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PCR- based detection of *Mycobacterium tuberculosis* complex (MTBC) in different bovine samples from Guwahati, Northeast India

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PCR- based detection of *Mycobacterium tuberculosis* complex (MTBC) in different bovine samples from Guwahati, Northeast India

Abstract

Bovine tuberculosis (bTB) is a chronic and often overlooked zoonotic disease caused by *Mycobacterium bovis*, a globally distributed pathogen within the group *Mycobacterium tuberculosis* complex (MTBC). The present study was conducted to detect *Mycobacterium tuberculosis* complex (MTBC) at the molecular level in clinical and slaughterhouse samples from bovines. A total of 620 animal samples (milk-360, nasal swabs-150, and tissue-110) were screened using polymerase chain reaction (PCR) with primers specific for the *Mycobacterium* genus-specific *hsp65* gene, MTBC-specific *IS6110* and *IS1081*. Tissue samples were initially processed for microbiological analysis using Ziehl-Neelsen (Z-N) staining to detect acid-fast bacilli, followed by isolation of mycobacteria from the Z-N positive samples. Out of 620 samples, the *hsp65* gene was detected in 43 (6.9%) samples and further molecular analysis confirmed that 24 of these were MTBC-positive. Of the 110 tissue samples examined by Ziehl-Neelsen (Z-N) staining, 12 (10.9%) were positive for acid-fast bacilli. However, only two samples were successfully isolated in Lowenstein-Jensen (L-J) media and detected as MTBC by PCR and confirmed through sequencing. This study highlights the widespread presence of bovine tuberculosis in these understudied dairy farms in Guwahati, the largest city in Northeast India. The findings emphasize the need for a comprehensive epidemiological study and the implementation of a One Health approach for the strategic control and prevention of bTB in the region.

KEYWORDS: Bovine tuberculosis, isolation, PCR, prevalence, sequencing

Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle caused by *Mycobacterium bovis*, a member of *Mycobacterium tuberculosis* complex (MTBC) that impacts productivity and represents a major public health threat through contaminated raw milk, meat and its products [1,2].The animal adapted MTBC members include *M.bovis, M. orygis, M.caprae, M. africanum, M. microti, M. pinnipedii, M.mungi* and *M.suricattae,* which are widely distributed in domesticated animals and wildlife [3]. *M. bovis* has an exceptionally broad host range, infecting nearly all mammals, with the highest incidence observed in cattle, which serve as the primary reservoir [4]. bTB is considered a neglected zoonotic disease and is responsible for a substantial burden of human tuberculosis (TB), with an estimated 140,000 new cases worldwide and approximately 11,400 (8.1%) deaths in 2019 [5]. Beyond its public health implications, bTB causes significant global economic losses, estimated at $3 billion annually, due to reduced cattle productivity, culling, and trade restrictions [6]. The disease remains endemic in many low- and middle-income countries, including India, where economic constraints and socio-religious factors hinder the implementation of test-and-cull strategies [7]. The endemicity of bTB is further exacerbated by the intensification of cattle farming in land-constrained, rapidly growing cities, where the high demand for milk and meat accelerates its spread.

The diagnosis of bovine tuberculosis relies on clinical signs, incidental findings during necropsy, tuberculin skin testing, and culture-based isolation methods. Additionally, immunological assays such as lymphocyte proliferation tests, the gamma interferon assay, and indirect enzyme-linked immunosorbent assays (ELISA) are also available [8]. Molecular diagnostic techniques, such as polymerase chain reaction (PCR), have been successfully applied to various sample types from live animals, including

lymph node aspirates [9], milk [10], blood [11], and nasal secretions [12]. These methods offer rapid and accurate detection and show considerable promise for early diagnosis. However, no single diagnostic test currently meets all the criteria necessary to identify all infected animals. As a result, a combination of diagnostic approaches is often required to achieve reliable detection [13]. Effective disease control relies on the timely identification and removal of infected animals to prevent transmission within and between herds. The incidence of bovine tuberculosis is reported to be rising, particularly in parts of Asia and Africa, where socioeconomic conditions and veterinary infrastructure remain limited [14].

Given these challenges, it is crucial to assess the status of bTB in urban and peri-urban areas to provide policymakers with accurate data for developing effective control strategies. There was no previous report on systematic molecular detection of MTBC in milk, nasal swab and tissue in the study area. In this context, a cross-sectional, slaughterhouse and farm-based molecular study was conducted to determine the distribution of MTBC among dairy cattle in Guwahati, a major city in Northeast India. This study represents the prevalence of bTB in Assam, confirmed through bacterial isolation, Ziehl-Neelsen staining, molecular detection and sequencing.

Methods

Ethical approval

The study was conducted in accordance with the animal ethics approval granted by the Institutional Animal Ethics Committee of the College of Veterinary Science, Assam Agricultural University (Approval No. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC/19-20/739, dated December 23, 2019).

Study area

The study was conducted on dairy farms located in and around Guwahati, the metropolitan city of Assam, India. The majority of livestock farmers in Guwahati rely on dairy farming as their primary source of income, and all follow an intensive livestock production system [15]. Within the study area, several small,

unorganized slaughter points are present, some of which share boundaries with the neighboring state of Meghalaya. These slaughter points receive large ruminants from local dairy farms and nearby districts, where they are processed, and their carcasses are sold in markets. The Global Positioning System (GPS) coordinates (latitude and longitude) of the selected areas were recorded using the GPS Map Camera Lite application (version 1.3.3). The study area is illustrated in the GPS map (Fig. 1).

Sampling and processing for molecular detection

A cross-sectional study was carried out in the dairy farms and slaughtered points in and around Guwahati city from December 2020 to November 2021. A total of 36 farms were tested in the present study which comprised of both small-scale farm (less than 30 animals) and large-scale farms (more than 30 animals). From each farm, 10 animals were selected which included both clinically ill (chronic coughing, fever, reduced milk production and emaciation) as well as apparently healthy (in contact animals). The selected animals were initially screened by single intra dermal comparative tuberculin test (SICTT) [16]. For the molecular detection of bovine tuberculosis (bTB), a total of 620 samples were collected, including 360 milk samples and 150 nasal swabs from the SICCT screened dairy farms, along with 110 tissue samples from 110 different slaughtered cattle were collected from the slaughtered points. Milk samples (n = 360) were obtained by pooling 50 mL of milk from all four quarters of each cow. The milk samples were collected aseptically in Falcon tubes (Tarsons, Kolkata, India) after proper udder disinfection and discarding the first few jets of milk. Nasal swabs (n = 150) were collected from deep within the nostrils using sterile nasal swab containers (Himedia, Mumbai, India). Tissue (lungs) samples (n = 110) were obtained from 110 different animals either from slaughtered cattle or post-mortem examinations and placed in sterile clinical sample containers (Himedia, Mumbai, India). All tissue samples were collected aseptically, transported under chilled conditions, and freshly collected samples were processed for microbiological analysis (acid fast staining, culture and DNA isolation) within 24 hours in the laboratory and the remaining samples were stored at -80°C for further molecular analysis. Sample processing was carried out under Class-III biosafety cabinet (ESCO, Singapore). Milk samples were digested and

decontaminated following the method described by Dundee et al [17]. Tissue and nasal swab samples were digested and decontaminated using the protocol established by Petroff [18]. One part of sample (nasal swab/ tissue) was mixed with 3 parts of 2-4% sodium hydroxide solution (NaOH) and incubated at room temperature for 10-15 mins to digest organic material and decontaminate the sample. The mixture was centrifuged thereafter and the pellets were washed 2-3 times with PBS. After washing, the pellets were again mixed with 1ml sterile PBS and processed for DNA extraction. DNA was extracted from milk, nasal swab, and tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted DNA samples were then stored at -20°C for further molecular analysis.

Reference Strains used for PCR standardization

The reference strain of *M. bovis* (AN-5) was available in the laboratory of the Division of Animal and Fisheries Sciences, ICAR RC NEH, Umiam, India, while *M. phlei* (Microbial Type Culture Collection, MTCC 1724) was obtained from the CSIR–Institute of Microbial Technology, Chandigarh, India.

Growth Media used for Mycobacteria Isolation

For the isolation of *Mycobacteria*, two solid media were used: Lowenstein-Jensen (LJ) slants with glycerol and Lowenstein-Jensen with Glycerol-Pyruvate (LJ-GP) slants (Hi-Media, Mumbai, India). Only selected suspected tuberculosis nodules were processed for isolation. Approximately 100 µL of inoculum was seeded onto Lowenstein-Jensen media (Hi-Media, Mumbai, India) supplemented with glycerol and glycerol-pyruvate, the latter favoring the growth of *M. bovis*. The cultures were incubated at 37°C for 8–12 weeks, with weekly observations for bacterial growth.

Molecular Identification and strain differentiation by PCR

To achieve the study's objectives, a combination of PCR primers with proven efficiency was

used. Due to the close genomic relationship among MTBC members, primers targeting both the *Mycobacterium* genus and *Mycobacterium tuberculosis* complex specific genes were selected, based on well-established literature references (Table 1). The primers used were mycobacterial genus specific - *hsp*65 amplifying 441bp [19]; two different MTBC specific primers -IS6110 amplifying 123bp [20] and IS1081 amplifying 248bp [21] to differentiate the MTBC from NTM (Nontuberculous Mycobacteria).

Reaction preparation

All reactions were performed in thin-walled 200 µL PCR tubes. The study utilized Thermo Scientific DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, MA, United States), a ready-to-use solution containing DreamTaq DNA Polymerase, dNTPs, $MgCl_2$, and an optimized buffer. The total reaction volume was 20 µL, consisting of 10 µL DreamTaq Green Master Mix, 2.0 µL template DNA, 1.0 µL of each primer (10 pmol), and 6.0 µL nuclease-free water. Each primer was optimized and positive and negative controls were included in every experiment to ensure accuracy and reliability. The DNA concentration of the template used for screening was estimated to be between 4.7 and 24.5 ng/µl.

PCR standardization and sequence-based analysis

The *hsp65* PCR assay was initially standardized using 2 µL of template DNA isolated from *M. bovis* AN5 and *M. phlei* (MTCC1724) cultures. Since *hsp65* can amplify all *Mycobacterium* species, so in addition to *M.bovis* AN5, *M. phlei* was also used as a positive control for this assay. For the remaining PCR assays—MTBC-specific (*IS6110* and *IS1081*) *M. phlei* was used as a negative control, while *M. bovis* AN5 served as a positive control. PCR conditions for each primer set were as follows: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation (95°C for 35 seconds), annealing (X°C for 30 seconds), and extension (72°C for 30 seconds), with a final extension at 72°C for 7 minutes. The annealing (X) for *hsp65,* fragment was 59^0C; for IS*6110* was 69^0C and for IS*1081* was 70^0C. Samples positive for *hsp65*, *IS6110*, and *IS1081* were further confirmed through Sanger sequencing. The

sequenced products were then subjected to nucleotide BLAST analysis in the NCBI database for validation.

Results

Gross pathological examination results of slaughtered cattle

Of the 110 clinically suspected tissue samples, 28 exhibited gross tuberculous-like lesions. The majority of the lesions observed were characteristic of tuberculous pathology, defined by centrally located, round, oval, or irregular foci of caseous necrosis and calcification, primarily in the lungs of slaughtered cattle (Fig. 2, A). Grayish white to yellowish nodules of varying sizes with fibrotic lesions were also recorded (Fig.2, B). Carcasses were evaluated following the guidelines of the World Organization for Animal Health [22].

Detection of acid-fast organism by microscopic examination from direct tissue

All 110 tissue samples were initially screened using the acid-fast staining test to detect acid-fast bacilli, with 10.90% testing positive (Table 2). Unlike the long, slender, beaded rods typically observed in culture, the organisms in tissue samples appeared as slender rods, either straight, curved, or club-shaped.

Isolation of *Mycobacterium spp.* from tissue samples of slaughtered cattle

In the present study, 14 numbers of freshly collected suspected bTB tissue samples were processed for the isolation of *Mycobacterium spp*. After 2 to 4 months of incubation on Lowenstein-Jensen (L-J) media, both glycerol-based and glycerol-pyruvate-based, two tissue samples (14.28%) exhibited small rough or granular bacterial growth, which tested positive for *MTBC* in culture. The culture-positive tissue samples showed a high degree of positivity in Ziehl-Neelsen (ZN) staining and were further confirmed through conventional PCR targeting *hsp65*, *IS6110* and *IS1081* using DNA extracted from the colonies.

Molecular detection of mycobacterial DNA in different bovine samples

The study employed adjuncts of several molecular approaches for detection and differentiating the mycobacterial infection in the samples (milk, nasal swab and tissue). Overall, 43/620 (6.9%) samples were positive for mycobacterial genus specific *hsp*65 PCR (fig-3) and of these 43 samples, 24 were amplified by both IS1081 PCR (fig- 4) and/or IS6110 PCR (fig-5) indicating MTBC (Table 2). The sequence-based confirmation of the samples was conducted by determining the amplicon sequences using Sanger dideoxy sequencing, followed by an nBLAST analysis of the *hsp65*, *IS6110*, and *IS1081* genes from the selected samples. This analysis identified mycobacterial species in the positive sample with 100% identity. Specifically, *Mycobacterium tuberculosis* variant *bovis* was identified in sample CVSC_AAU_BTB (accession No. OQ417940) based on *hsp65* and KNL (accession nos. OQ417941 for *IS6110),* while *Mycobacterium tuberculosis* was confirmed in sample MTBC_knl_IS1081, with accession nos OQ417942 for *IS1081* in nBLAST.

Discussion

Bovine tuberculosis infections in dairy herds are a major concern in both underdeveloped and developing countries, including India, as they impact the economy and pose a risk to human health [7]. Detecting and confirming mycobacterial infections in animals remains challenging since no available diagnostic test is 100% sensitive or specific [13]. Additionally, these tests require specialized facilities, trained personnel, and significant costs. These challenges highlight the need for improved methodologies that can accurately detect bTB infections in both live and post-mortem samples while differentiating them from non-tuberculous mycobacteria (NTMs). Such advancements are crucial for developing effective control and prevention programs in the future.

To date, nearly 200 species of mycobacteria have been identified, the majority of which are either non-pathogenic or opportunistic non-tuberculous mycobacteria (NTMs). Aside from *M. leprae* and the NTMs, the primary pathogenic species belong to the *Mycobacterium tuberculosis* complex (MTBC), with *M.*

tuberculosis and *M. bovis* being most relevant to human and bovine health, respectively. *M. bovis* is of particular concern due to its zoonotic potential. However, the close phenotypic and genotypic similarities among mycobacterial species present significant challenges for accurate speciation. While whole-genome sequencing offers the highest resolution for identification, it is not always practical for routine diagnostic use. In clinical and field settings, a more feasible diagnostic approach involves integrating clinical signs with immunological tests, culture, and molecular assays. Currently, no single diagnostic test offers complete sensitivity and specificity for animal tuberculosis, and most methods are time-consuming, labor-intensive, and costly- limitations that are particularly acute in developing and underdeveloped countries. In veterinary diagnostics, where research infrastructure and laboratory capabilities are often more limited, there is a pressing need for simplified, affordable, and scalable diagnostic strategies to effectively detect and control mycobacterial infections in both animals and humans.

In the present study, gross tuberculous-like lesions of grayish white to yellowish nodules of varying sizes with round, oval, or irregular foci of caseous necrosis of bovine tuberculosis were recorded in animals. These findings align with previous studies conducted by Olea-Popelka et al [23] and Pascual-Linaza et al [24] in Ireland. In bovine tuberculosis, *Mycobacterium* primarily affects the respiratory system of immunocompromised animals. Consistent with this, the present investigation found that the highest numbers of tuberculous-like lesions were observed in the lungs. However, postmortem examination for detecting lesions is not 100% sensitive and a thorough examination of the lungs and lymph nodes can identify up to 95% of cows with visible tuberculosis lesions [25]. According to previous findings, reported by Ewnetu et al [26], gross TB lesions were primarily located in the lungs (69.79%), head lymph nodes (18.75%), mesenteric lymph nodes (9.38%), and hepatic lymph nodes (2.08%). In this study, although the majority of the tissue samples exhibited visible tuberculous-like lesions in the lungs, only 12 (10.9%) tested positive by acid-fast staining. This could be due to a low bacterial load in the samples or the presence of similar lesions, such as abscesses [27]. These findings are consistent with previous studies that relied solely on the Ziehl-Neelsen (Z-N) staining technique [28]. Though 28 samples exhibited the

gross tuberculous-like lesions, indicative of tuberculosis but we proceeded with only 14 samples for isolation and identification due to COVID-19 pandemic restrictions limiting us access to laboratory. For isolation of mycobacteria, the tissue samples should be fresh and it should be processed within 24 hrs in the laboratory. Although to carry out the isolation of mycobacteria is very difficult and time-consuming process, but in between an attempt was made with fresh samples during COVID-19 when we got access to laboratory. Out of 14 samples, only two samples (14.28%) could lead to isolation of pure colonies of mycobacterium, rest of the samples yielded no growth or contaminated growth. The extremely low culture yield from tissue samples (only two isolates from fourteen attempts) represents a key limitation, as culture remains the gold standard for confirming *Mycobacterium* infection. The poor recovery may be attributed to the slow growth rate of the organism, possible contamination, and low bacillary load in tissues. Colonies appeared on L-J slants after 9–10 weeks of post-inoculation from granulomatous lung lesions. This finding suggests that lung samples with nodules and pus formation are the most suitable specimens for isolating MTBC. Similar results were reported by Thakur et al (2016), who successfully isolated *M. bovis* colonies from lung and lymph node samples and validated their findings using biochemical tests [29]. According to Srinivasan et al (2018), the prescapular lymph gland is the most suitable specimen for isolating *Mycobacterium bovis* from infected cattle. However, collecting lymph node aspirates is a challenging technique, particularly in unorganized dairy farms, as it requires the expertise of a trained veterinarian.

In the present investigation, the *hsp65* gene proved to be an excellent marker for detecting mycobacterial infections in various sample types, including milk (16/360, 4.44%), nasal swabs (3/150, 2%), and tissue samples (24/110, 21.81%). The lower detection rate in milk is consistent with findings from other studies, likely due to the intermittent shedding of bacilli in milk and challenges associated with DNA isolation from direct milk samples [30]. The current findings align with those of other published report, who analyzed 300 bovine milk samples and identified *Mycobacterium* spp. in 24 samples using the *hsp65* gene [31]. The lower sensitivity of PCR for detecting *Mycobacterium* spp. in nasal swabs may be attributed to

the presence of a low bacterial load in these samples. However, nasal swabs remain a valuable tool for indicating airborne contamination within herds. They are particularly useful in cases of generalized tuberculosis, where some animals may not respond to skin tests, yet *M. bovis* can be detected in nasal secretions [32].

All 43 samples positive for *hsp65* PCR were further examined for amplification of MTBC-specific *IS6110* and *IS1081*-based PCR. Of these, 24 samples (n=24/43, 55.8%) that tested positive for at least one of these PCRs were classified as MTBC members, in milk (4/16, 25%), nasal swab (2/3, 66.67%) and tissue (18/24,75%). However, the *IS6110* and *IS1081* sequences specific to MTBC cannot differentiate between members of the complex, highlighting the need for additional primers. This study is the first to report the direct detection of MTBC DNA in milk, nasal swabs, and tissue samples from dairy cattle in Guwahati city, Assam. The presence of MTBC in milk poses a potential risk to humans, particularly due to the consumption of raw or unpasteurized milk in some communities [16, 33]. The presence of TB bacilli in tissue samples also poses a threat to carnivores in zoological gardens and wildlife, as they consume raw meat. Guwahati city has a zoological garden, and its meat supply comes from nearby slaughter points, which may increase the potential risk of bTB transmission.

We employed a two-group primer strategy to systematically confirm the presence of mycobacteria in samples. Initially, *hsp65* was used to detect mycobacteria, followed by *IS6110/IS1081* to identify members of the MTBC. However, a key limitation of this approach is the emerging theory that all *M. bovis* detected in India may actually be *M. orygis* [34]. To differentiate between *M.bovis* and *M.orygis* PCR based methods are not available while whole genome sequencing can differentiate.

Sequence-based confirmation of *hsp65, IS6110,* and *IS1081* genes through Sanger dideoxy sequencing, followed by BLASTn analysis, identified mycobacterial species in positive samples with 100% identity to *Mycobacterium*, *M. bovis* (also referred to as *M. tuberculosis* variant *bovis*), and *M. tuberculosis*. *hsp65*, *IS6110*, and *IS1081* can only confirm up to the genus or MTBC level. This is particularly valuable given the existence of over 200 mycobacterial species, including eight MTBC members, with new

additions frequently being recorded in the database [35, 36].

The findings of this study, based on gross tuberculous lesions, molecular detection, culture, and sequencing confirmation, provide strong evidence of bovine tuberculosis (bTB) prevalence in the Guwahati Metropolitan City, Assam, India. The study also suggests that older animals, those with poor health conditions, females, and certain crossbreeds may be more susceptible to bTB, although statistical significance was not established. The combination of body condition assessment, gross lesion evaluation, and immune and molecular assays is essential for accurate bTB diagnosis. The use of *hsp65* along with MTBC or species-specific genes offers a reliable approach for detecting pathogenic mycobacteria responsible for bTB.

The inference of the present study is limited to the representative farms and animals tested; however, further large-scale studies are required to generalize these findings. In addition, COVID-19 restrictions limited the scope of further analysis.

Conclusion

This study highlights the presence of bovine tuberculosis in the study area and underscores the need for targeted investigations in dairy herds, along with follow-up actions to remove infected animals from the herd. Raising awareness among dairy farmers and consumers about bTB is crucial. A collaborative effort involving the government, academia, and research institutions under the "One Health" approach is essential to reduce the burden of bTB in animals and, in turn, mitigate its impact on human populations. The study was also unique in employing a reliable combination of assays for the detection and confirmation of *Mycobacterium tuberculosis* complex (MTC) in the study area.

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Author contributions

All authors listed have made a substantial, direct and intellectual contribution to this research work. Sample collection, Laboratory investigation, Writing- original draft preparation by N.A.K. Conceptualization, Supervision, laboratory investigation, Writing- review & editing, funding acquisition and final approval of the manuscript by S.D. The data of the manuscript was checked and edited the draft by A.G.B, R.A.H, D.P.B, S.T**,** M.C.B.P, Z.H**,** K.S**,** A. A.P.M and S.G. All authors read and approved the final manuscript for publication.

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Conflict of Interest Statement

Authors have no conflict of interest in this study.

Data availability

All datasets generated or analyzed during this study are included in the manuscript. For *hsp65* (accession No. OQ417940), for *IS6110* (accession nos. OQ417941*),* and for *IS1081* (accession nos OQ417942) are available at NCBI database.

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425

426 **Figure legends**

427 Fig.1: Showing the sampling area from the Urban and Peri-urban area of Guwahati, Assam

428 Fig.2: (A) Cut section of lungs showing granulomatous tissue, yellowish-white areas with caseous
429 necrosis (B) showing miliary to large tuberculous nodules with fibrotic lesions in the lungs

430 Fig.3: Amplified 441bp of *hsp65* gene product of positive milk, tissue and nasal swab samples in 1.8%
431 agarose gel. L-Ladder (100bp DNA Ladder, Thermo Fisher Scientific GeneRuler, MA, United States);P-
432 positive control (*M.phlei* MTCC1724); samples (S1-S7); N-negative control

433 Fig.4: Amplified 248bp of IS1081 gene product of positive tissue samples in 1.8% agarose gel
434 L-Ladder (Thermo Scientific GeneRular 100bp DNA Ladder); P-positive control (*Mycobacterium bovis*
435 *(AN-5)*; samples (S1-S9); N-negative control

Fig.5: Amplified 123bp of IS6110 gene product of positive milk and tissue samples in 1.8% agarose gel

L-Ladder (Thermo Scientific GeneRular 100bp DNA Ladder); P-positive control (*Mycobacterium bovis (AN-5)*; samples (S1-S3); N-negative control

Figures



Fig.1: Showing the sampling area from the Urban and Peri-urban area of Guwahati, Assam



Fig.2(A)

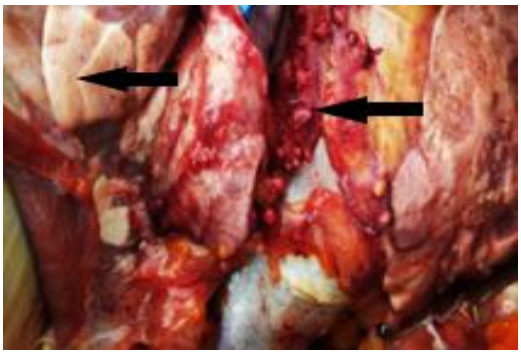


Fig.2(B)

Fig.2: (A) Cut section of lungs showing granulomatous tissue, yellowish-white areas with caseous necrosis (B) showing miliary to large tuberculous nodules with fibrotic lesions in the lungs

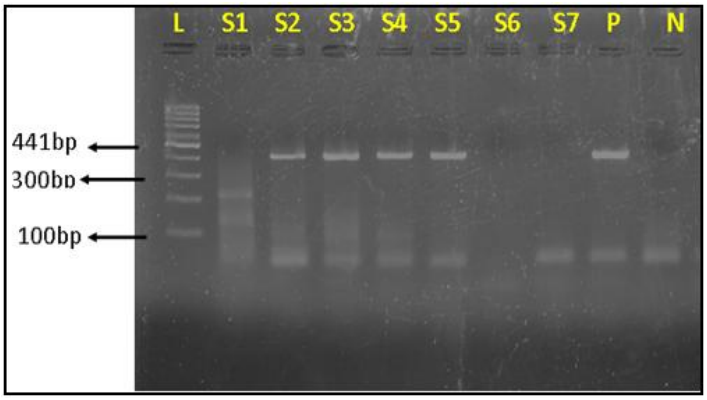


Fig.3: Amplified 441bp of *hsp65* gene product of positive milk, tissue and nasal swab samples in 1.8% agarose gel. L-Ladder (100bp DNA Ladder, Thermo Fisher Scientific GeneRuler, MA, United States);P-positive control (*M.phlei* MTCC1724); samples (S1-S7); N-negative control

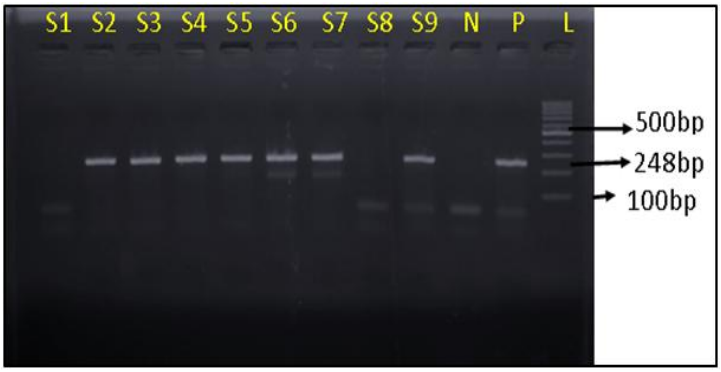


Fig.4: Amplified 248bp of IS1081 gene product of positive tissue samples in 1.8% agarose gel

L-Ladder (Thermo Scientific GeneRular 100bp DNA Ladder); P-positive control (*Mycobacterium bovis (AN-5)*; samples (S1-S9); N-negative control

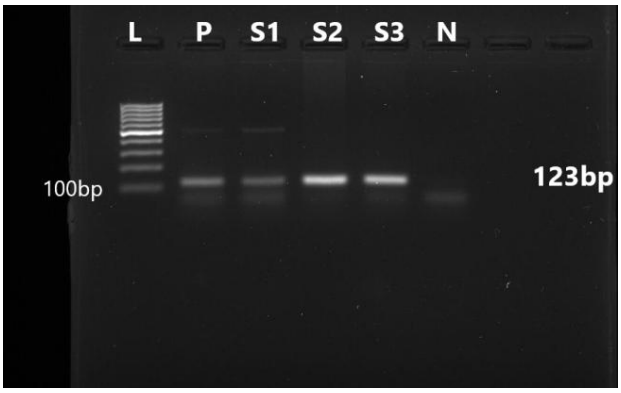


Fig.5: Amplified 123bp of IS6110 gene product of positive milk and tissue samples in 1.8% agarose gel

L-Ladder (Thermo Scientific GeneRular 100bp DNA Ladder); P-positive control (*Mycobacterium bovis (AN-5)*; samples (S1-S3); N-negative control

Tables

Table 1- The details of primers used in the present study

Primer Name	Forward primer (5'-3')	Reverse primer (5'-3')	Product size	Reference
Hsp65	ACCAACGATGGTGTGTCCAT	CTTGTCGAACCGCATACCCT	441bp	Telenti et al., 1993
IS6110	CCTGCGAGCGTAGGCGTCGG	CTCGTCCAGCGCCGCTTCGG	123bp	Eisenach et al., 1990
IS1081	ACAGGCGAGCCCGGATCTGCTG	GTTCAGCTCGCTTGCGGCGCTG	248bp	Wards et al., 1995

509 **Table-2: Results of percent positivity of bTB under following tests**

Type of sample	Total number of samples tested	Z-N staining positive	Isolation	hsp65 PCR positive	IS1081/ IS6110 PCR positive
Milk	360	N.D	N.D	16/360 (4.44%)	4/16 (25%)
Nasal swab	150	N.D	N.D	3/150 (2.00%)	2/3 (66.67%)
Tissue	110	12/110 (10.90%)	2*/14 (14.28%)	24/110 (21.8%)	18/24 (75%)
Total	620	12/110 (10.90%)	2*/14 (14.28%)	43/620 (6.93%)	24/43 (55.81%)

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511 Note- N.D is not done and (*) stands for only selected tissue samples with nodules which showed pure
512 colonies.

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