ORIGINAL ARTICLE

Molecular Identification of *Mycobacterium bovis* in Human Pulmonary Tuberculosis: Insights from a Tertiary Care Hospital in Gujarat, India

Dipal Bhatt[1*], Suman Singh[2], Piyush Chudasama[3]

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Abstract

Background: *Mycobacterium bovis*, the causative agent of bovine tuberculosis, is a zoonotic pathogen capable of infecting cattle and humans. Human contraction of bovine tuberculosis, particularly pulmonary infection, remains a significant public health concern. The differentiation between *Mycobacterium bovis* and *Mycobacterium tuberculosis* is challenging due to limitations in conventional diagnostic methods, leading to an underestimated burden of *M. bovis* in human population. This study focuses on the prevalence of *M. bovis* in cases of pulmonary tuberculosis in a tertiary care teaching hospital located in Karamsad, Anand, a rural district of Gujarat.

Methods: In this cross-sectional study, 1,000 sputum samples from patients clinically suspected of having pulmonary tuberculosis were collected at the Department of Respiratory Medicine from November 2017 to June 2018. All samples underwent Ziehl–Neelsen staining for Acid Fast Bacilli detection, followed by molecular testing using primers targeting the *HupB* gene (a histone-like protein), to differentiate between *M. tuberculosis* and *M. bovis*.

Results: Of the 1,000 sputum samples, 100 (10%) tested positive for Acid Fast Bacilli. Molecular analysis revealed that 90% of these positive samples were *M. tuberculosis*. Among the remaining samples, 4% were positive for *M. bovis*, and 6% indicated a mixed infection with both *M. tuberculosis* and *M. bovis*.

Conclusion: The study found the prevalence of *M. bovis* in 10% cases of pulmonary tuberculosis in the Anand district of Gujarat. The findings highlight the limitations of conventional diagnostic methods in identifying *M. bovis* infections and demonstrate the efficacy of molecular techniques, explicitly targeting the *HupB* gene, for accurate detection and differentiation of *M. tuberculosis* and *M. bovis*. The evidence of coinfection in 6% patients further emphasizes the complexity of tuberculosis diagnosis in endemic areas.

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Introduction

Tuberculosis, a global health crisis, continues to affect millions every year. A significant contributor to this burden is *Mycobacterium bovis*, the causative agent of bovine tuberculosis in humans and animals. This form of tuberculosis is especially prevalent in developing countries, where there is a high degree of interaction between humans and animals in rural areas, raising public health concerns and causing economic losses.[1]

In 2019, the World Health Organization estimated that *M. bovis* was responsible for approximately 143,000 new tuberculosis cases and 12,300 deaths.[1] However, the reporting and surveillance of *M. bovis*-related tuberculosis are not uniform across countries. This inconsistency is evident as only 16 countries provided specific data on this issue. The estimated number of cases varied widely, ranging from 71,000 to 240,000, accounting for about 1.4% of the 10 million global pulmonary tuberculosis cases.[2] A focused study in Mexico City from 2000 to 2014 showed that *M. bovis* represented 26% of isolates from all sites and 16% of pulmonary samples, indicating its significant regional impact.[3]

The transmission of bovine tuberculosis to humans typically occurs through inhalation of aerosols containing the bacteria, direct contact with infected animals, or consumption of contaminated products.[4,5] The severity of lung involvement in pulmonary tuberculosis varies, influenced by several factors, including the bacterial strain, the host's immune response, and the timeliness of diagnosis and treatment.[5,6]

Clinically, *M. bovis* cannot be easily distinguished from *M. tuberculosis* in patients, as they present similarly radiologically and pathologically. Most conventional microbiological techniques designed for diagnosing tuberculosis identify members of the Mycobacterium tuberculosis complex but do not differentiate between specific species, including *M. bovis*. The introduction of nucleic acid-based methods, particularly polymerase chain reaction (PCR), has greatly improved the ability to identify and differentiate mycobacterial species, a vital step in cases where *M. bovis* infection is suspected.[7–9]

In resource-limited settings, obtaining comprehensive national data on the frequency of human tuberculosis caused by *M. bovis* is challenging.[10] Consequently, the full extent of *M. bovis*-related tuberculosis in certain regions remains largely unknown. In Gujarat, for instance, the burden of *M. bovis* is not well-documented, often leading to misdiagnosis.[11] Therefore, our study focuses on determining the prevalence of *M. bovis* in human pulmonary tuberculosis cases. We selected a tertiary care teaching hospital as our study site, where we collected sputum samples from patients exhibiting tuberculosis symptoms. These samples were subjected to molecular techniques, primarily nucleic acid-based methods, to identify and differentiate the *Mycobacterium* species present.

By conducting this study, we aim to find the prevalence of *M. bovis* in pulmonary tuberculosis cases in this region of Gujarat.

The findings could provide valuable insights into the regional impact of this disease and aid in developing targeted strategies for diagnosis, treatment, and prevention.

Methods

Ethics Statement

This study was conducted with the approval of the Institutional Ethics Committee of Shree Krishna Hospital, Karamsad, Gujarat, under the

[1]Assistant Professor, Smt. LP Patel Institute of Medical Laboratory Technology, Bhaikaka University; [2]Professor, Department of Microbiology, Pramukhswami Medical College, Bhaikaka University, Karamsad; [3]Research Scientist, Department of Research and Development Unit, Sat Kaival Hospital Pvt Ltd, Anand, Gujarat, India; *Corresponding Author

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HREC clearance number HMPCMCE/HREC/14/SESSION-2/12. Adherence to ethical standards was a priority; thus, written informed consent was obtained from all participants, ensuring their autonomy and confidentiality. The research team took great care to ensure that no personal information of the patients was disclosed during or after the study.

Study Design and Participants

The research design was a prospective cross-sectional study carried out from November 2017 to June 2018. The participants included patients who exhibited clinical symptoms suggestive of pulmonary tuberculosis. These individuals sought medical attention at the Outpatient Department of Respiratory Medicine at Shree Krishna Hospital, Karamsad. However, patients diagnosed with extrapulmonary tuberculosis were excluded from the study. Comprehensive demographic data and other relevant information, such as occupation, animal-handling habits, and consumption of raw, unpasteurized milk, were meticulously gathered from each participant using a structured proforma.

Sample Collection and Processing

In this study, 1,000 patients suspected of having pulmonary tuberculosis were enrolled. The collection of sputum samples involved obtaining preferably one early morning specimen and two spot specimens. These were collected in sterile, leak-proof containers, with patients receiving detailed instructions on the collection process. The collected specimens were then transported to the microbiology laboratory for further processing.

For the detection of acid-fast bacilli (AFB), the Ziehl–Neelsen staining method was utilized. Sputum smears, prepared from the purulent part of the sample, were stained and examined under an oil immersion lens of a light microscope. The presence and quantity of AFB were graded following the Revised National Tuberculosis Control Programme (RNTCP) guidelines.[12] The sputum samples underwent a decontamination process, were homogenized, and liquefied using the modified Petroff's concentration method to effectively release the bacilli trapped in the mucus. The sputum sample was stored at –20°C for molecular procedure.

Identification of *Mycobacterium bovis* using Molecular Technique

DNA extraction from the concentrated sputum samples was performed using the QIAGEN DNA mini kit.

Polymerase Chain Reaction Amplification

The study utilized specific primers targeting the *HupB* gene (coding for histone-like protein), as detailed in Table 1.[11,13] The primer sequences were validated using the primer-BLAST database of NCBI and synthesized by Eurofin Genomics India Pvt. Ltd. Each PCR mix, with a total volume of 25.0 µL, included 12.50 µL of Emerald master mix, 7.50 µL of PCR water, 1.00 µL each of reverse and forward primers, and 3.00 µL of the DNA template.

The PCR protocol for amplifying the *hupB* gene target included an initial denaturation at 95°C for 10 minutes, followed by 35 cycles comprising denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, with a final extension phase at 72°C for 7 minutes. Postamplification, 10 µL of the amplicon was mixed with 3 µL of DNA loading dye and loading buffer for electrophoresis on a 2% agarose gel prepared with ethidium bromide. The results were visualized using a Gel Doc system.

The data was collected in a predesigned proforma and entered into an Excel sheet. The Statistical Package of Social Sciences (SPSS), IBM version 22, was used for the statistical analysis of data. Categorical measurements were summarized as the number and percentage, and descriptive statistics were used for numerical variables wherever applicable.

Results

In this study involving 1,000 enrolled patients, we identified 100 individuals who tested positive for AFB, as detailed in Table 2. These positive cases exhibited varying AFB presence according to the RNTCP guidelines. Notably, most of these cases (41 out of 100) demonstrated a 1 + grade level of AFB in their sputum samples. On the lower end of the spectrum, 10 patients showed only scanty amounts of AFB.

Demographically, as illustrated in Table 3, the predominant age group affected was between 26 and 40 years, accounting for 29% of the cases. Individuals above the age of 70 closely followed this. The mean age across the patient cohort was 17.18 years, with a standard deviation of ±9.27 years, encompassing a wide age range of 10–85 years. Regarding gender distribution, the study observed a higher prevalence in males, with 73 male patients compared to 27 females.

Further insights into the clinical profile and risk factors of these patients are presented in Table 4. A common characteristic among the participants was the duration of respiratory symptoms, which, in all cases, had persisted for over a month, indicative of active tuberculosis. The aspect of this study was the high percentage of patients engaged in certain occupations or activities that could potentially increase their risk of TB exposure. A significant 61% of the patients were involved in animal handling, while 40% had a history of contact with known TB cases. Additionally, lifestyle factors such as dietary habits were also notable; 45% of the patients reported consuming raw milk, and 30% were employed in farming.

This demographic and clinical data provide valuable insights into the patterns and risk factors associated with tuberculosis in this patient population, offering crucial information for targeted public health interventions and further research.

Detection and Identification of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in Sputum Samples by PCR

In this study, DNA was extracted from the sputum samples of 100 patients and subsequently subjected to PCR analysis. This technique was employed to ascertain the specific species of mycobacteria in each sample. The determination of the

Table 2: RNTCP grade in AFB-positive sputum samples (n = 100)

RNTCP grade (AFB)	*No. of sputum samples*
Scanty	10
1+	41
2+	29
3+	20
Total	100

Table 1: Characteristics of the primer used for detection of *M. bovis*

Target genes	*Primers*	*Primer sequence*	*Product size (bp)*	*Reference*
HupB	*HupB* (F) *HupB* (R)	5′GCAGCCAAGAAGGTAGCGAA-3′ 5′GTATCCGTGTGTCTTGACCTATTTG-3′	336 *M. tuberculosis* and 309 *M. bovis*	Prabhakar et al.[13]

Table 3: Age and sex distribution of participants ($n = 100$)

Age (in year)	*Male*	*Female*	*No. of participants (%)*	*Mean age (years)*	*Std dev ± 2*
10–25	15	08	23 (23)	17.18	9.27
26–40	23	06	29 (29)		
41–55	17	08	25 (25)		
56–70	15	03	18 (18)		
70 and above	03	02	5 (5)		
Total	73	27	100 (100)		

Table 4: Clinical profile and risk factors in pulmonary tuberculosis patients ($N = 100$)

Characteristics	*Mycobacterium tuberculosis (n = 90)*	*Mycobacterium bovis (n = 4)*	*Mixed (n = 6) (Mycobacterium tuberculosis and Mycobacterium bovis)*	*N = 100*
Fever	82 (91%)	3 (75%)	2 (33%)	87
Cough	87 (97%)	3 (75%)	6 (100%)	96
Weight loss	66 (73%)	2 (50%)	5 (83%)	73
Hemoptysis	23 (26%)	2 (50%)	2 (83%)	30
HIV	2 (2.2%)	0	0	2
Previous contact with a TB case	34 (38%)	4 (100%)	4 (67%)	40
Raw milk consumption	51 (57%)	4 (100%)	6 (100%)	45
Animal handler	36 (40%)	4 (100%)	5 (83%)	61
Farmer	24 (27%)	2 (50%)	4 (67%)	30

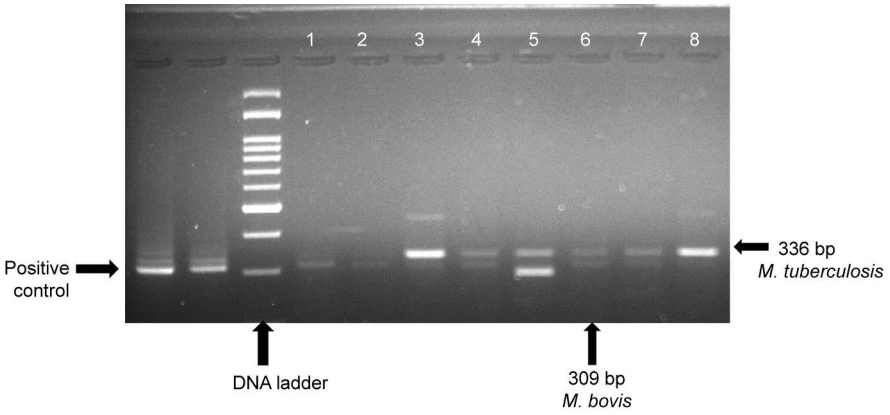

Fig. 1: PCR result for the identification of *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

mycobacterial species was achieved by analyzing the molecular size of the PCR products. These products were then subjected to electrophoresis on a 2% agarose gel alongside appropriate controls to ensure the accuracy and reliability of the results.

The findings of the PCR assays are detailed in Figure 1. Most of the samples, accounting for 90%, were identified as containing *M. tuberculosis*, the primary causative agent of tuberculosis in humans. In addition, *M. bovis*, a species more commonly associated with tuberculosis in cattle but capable of infecting humans, was detected in 4% of the samples. Interestingly, a small percentage of the samples, amounting to 6%, contained both *M. tuberculosis* and *M. bovis*.

These results highlight the prevalence of *M. tuberculosis* in this patient cohort while also pointing to the presence of *M. bovis* in a notable fraction of cases. Detecting both species in some samples underscores the complexity of tuberculosis infections and the necessity for accurate species-level identification for effective diagnosis and treatment.

Discussion

The earliest recorded case of bovine pulmonary tuberculosis in a human, identified through bacteriological testing, was reported in 1909. This landmark discovery opened avenues for extensive research over the subsequent decades, revealing that *M. bovis* was the causative agent in 1–3% of human cases of pulmonary tuberculosis.[14] The current study aimed to assess the prevalence of pulmonary tuberculosis due to *M. bovis* in a tertiary care teaching hospital in Gujarat, India, a region where tuberculosis remains a significant public health challenge.

In this study, we collected 1,000 sputum samples from patients suspected of having pulmonary tuberculosis and conducted tests for AFB. Of these, 100 samples were found to be positive for pulmonary tuberculosis. This 10% positivity rate is in line with the findings from Peshawar, Pakistan,[8] where a similar study reported that 100% of the samples tested were positive for pulmonary tuberculosis, underscoring the persistent prevalence of the disease in South Asia.

Of the 100 positive cases in our study, 73 (73%) were male. This gender disparity echoes the findings of Tchatchouang et al.,[15] who observed a higher infection rate in men (53.16%) than women (46.84%). Similarly, Bapat et al.[9]noted that the disease primarily affects men aged between 20 and 45 years. These observations suggest that occupational factors, possibly linked to increased exposure risks in certain professions predominantly held by men, may play a role in disease distribution.

To enhance the specificity of our diagnostic approach, we employed PCR, a technique known for its rapidity and sensitivity, particularly compared with traditional methods such as Ziehl–Neelsen staining and culture.[8] For this purpose, purified genomic DNA from the sputum samples was subjected to PCR using sequence-specific primers targeting the *HupB* gene. The resulting PCR products were 309 bp (corresponding to *M. bovis*) and 336 bp (corresponding to *M. tuberculosis*), facilitating

the differentiation between these closely related mycobacterial species.

Mycobacterium tuberculosis's histone-like protein gene (*HupB* [*Rv2986c*]) has been recognized as a critical marker for distinguishing *M. tuberculosis* and *M. bovis* within the MTB complex via a PCR assay. In our analysis of the 100 samples, 90 (90%) were positive for *M. tuberculosis*, 4 (4%) for *M. bovis*, and 6 (6%) indicated coinfection with both species. These findings are consistent with those reported by Prasad,[10] highlighting the presence of both *Mycobacterium* species in pulmonary cases.

The study by Bapat et al.[9] obtained 32 culture isolates from 347 samples using traditional culture techniques. While effective in growing mycobacteria, these methods lacked the specificity required for species-level identification. To overcome this, PCR assays were applied to the same samples, revealing 9 cases (2.59%) of *M. bovis* and 60 cases (17.29%) of *M. tuberculosis*. These results demonstrate the enhanced accuracy of PCR in identifying members of the *Mycobacterium tuberculosis* complex (MTC).

This pattern was further evidenced in the study by Nawaz,[16] where 100 sputum samples examined using PCR identified 37 (37%) as positive for *M. tuberculosis* and 5 (5%) for *M. bovis*. The present study's findings align with these results, confirming the coexistence of both *M. tuberculosis* and *M. bovis* in the sputum samples identified through the advanced molecular technique of PCR.

In our research, 6 (6%) patients presented with coinfections by *M. tuberculosis* and *M. bovis*, a scenario similar to the findings of Silva,[17] who reported a 1.6% rate of *M. bovis–M. tuberculosis* coinfections. The application of the *HupB* gene as a PCR target was instrumental in these identifications. The study also analyzed 100 mycobacterial strains, finding the *HupB* gene-specific primers suitable for distinguishing MTB complex members. This technique aligns with the discoveries made by Prabhakar et al.,[13] who observed variations in *HupB* gene product sizes between *M. tuberculosis* (645 bp) and *M. bovis* (618 bp).

Our study revealed that the *HupB* gene was found in 6% of mixed infections by both species, 4% of *M. bovis*, and 90% of *M. tuberculosis* cases. Multiple copies of the target gene in the mycobacteria genome likely account for the high PCR activity observed. However, integrating additional markers such as IS6110, oxyR, and rpoB in future studies could provide further confirmation and insight into the genomic complexity of these pathogens.[15]

It is noteworthy that reported incidence rates of zoonotic TB in developed regions such as Europe, the United States, Australia, and New Zealand have consistently remained below 1 per 100,000 population per year.[18,19] In contrast, the incidence rates in several other regions, including parts of Asia and Africa, are not as well documented. In the Indian context, studies like those conducted by Shah et al.[20] and Prasad et al.[10] have provided crucial insights into the occurrence of *M. bovis* in humans and cattle. These studies and the present research underscore the importance of comprehensive screening and accurate differentiation of *M. tuberculosis* complex members in humans and livestock, as Mittal et al. emphasized.[21]

In summary, this study successfully demonstrates the presence of pulmonary infections caused by *M. bovis* and *M. tuberculosis* in a tertiary care hospital in Anand, Gujarat. The results, confirmed via advanced molecular techniques, highlight the utility and necessity of PCR in differentiating *M. tuberculosis* and *M. bovis*. This distinction is crucial for appropriate disease management and the formulation of targeted public health strategies. Our findings contribute to the growing body of evidence on the prevalence and characterization of tuberculosis-causing mycobacteria, reinforcing the need for continued research and surveillance in this critical area of public health.

Orcid

Dipal Bhatt https://orcid.org/0000-0002-6895-1836
Suman Singh https://orcid.org/0000-0002-2440-8049

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