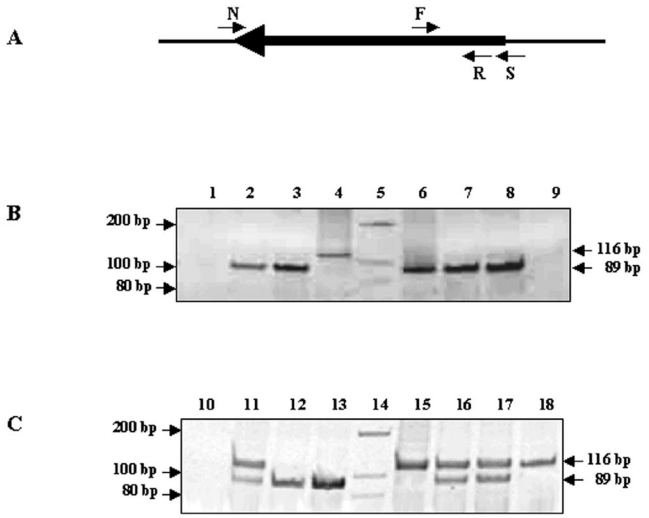

FIG. 1. N-PCR for detecting and differentiating *M. tuberculosis* and *M. bovis* in CSF samples. (A) Positions of the primers in the *hupB* gene (Rv2986c in *M. tuberculosis*, Mb3010c in *M. bovis*) sequence are depicted. Primers N and S (S-PCR) are specific for the *hupB* gene, and internal primers 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 nondenaturing 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.

TABLE 2. Comparative efficiencies of N-PCR and auramine O-stained smear examination in CSF samples for detection of mycobacteria

Method	No. (%) of samples					
	Tested	Positive	*M. tuberculosis*	*M. bovis*	Dual infection	Negative
N-PCR	112	37 (33.0)	3 (2.7)	19 (17.0)	15 (13.4)	75 (67.0)
AFB smear	112	14 (12.5)	CND[a]	CND	CND	98 (87.5)

[a] CND, could not differentiate mycobacterial species.

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 amplified 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 ($n = 5$), and 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

Patient category[a]	Total no.	No. of N-PCR results		No. of microscopy results	
		Positive	Negative	Positive	Negative
Definite TBM	9	9	0	9	0
Highly probable TBM	3	2	1	0	3
Probable TBM	5	3	2	0	5
Possible TBM	8	1	7	0	8
NTBM	44	5	39	0	44
Total	69	20	49	9	60

[a] Patients were categorized on the basis of the criteria described in Table 1.

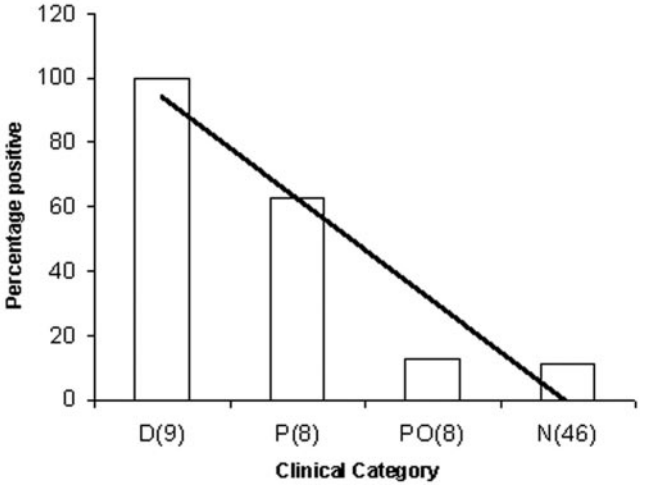

FIG. 2. Correlation between percent N-PCR positivity and clinical categorization of patients. Clinical categorization was based on previously described criteria (Table 1) (1). The bars represent the percent positivity in each category of patients. The straight line connecting the bars denotes the trend in positivity by N-PCR in each of the groups. (D, definite TBM; P, highly probable and probable TBM; PO, possible TBM; N, non-TBM). The value in parentheses is the number of patients in each category.

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

Assay	Result	No. of patients with clinical diagnosis[a] of:		Sensitivity[b] (%)	Specificity[c] (%)
		TBM ($n = 25$)	NTBM ($n = 44$)		
N-PCR	Positive	15	5	60.0	88.6
	Negative	10	39		
Microscopy	Positive	9	0	36.0	100
	Negative	16	44		

[a] 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] $TN/(TN + FP) \times 100$.

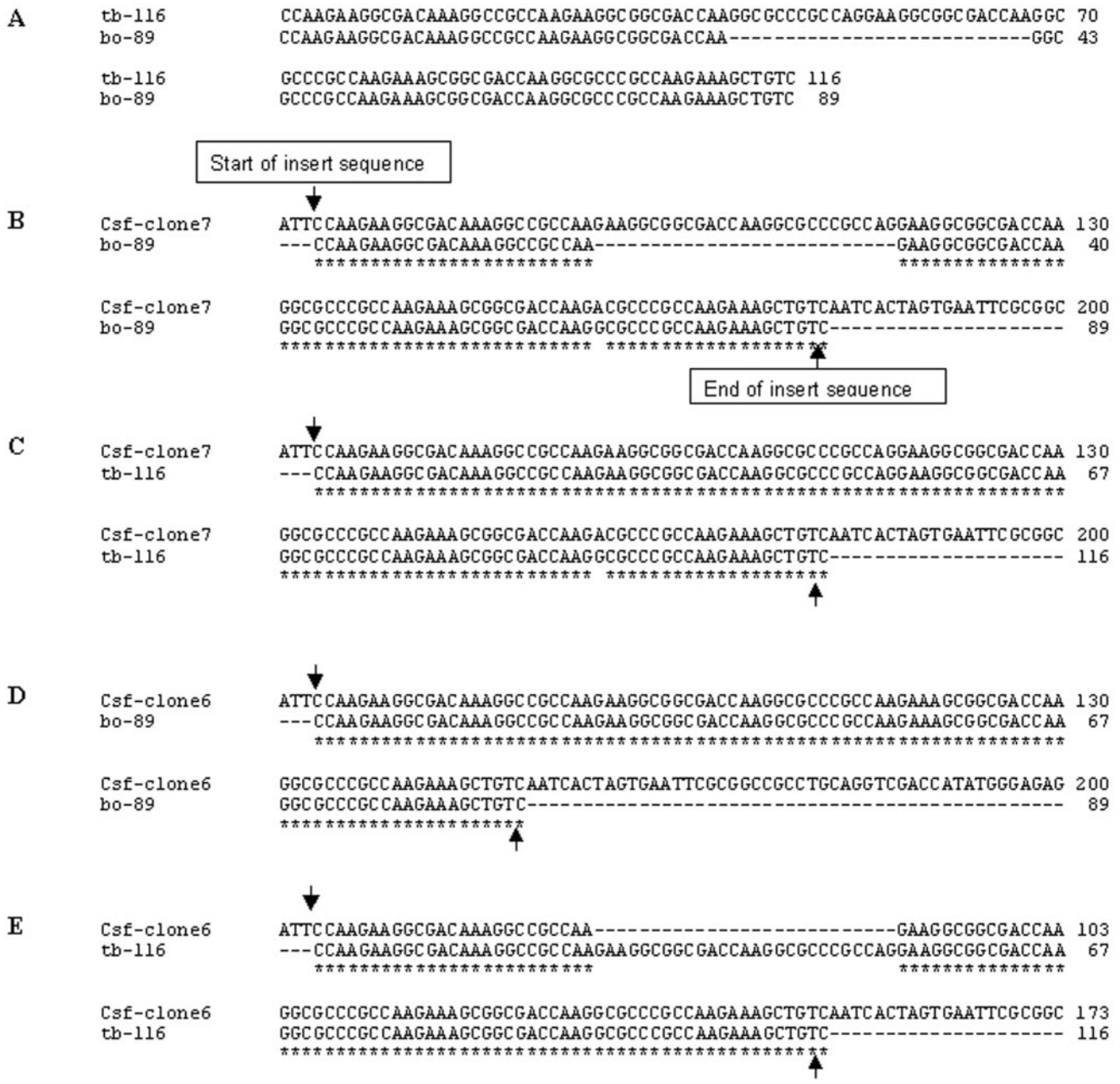

FIG. 3. Sequence alignment of the clones obtained from a CSF sample which showed mixed infection. The 116- and 89-bp amplicons were cloned separately into vector pGEMT and sequenced. Panel A depicts a comparative alignment of the sequences of N-PCR products of the *hupB* gene of *M. tuberculosis* (Rv2986c, tb-116, accession no. NC_000962) and *M. 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.

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. tuberculosis* (tb-116) and *M. bovis* (bo-89). Figure 3A depicts the sequence alignment of the N-PCR products of the C-terminal parts of the *hupB* gene of *M. tuberculosis* and *M. bovis*, showing the 27-bp difference between the two products (32).

Figure 3B and C show a comparative sequence analysis of the clone containing the higher-molecular-weight N-PCR product (116 bp) as an insert. The sequence has been compared with those of *M. tuberculosis* (tb-116, panel C) and *M. bovis* (bo-89, panel B). Complete identity was seen with that of

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, viz., *M. tuberculosis* and *M. bovis* (32). The utility of the *hupB* gene target for differentiating *M. tuberculosis* and *M. bovis* not only in isolates but also directly in bovine specimens has been previously demonstrated (26). This is an advantage over the other assays, which target multiple loci and genes to differentiate *M. tuberculosis* from *M. bovis* (19, 34). Identification to the species level of members of the MTC present in a sample would not be important from the viewpoint of chemotherapy, as both *M. tuberculosis* and *M. bovis* have been shown to respond to standard chemotherapeutic regimens, with the exception of pyrazinamide (29). However, identification to the species level would be important in determining the transmission chain, reservoirs of infection, and prevalence of mycobacterial pathogens in suspected cases of TBM. This, in turn, would be useful in formulating preventive therapies and strategies in the interest of public health. The differential ability of BCG immunization to generate effective immunity against TBM among children in particular (23, 36) and its inability to provide protection against pulmonary TB in adults (8) indirectly indicate the role of *M. bovis* as a causative agent of pediatric TBM, as substantiated by the present study.

The notable aspect of the N-PCR assay has been the detection of *M. tuberculosis* and *M. bovis* infections together in CSF samples. Detection of mixed infection with more than one strain of *M. tuberculosis* in human clinical samples by molecular techniques has been reported (5, 31, 38, 43). Mixed infections in these reports were established by the definite patterns in DNA fingerprints observed in isolates from a single sample. Besides DNA fingerprinting, spoligotyping and amplification of specific gene targets have been used to identify *M. tuberculosis* and *M. bovis* in archival tissue (45). Kidane et al. (21) have detected mixed infection with *M. tuberculosis* and *M bovis* in human lymphadenitis. In this study, the patients in whom *M. bovis* or mixed infection was detected by the N-PCR assay were equally distributed in rural and urban areas. The increased risk of TBM in these individuals perhaps relates to not only their socioeconomic standards, reflecting their nutritional status, but also their poor living standards, overcrowding, and the prevailing unsanitary conditions. In our earlier study (26), we had demonstrated both by culture and N-PCR assay the prevalence of *M. tuberculosis*, *M. bovis*, and mixed infections in bovines. As both in the villages and cities no restriction is placed on the movement of dairy or stray cattle, owing to economic compulsions and religious norms prevalent in our country and the high frequency of individuals coming in contact with these animals, there is a potential for transmission of these pathogens from

infected 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*.

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