PREVALENCE AND MOLECULAR DIAGNOSIS OF BOVINE TUBERCULOSIS

THESIS

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In partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

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BY

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2023

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled, **PREVALENCE AND MOLECULAR DIAGNOSIS OF BOVINE TUBERCULOSIS** or part thereof has not been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis/publication of any university or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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BANKAR ANIKET SARJERAO has satisfactorily prosecuted his course of research for a period of not less than one semester and that the thesis entitled, **PREVALENCE AND MOLECULAR DIAGNOSIS OF BOVINE TUBERCULOSIS** submitted by him is the result of research work and is sufficient to warrant its presentation to the examination in the subject of **Veterinary Pathology** for the award of Master of Veterinary Science degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

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CERTIFICATE

This is to certify that the thesis entitled, **"PREVALENCE AND MOLECULAR DIAGNOSIS OF BOVINE TUBERCULOSIS"** submitted by Shri **BANKAR ANIKET SARJERAO** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **M.V.Sc** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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ABBREVIATIONS

Abbreviations		Full Form
%	:	Percent
Gm	:	Gram
Cm	:	Centimetre
ELISA	:	Enzyme linked Immunosorbent assay
Fig	:	Figure
DNA	:	Deoxyribose nucleic acid
DW	:	Distilled water
Ag	:	Antigen
SCITT	:	Single Comparative intradermal tuberculin test
INF	:	Interferon
bp	:	Base pair
pm	:	Picomole
Min	:	Minute
Hr	:	Hour
ml	:	Milli Litre
PPD	:	Purified Protein Derivative
IS6110	:	Insertion sequence
OIE	:	World Organization for Animal Health
RD	:	Region Difference
PBS	:	Phosphate Buffer Saline
RPM	:	Revolution per time
V	:	Volt
UV	:	Ultra violet
BCG	:	Bacillus Calmette -Guerin
Et al	:	Ethically all
Mm	:	Millimolar
TAE	:	Tris -glacial acetic acid
°C	:	Degree Celsius
µL	:	Microliter

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease caused by *Mycobacterium bovis* leading to a loss in productivity and signifies a crucial public health risk. *Mycobacterium bovis* has an extensive host range and is the principal agent responsible for tuberculosis in domestic and wild animals (Parmar *et al.,* 2014; Aswathy *et al.,* 2019). Regardless of the zoonotic threat and significant economic costs associated with the disease, precise estimates of bTB prevalence are deficient in many countries, including India, where national control programs are yet to be instigated. The true burden of the disease remains unknown due to the lack of routine surveillance data from most developing countries. India is progressing well towards attaining the end TB goal, yet bTB remains largely hidden. Bovine tuberculosis is endemic in India, where cattle and humans coexist, creating a huge economic impact on agricultural industries, animal productivity, threatening the livelihoods of farmers and ranchers. India is home to over 300 million bovines and has the largest cattle population, leading to the planet's highest milk production (~67 150MT). Bovine tuberculosis is also a significant economic concern, estimated to cost 3 billion Doller annually worldwide due to losses from reduced productivity, culling and movement, and trade restrictions (Waters *et al.,* 2012). A meta-analysis study reported a pooled prevalence estimate of 7.3% (21.8 million cattle) affected by bovine tuberculosis in India (Srinivasan *et al.,* 2018). The spread of the disease in cattle has been controlled well, with extremely low prevalence in most of the developed countries through the implementation of test and slaughter control programs (Olmstead and Rhode, 2004). As cow slaughter is banned in India, most of the bovine tuberculosis cases remain undiagnosed, and such infected animals act as a source of infection.

Traditional control programs for bovine tuberculosis are based on the skin testing of all cattle. Tuberculin purified protein derivative (PPD), which is used for diagnostic purposes, is a blend of dominant mycobacterial proteins from specific strains of *M. bovis* (Inwald *et al.,* 2003). Bovine purified protein derivative (PPD) has proved to be a reliable screening test for detecting cattle with TB (Tweddle and Livingstone, 1994). The first study of screening for bovine

tuberculosis in live cattle based upon tuberculin test was done in Madras and Pune. (Sahai *et al.,* 1941; Dhanda *et al*., 1942). However, false positive reactions can occur with a range of microbial infections, including subspecies paratuberculosis. So, *Mycobacterium avium* (*M. avium*) tuberculin is incorporated into the comparative intradermal test (Monaghan *et al.,* 1994). As the sensitivity of the Single Comparative Intradermal Tuberculin Test (SCITT) is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test (World Organisation for Animal Health). When SCITT and Interferon Gamma Release Assay (IGRA) are used in combination, a greater number of infected cattle have been identified (Bernitz *et al.,* 2018). Interferon gamma release assay is helpful for the early detection of infection (Mareledwane *et al*., 2022). IFN-γ test can be a very useful tool for identifying infected animals that are missed (false-negative) by the skin test (Clegg *et al*., 2019). Culture is still viewed as the gold standard for diagnosis of bovine tuberculosis, but due to the slow growth of organisms, and low availability of biosafety labs (BCL3) across the developing country like India, it is laborious and requires very good experience (Corner *et al*., 2012; Courcoul *et al.,* 2014). For better diagnosis of bovine tuberculosis, an array of tests, including comparative intradermal test, interferon gamma release assay, and PCR have been suggested (Das *et al*., 2018).

Although a substantial economic expenditure is addressed to ensure efficient surveillance systems and control programs, the detection and confirmation of bTB infection in cattle herds should be more reliable and swifter. Enzyme-linked immunosorbent assay (ELISA) is an antibody-antigen detection test that can be considered a supplementary test to other cell-mediated immune tests of bovine tuberculosis. It is the more reliable and less time-consuming test as compared to tuberculin. It may be advantageous for the identification of anergic animals by using species-specific antigens in an ELISA, by which the rate of false positive cases can be reduced (Gupta and Ram, 1997). Rapid as well as accurate diagnostic tools, such as Real-Time PCR (qPCR) and ELISA need to be implemented as a confirmatory test in the framework of bovine tuberculosis

surveillance and control programs for shortening the turnaround time to confirm bTB infection.

So, the present study is planned with the following objectives:

1. To study the prevalence of bovine tuberculosis in Maharashtra, Chhattisgarh, and Madhya Pradesh states.
2. Diagnosis of bovine tuberculosis by comparative intradermal skin test, interferon-gamma release assay, conventional PCR, and Real-time -PCR.
3. To standardize an in-house ELISA for diagnosis of bovine tuberculosis.

REVIEW OF LITERATURE

2.1 Prevalence of bovine tuberculosis in India

Mukherjee (2006) studied the prevalence of bovine tuberculosis in northern India by a comparative intradermal test on two farms. Five out of 9 animals were found as positive in one farm (15.76%) in another farm prevalence was between 0.65% and 1.85%.

Thakur *et al.,* (2010) screened total of 440 dairy cattle from 6 dairy farms for bovine tuberculosis in Palampur, Himachal Pradesh by tuberculin test (TST). Overall animal prevalence was found 14.31% (63/440). Study reported more prevalence in more than 6-year-old animals than 1-6 yr old animals and variation in prevalence according to the region to region and pure breed and cross breeds.

Sharma *et al*., (2011) studied the prevalence of bovine tuberculosis in a different parts of Panjab state over a 23-year period (1986-2009). A total of 15,737 animals were tested for Bovine tuberculosis using a single intradermal tuberculin test. A total of 847 animals showed positive reactions with an overall prevalence rate was 5.38% (Range from 1.53 to 13.91% in different years). The epidemiological history of animals (n=5208) screened in the last eight years (2002-2009) was recorded and risk factor analysis for species, age, and sex was made. Bovine tuberculosis prevalence was found significantly higher ($\chi 2$ test; $P < 0.01$) in cattle (5.02%) as compared to buffaloes (3.08%).

Trangadia *et al.,* (2013) conducted a study to investigate the prevalence of bovine tuberculosis in three organized herds using a tuberculin test from August 2004 to May 2006. Farm A and B were located in Gujarat state, whereas farm C was in Uttar Pradesh. A total of 765 animals in farm A, 1545 animals in farm B, and 338 animals at farm C were included in the study. The overall prevalence of tuberculosis in farms A, B, and C was recorded to be 2.39 %, 2.22 %, and 0.59% respectively. A higher prevalence of tuberculosis was found to be 2.58% in the Jersey breed and its crosses followed by 2.40% in Holstein Friesian and its crosses as compared to indigenous cattle and buffaloes. The prevalence of

tuberculosis in females (2.90%) was higher as compared to male animals (1.49%). Age-wise data revealed the prevalence as 80.36% (45/56) in animals older than 2 years of age as compared to younger animals.

Didugu *et al.,* (2016) investigated seroprevalence for *Mycobacterium bovis* among bovines in Krishna district of Andhra Pradesh state. Total 456 serum samples were collected (48 from non-descript cattle, 62 from crossbred cattle and 346 from buffaloes). The IDEXX® M. bovis antibody test kit was used to detect antibodies against *Mycobacterium bovis*. Among 456 bovine samples, 4 (0.87%) were positive against *Mycobacterium bovis* antibodies. Authors recorded significantly lower seroprevalence of bovine tuberculosis (2.08% in non-descript cattle, 3.22% in crossbred cattle, and 0.29% in buffaloes).

Das *et al.,* (2018) studied total 173 cases in Gangetic delta region of West Bengal, India. In total, 36 (25.4%) animals from the organized and one (3.2%) from the backyard farming sector were found positive for bTB. The prevalence of bTB in exotic crossbred animals (34.6%) was significantly higher ($p<0.001$) compared to indigenous cattle (10.5%). Further, gender-wise analysis of data with respect to bTB revealed higher positivity y ($p<0.05$) among cows/heifers (25.8%) compared to bulls/bullocks (7.3%).

Srinivasan *et al.,* (2018) studied the prevalence of bovine tuberculosis and identified 285 cross-sectional studies on bTB in cattle in India across four electronic databases and handpicked publications. Of these, 44 articles were included, contributing a total of 82,419 cows and buffaloes across 18 states and one union territory in India. Based on a random-effects (RE) meta-regression model, the analysis revealed a pooled prevalence estimate of 7.3% (95% CI: 5.6, 9.5), indicating that there may be an estimated 21.8 million (95% CI: 16.6, 28.4) infected cattle in India, a population greater than the total number of dairy cows in the United States.

Sharma *et al.,* (2019) studied prevalence of bovine tuberculosis in animals reared under different farming systems in 33 panchayats of 5 districts in Himachal Pradesh, India. Total 997 animals comprising of 143 Jersey/Holstein Frisian (HF,

all females), 611 Jersey/HF crosses (23 males and 588 females), 110 Red Sindhi crosses (all females) and 133 local pahari (46 males and 87 females) cattle were tested for tuberculosis. One hundred seventy-seven (17.8%) tested animals (7 Jersey/HF, 113 Jersey/HF crosses, 9 Red Sindhi crosses and 48 non-descriptive animals) were owned by families of 100 registered tuberculosis patients. The tested 997 animals were reared under different farming systems, organized government dairy farms (50), organized private dairy farms (95) and private unorganized farms (852). The overall prevalence of 34.0% in organized government dairy farms was recorded.

Kowalli *et al.,* (2019) studied the prevalence of bovine tuberculosis in Karnataka for eight years between 2002-03 to 2009-10. A total of 3286 cattle in 36 organized farms spread across Belagavi, Dharwad and Bagalkote districts of north Karnataka were screened using single intradermal tuberculin test. Overall percentage prevalence of tuberculosis among cattle was found to be 1.76% Percentage prevalence of tuberculosis was higher in female (1.85%) than in male (0.78%). The breed wise percentage prevalence was higher in Holstein Fresian breed (HF) (2.66 %) than Jersey (1.37 %) cattle and there are no positive cases among Indigenous breed of cattle.

2.2 Single Comparative Intradermal Tuberculin Test (SCITT) for diagnosis of bovine tuberculosis

Ameni *et al.,* (2007) conducted a comparative study on the prevalence and pathology of bovine tuberculosis on 5,424 cattle (2,578 zebus, 1,921 crosses, and 925 Holsteins) using bovine and avian PPD. Study reported 4.8% of animals responded positively to both PPD-A (avium) and PPD-B (bovine) and 8.7% of them reacted only to PPD-B, while 1.3% reacted only to PPD-A.

Boukary *et al.,* (2011) carried household survey in Torodi, (Niger), West Africa by using comparative intradermal test on 393 cattle of different breeds different age group by using bovine and avian PPD. 3.6 % animals were found reactor to tuberculin test.

Noorrahim *et al.,* (2015) carried out comparative intradermal tuberculin test for estimation of prevalence of bovine tuberculosis in three tehsils of Charsadda district of Pakistan. A total of 100 animals per tehsil was screened. Total 13 animal was found to be positive by comparative intradermal test.

Mugambi *et al.,* (2016) tested 391, 169, and 401 cattle for Bovine tuberculosis by using CITT in the southern highlands zone (SHZ), eastern zone (EZ), and northern zone (NZ) of Tanzania, respectively. Bovine tuberculosis was more common in EZ (n = 169) than in SHZ (n = 391) or NZ (n = 401), where there were no encouraging findings. Results for 33, SHZ animals, and seven EZ cattle were ambiguous. Total 625 cattle from four different agro-pastoral and pastoral production systems in Kenya were evaluated. In one region of Mwingi County in eastern Kenya, all 161 cattle tested negative, while CITT discovered a frequency of 4-6% in the other three Migori sites in Nyanza, West Pokot, and Laikipia in the Rift Valley.

Filia *et al.,* (2016) screened total 121 animals (93 females and 28 males) of 1 year and above using single intradermal comparative cervical tuberculin (SICCT) test by using bovine and avian PPD. Author observed that reaction to both PPD-B and PPD-A exceeded 2 mm, but the difference between the bovine and avian reaction was <4mm.

Barua *et al.,* (2017) examined total 199 animals in Assam and Meghalaya by using bovine and avian PPD. Out of 199 cases examined, 33 (16.58%) showed positive for single intradermal comparative cervical tuberculin (SICTT).

Rodrigues *et al.,* (2017) screened 53 animals from three dairy herds that in the state of Rio Grande do Sul's target area for bovine tuberculosis by the CITT. Tissues were cultured, and PCR and DNA sequencing were used to verify the colonies that formed. In the 53 animals that were subjected to the CITT analysis, 32 (60.4%) were negative, 14 (26.4%) animals were positive, and seven (13.2%) results were inconclusive. Of the 39 animals with a culture-confirmed *M. bovis* infection, the CITT identified 11 as positive. Eight of the 14 uninfected animals

based on cultures was negative. The CITT showed sensitivity of 28.2% and specificity of 57.1%. 24/32. and 75.0% Negative CITT results were declared false negatives based on the CITT since they were culture positive (confirmed by PCR). Since they can spread infection, keeping these false-negative animals in herds has major ramifications for disease control. So, author suggested complementary testing may improve the chances of a successful diagnosis by assisting in the identification of these animals.

Brahma *et al.,* (2019) studied prevalence of bovine tuberculosis in Punjab by using bovine and avian PPD. Out of total 202 animals screened for TB, 40 animals (19.80%) were found to be positive by Comparative Intra dermal Tuberculin Test (18 cattle and 22 buffaloes). Out of these only 30 animals (13 cattle and 17 buffaloes) showed an exclusively positive reaction to CITT by showing an increase in the thickness > 4 mm.

2.3 Interferon Gamma Release Assays (IGRA) for diagnosis of bovine tuberculosis

Ryan *et al.,* (2000) carried out gamma interferon release assay. A total of 163 cattle from 21 herd infected with *Mycobacterium bovis* were used to determine the test's sensitivity. The specificity was calculated using 213 cattle from 82 herds that exhibited no signs of *M. bovis* infection but had responded to a caudal fold test. The IFN-test had a sensitivity and specificity of 85% and 93%, respectively. Blood samples that were cultivated the day of collection compared favourably to those that were cultured the day after collection in terms of the test's sensitivity and specificity. These results lend credence to the IFN-gamma tests as a useful serial test that may be utilised in conjunction with the caudal fold skin test.

Carla *et al*., (2010) studied gamma interferon assay for finding efficacy of assay. A Comparative Intradermal Tuberculin Test (CITT) was performed in 50 cows from a dairy herd known to be infected with TB. Blood samples for IFN testing were collected concurrent with the CITT, as well as seven and 21 days later. At 30 days after the CITT, all cattle deemed reactive to this test were

slaughtered and samples were processed by both bacteriological culture and PCR. The sensitivity of IFN as a diagnostic tool was 91.4%, whereas specificity was 86.7%.

Thakur *et al.,* (2010) conducted gamma interferon test on 23 (positive for bTB by tuberculin test) blood samples in Himachal Pradesh using bovine and avian PPD as stimulating agent. Out of 23 animals 18 were found positive and 5 animals were negative. These 23 animals were previously found as positive for tuberculin test.

Lopes *et.al.,* (2012) conducted study on bovine tuberculosis by using comparative intradermal test and gamma interferon release assay. A total of 350 cattle from two TB-free and two TB-infected herds were submitted to CITT and 102 animals were selected for gamma interferon assay. Twenty nine (29) animal was positive skin by CITT and 73 was negative. Gamma interferon test was done in two phases day 0 and day 3 but no significant difference was found. Sensitivity of parallel testing was over 97.5%, while specificity of serial testing was over 99.7%.

Neeraja *et al.,* (2014) compared between single intradermal test, gamma interferon release assay and indirect ELISA for diagnosis of bovine tuberculosis. Forty-five (45) animals from known prevalence farm were tested. Out of these 45 animals, 10 animals were found positive by gamma interferon assay, 10 by tuberculin test and none of the animals were found positive by ELISA. Author suggested that cell mediated test like tuberculin test and gamma interferon assay together are better than ELISA and no single test having 100 % sensitivity and specificity. There sensitivity depends upon progressive disease stages and it may be varied.

Katale *et al.,* (2017) tested 102 African buffalo from 16 herds by an interferon gamma assay on harvested plasma samples using sandwich enzyme linked immunosorbent assay with Avian and bovine PPD as stimulating agent for this study. Out of the 102 animals, two (2%) African buffalo tested positive for bovine tuberculosis.

De Lisle *et al.,* (2017) carried out gamma interferon release assay for detection of bovine tuberculosis, groups of experimentally infected cattle (n = 10), naturally infected (n = 11), and uninfected animals (n = 12) that were examined with a caudal fold skin test. Blood was taken on the day of tuberculin injection, 3 days later when the skin tests were read, and 11–19 days post–tuberculin injection, and was processed for the IFN-γ test at 8, 30, and 36 hours post collection. Authors observed that there is no link between collection time after tuberculin test because statistically same result was found but suggested that samples should not be processed if in transit for >30 hr

Raffoa *et al.,* (2018) tested 609 animals from 17 herds for blood interferon gamma release assay and with the caudal fold tuberculin test (CFT). Animals which showed CFT positive results were sent to the slaughterhouse for post mortem confirmation testing. The analysis of the *in vivo* tests reported that CFT results classified 268 animals (44%) as tuberculin reactors, however, on the same population, Interferon test showed only 99 samples as positive (16.3%) using the standard interpretation, and when severe interpretation was used, the number rose to 118 animals (19.37%).

Keck *et al.,* (2018) studied specificity and sensitivity of gamma interferon test in around 250 bullfighting cattle herds in the Camargue region (around 30,000 animals) using avian and bovine PPD as stimulating agent. The sensitivity (Se) and specificity (Sp) were evaluated with their 95% exact binomial confidence interval (CI) at the individual level but also at the herd level. Authors concluded that gamma-IFN assay can be used either as a serial test to the skin test where its use is to enhance overall disease detection specificity for screening programs in low prevalence areas or in parallel with the intradermal test in order to increase overall disease detection sensitivity in infected herds.

Jang *et al.,* (2020) performed gamma interferon test using avian and bovine PPD as stimulating agent and PBS as non-stimulating agent on cattle (*n* = 120) with bovine tuberculosis and cattle (*n* = 426) from bovine tuberculosis free herds from 2012 to 2013 to evaluate the sensitivity and specificity of the assay respectively depending on various cut-offs (0–3.5). When optical density of the

cut-off was 0.1, the sensitivity and specificity were found to be 81.7% (74.7–88.6) and 99.5% (98.9–100.0).

Mareledwane *et al.,* (2022) performed gamma interferon assay on total of 410 fresh blood samples collected from slaughter livestock (369 cattle and 41 sheep) from 15 abattoirs, and analysed using Bovigam® test kit with bovine, avian and Fortuitum purified protein derivatives (PPD) as blood stimulating antigens. The estimated prevalence of bTB in cattle was 4.4% (95% CI: 2.4%–7.3%).

2.4 Comparison of interferon gamma release assay and single comparative intradermal test for diagnosis of bovine tuberculosis

Gormley *et al.,* (2013) tested 2157 animals that were negative to both the Single Intradermal Comparative Tuberculin Test. Single (SICTT) and at post mortem by Gamma interferon (IFN-γ) assay by using avian and bovine PPD as stimulating agent and PBS as non-stimulating agent. Out of 2157 animals 199 were positive to the IFN-γ test. Based on this subset of animals, the estimated specificity of the IFNγ test was 90.77% (95% CI: 89.46% - 91.94%).

Ahir *et al.,* (2016) investigated two hundred lactating animals in Ludhiana and Jalandhar district of Punjab (158 cattle and 42 buffaloes) of organized and unorganized farms for the bovine TB using comparative intradermal tuberculin test (CITT) and IFN-γ assay using avian and bovine PPD and PBS as non-simulating agent. Overall, 14.5% animals were found positive by Comparative intradermal Tuberculin Test (CITT). Author concluded that when both CITT and IFN-γ assay used together lead to more accurate screening for bovine TB in dairy herd.

Bernitz *et al.,* (2018) studied *Mycobacterium bovis* infection in 71 African buffaloes *(Syncerus caffer)* by single comparative intradermal tuberculin test (SCITT) and interferon gamma release assays (IGRAs) using bovine and avian PPD as stimulating agent and measured using Bovigam® peptide IGRIA.

Bovigam® IGRA identified 64/71 buffaloes as positive. When SCITT and IGRIA tests are used in combination (70/71) buffalo were positive.

Clegg *et al.,* (2019) tested total of 8357 animals by both SICTT and IFN-*γ* using avian and bovine PPD as stimulating agent and PBS as non-stimulating agent in National tuberculosis eradication programme in Ireland in 2016–2017. The combined sensitivity for the two tests (IFN-*γ* and the SICTT) relative to post-mortem was 98.6% at a cut-off for IFN-*γ* of 0 and decreased to 96.4% when the IFN-*γ* cut-off increased to ≥100 and conclude that IFN-γ test can be a very useful tool for identifying infected animals that are missed (false-negative) by the skin test.

Elsohaby *et al.,* (2020) screened blood and milk samples from 245 Holstein dairy cows in 11 Egyptian dairy herds by PCR, mycobacterial culture and IFN-γ testing by using avian and bovine PPD as stimulating agent and PBS as non-stimulating agent. IFN-γ recorded higher Se [0.97(95% Posterior Credible Interval (PCI): 0.87–1.00)] than PCR [0.68 (95%PCI:0.53–0.95)] and culture0.22(95% PCI: 0.13–0.37)]. However, Sp estimates of PCR [0.98 (95% PCI:0.95–1.00)], culture [0.99(95%PCI:0.98–1.00)] and IFN-γ [0.97 (95% PCI: 0.88–1.00)] were comparable. As for milk samples, Se estimate of PCR [0.29 (95% PCI: 0.01–0.60)] was higher than that of culture [0.08 (95% PCI: 0.001–0.23)] and conclude that IFN-γ registered a similar overall performance to PCR but was superior to mycobacterial culture.

2.5 Conventional PCR for diagnosis of bovine tuberculosis

Vitale *et al*., (1998) studied 100 cattle in 12-month period using IS6110 PCR using nasal swab, milk, and lymph node aspirate from 60 skin test positive and 40 skin test negative cattle's 54 milk samples and 49 lymph node aspirates gave the 100% values for sensitivity, specificity, and positive and negative predictive values. PCR using 50 nasal swabs shown high specificity (100%) but a low sensitivity (58%); the positive predictive value was also 100% 28 milk samples, 30 lymph node aspirates, and 26 nasal swabs from 33 cows and 7 bulls

that were negative to the skin test were examined, A total of 11 milk, 10 lymph node aspirate, and 13 nasal swab samples were positive by PCR.

Bakshi *et al*., (2005) used *M. tuberculosis* strains (35) isolated from human patients with pulmonary TB from the Medical Hospital, IVRI, Izatnagar (UP), and (20) *M. bovis* strains isolated from clinical cases of tuberculosis in human and animals. 12.7 kb based PCR showed a unique amplicon of 168 bp for *M. bovis* and *M. bovis* BCG, while the reactions with the DNAs from *M. tuberculosis* strain showed a unique amplicon of 337 bp.

Bhanurekha *et al*., (2015) studied total 181 bovine raw milk samples and 123 pre-scapular lymph node biopsy samples. Four milk samples were found positive by PCR for Mycobacterium genus (IS6110 PCR). Four (4) milk sample that was positive for *Mycobacterium tuberculosis* Complex found positive by species specific PCR for *M. tuberculosis.*

Thakur *et al.,* (2016) used IS6110 conventional PCR for diagnosis of bovine tuberculosis using 71 pre- scapular lymph node aspirates (PSNL), 54 milk and 71 blood samples. Samples were collected from positive reactors after screening by SITT. Total 28 (39.43%) PSLN aspirate and 5 (9.25%) milk samples were positive for *Mycobacterium tuberculosis* complex (MTC) by PCR amplification for the IS6110 insertion sequence; however, blood samples were found negative. For species differentiation, multiplex-PCR using 12.7 kb primers was conducted. Out of 28 PSLN aspirate, *Mycobacterium bovis* was detected in 18 (64.28%) and MTB in 8 (28.57%), whereas 2 aspirate samples (7.14%) were positive for both the species. All the five milk positive samples were positive for *M. bovis.*

Verma *et al*., (2022) analysed 24 *M. bovis,* 39 *M. tuberculosis,* 21 fresh acid-fast positive sputum samples and standard mycobacterial strains with pncA, 12.7 Kb and IS6100 based PCR assays. PCR products of IS6110 and 12.7 Kb fragment showed 99-100% and 100% identity in amplicon products, respectively. To test reliability of primers, *M. tuberculosis* and *M. bovis* cultures were mixed and subjected to IS6110, pncA and 12.7 Kb PCR assay. PncA primers could not

successfully and reliably discriminate the mixed culture, however, 12.7 Kb fragment primers successfully discriminated the mixed culture of *M. tuberculosis* and *M. bovis*.

2.6 Real-time PCR for diagnosis of bovine tuberculosis

Comparative genomic analyses have revealed many regions of divergence

(RDs) between mycobacterial species. RD1 and RD4 both regions present in *Mycobacterium tuberculosis* but RD4 absent in *Mycobacterium bovis* and both regions absent in *M. bovis* BCG (Mahairas *et al.,*1996; Halse *et al.,* 2011; Ru *et al.,* 2017).

Halse *et al.,* (2011) used real-time PCR targeting IS6110 for testing total 727 samples. Out of 727 samples 192 samples found positive. Then tested with the MTBC-RD real-time PCR assay of these, 165 had paired Bactec MGIT 960 positive cultures with conventional PCR results to determine the MTBC species for comparison. A total of 155 (94%) correlated and were identified as follows (n): *M. tuberculosis* (145), *M. africanum* (4), *M. bovis* (2), *M. bovis* BCG (2), and negative for MTBC (2). A small subset, 10 specimens (6%), were determined to be negative (5) or inconclusive (5) by MTBC-RD real-time PCR but were later identified as having *M. tuberculosis* by conventional PCR and MTBC-RD real-time PCR once they became culture positive.

Thacker *et al.,* (2011) detected 20/30 samples from *M. bovis* infected animals and 0/18 control tissues by real time PCR.

Selim *et al.,* (2014) studied duplex real-time PCR assay targeting insertion elements IS1081 and IS6110 for detection of *Mycobacterium bovis* in lymph nodes of cattle. The specificity and sensitivity of the assay were evaluated for detecting serial dilutions of reference Mycobacteria strains as well as from spiked lymph node homogenate. Results revealed that microscopical examination of 600 lymph nodes with tuberculous-like lesion for detection of Acid-fast bacilli (AFB) showed a detection rate of 96.6%, compared to 98% *M. bovis* with duplex real-

time PCR. The assay was evaluated on 19 bacterial strains and was determined to be 100% specific for *M. bovis.*

Phom *et al.,* (2016) studied total 16 cases in Panjab by real time PCR targeting IS6110. Out of 16 sample 12 were positive for IS6110 PCR assay.

Cezar *et al.,* (2016) tested *Mycobacterium bovis* in milk (n = 401) and blood (n = 401) samples collected from 401 dairy cows of 20 properties located in the state of Pernambuco, Brazil, by real-time PCR targeting the region of difference 4 (RD4). One milk (0.25 %) and eight blood (2 %) samples were positive for *M. bovis* in the real-time PCR and their identities were confirmed by sequencing.

Sanchez-Carvajal *et al.,* (2021) screened 62 animals by qPCR targeting IS6110 showed an apparent diagnostic sensitivity and specificity values of 77.1% [95% confidence interval (CI): 66.5–87.6%] and 99.4% (95% CI: 98.3–100.6%), respectively, and a positive predictive value of 97.9% (95% CI: 93.9–102.0%) and negative predictive value of 92.3% (95% CI: 88.4–96.2%). Positive and negative likelihood ratios were 130.2 and 0.2, respectively and highlight that qPCR targeting IS6110 as a suitable complementary method to confirm bTB in animals.

Lorente-Leal *et al.,* (2021) analysed 985 bovine tissue samples using culture and real time (IS6110). Total 326 samples were MTB Complex positive by both microbiological culture and real-time PCR, and 12 were culture-MTB Complex positive and real-time PCR negative. Diagnostic sensitivity (Se) and diagnostic Specificity (Sp) relative to culture were 96.45% (95% CI, 93.90% to 98.15%) and 93.66% (CI, 91.51% to 95.29%), respectively.

2.7 Peptide based In-house ELISA for Diagnosis of Bovine tuberculosis

1. Pep A: MTB Pep A, belongs to the serine protease family of proteins. It is also known as Mtb32a. It is conserved across *M. tuberculosis, M. leprae, M bovis and M. avium paratuberculosis*. It has been identified in the supernatant of the MTB strain H37Rv and is required for the survival and pathogenicity of Mycobacterium *in vivo.*

2. Dap E: It is also called as succinyl-diaminopimelate desuccinylase. It is a 37 kDa membrane certain hydrolase enzyme that catalyzes the hydrolysis of N-succinyl-L, L-diaminopimelic acid (SDAP), forming succinate and LL-2,6-diaminoheptanedioate (DAP), an intermediate involved in the bacterial biosynthesis of lysine and meso-diaminopimelic acid. It is conserved throughout *M. tuberculosis, M. orygis, M. mungi. M. africanum and M. bovis* strain of TB.

3 Pep Q: MTB Pep Q is a cytoplasmic peptidase that is potentially conserved in *M. tuberculosis, M. leprae, M. bovis and M. M. avium paratuberculosis.* It has been identified by mass spectrometry in the Triton X114 extract from M. tuberculosis H37Rv and is thought to be essential for the survival and pathogenicity of Mycobacterium *in vivo*.

Whelan *et al.,* (2008) developed multiplex immunoassay for diagnosis of bovine tuberculosis in this ELISA. Author used 25 peptide antigen which simultaneously detected antibody activity for 25 antigens. The comparison of sera from 522 infected and 1,489 uninfected animals showed that a sensitivity of 93.1% and a specificity of 98.4% this study show that sensitivity and specificity of peptide ELISA for diagnosis of bovine tuberculosis can be increased when we use more than 2 antigens.

Lilenbaum *et al.,* (2011) used MPB-70 ELISA as a complementary test for bovine TB in Brazil. Blood samples were collected from all 50 cows at the time of the PPD injection. MPB70-ELISA was positive in 11 of the 32 CCTT-reactive animals. MPB70-ELISA was positive in five cows that were negative both by CCTT and IFN-gamma test. Both intradermal tests and the g-IFN test failed to detect five anergic animals, but the MPB-70 ELISA could detect them.

Farias *et al.,* (2012) tested sera from 90 cattle from different herds from the state of Mato Grosso do Sul, Brazil tested either positive (53 animals) or negative (37 animals) on the comparative intradermal tuberculin test (CITT) were evaluated through ELISA. recombinant MPB70 and recombinant p27 are promising for use in enzyme-linked immunosorbent assays for the detection of

antibodies against *M. bovis*, which, when associated to the tuberculin skin test, could improve the diagnostic coverage of bovine tuberculosis.

Infantes Lorenzo *et al.,* (2017) Evaluated specificity of ELISA for detection of specific antibody against mycobacterium tuberculosis complex in cattle sheep and goats this ELISA test is based upon multiprotein complex P22. 4%, 22%,4% prevalence was recorded using P22 multiprotein. Author concludes that P22 may be an alternative to PPD-B for serodiagnosis of bovine tuberculosis.

Cho *et al*., (2020) evaluated ELISA for diagnosis of bovine tuberculosis recombinant by using MPB70 and SahH (M70S) and native 20-KDa protein(20K) A total of 18 serum samples from *M. bovis*-positive cattle from 8 farms (confirmed by ISTs and *M. bovis* isolation) and 975 serum samples from *M. bovis*-negative cattle from 14 farms (confirmed by ISTs and absence of clinical signs of infection) in South Korea were tested using PPD, M70S, and 20K ELISA the sensitivity and specificity of M70S ELISA were 94.4% and 97.3%, respectively.

Sonekar (2021) carried out indirect ELISA for sample collected in and around Nagpur region using PEP A, PEP Q, and DAP E. Author found an overall seroprevalence of 16.61% from total 331 cattle and buffalo tested.

Hekal *et al.,* (2022) analysed sera from 108 positive comparative intradermal test positive animals using purified protein derivative (PPD-B) ELISA and commercial polypeptide ELISA 94 (87.03%) and 97(89.81) animal found positive for PPD-B ELISA and polypeptide ELISA.

MATERIALS AND METHODS

The present study on prevalence and molecular diagnosis of bovine tuberculosis was carried at Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur.

3.1 MATERIALS

3.1.1 Collection of samples

A total of 418 cattle having emaciated body conditions were screened by Single Comparative Intradermal Tuberculin Test (SCITT) from different regions of Maharashtra, Madhya Pradesh and Chhattisgarh state after the collection of detailed history. The details of samples collected for present study is given in table 3.1. Blood samples were also collected from 418 cattle in anticoagulant vials for DNA extraction and clot activator vials for the collection of serum to carry out interferon gamma release assay and standardization of peptide based in-house ELISA for diagnosis of bovine tuberculosis.

Table 3.1: Details of cattle samples collected during study

Sr. No.	State	Region	Blood	Serum
1	Maharashtra	Pune	42	42
		Solapur	47	47
		Ahmednagar	47	47
		Wadasa	32	32
		Jalgaon	24	24
		Akola	38	38
2	Chhattisgarh	Durg	50	50
3	Madhya Pradesh	Chhindawara	138	138
Total			**418**	**418**

3.1.2 Collection of blood/ serum

Blood samples were collected aseptically from the jugular vein of cattle in sterile EDTA vacutainers (Z- plus Gujarat, India) and clot activator vacutainers

(Z- plus Gujarat, India). Before collecting blood, sample identification numbers were allotted to each vacutainer. Five (5) ml blood samples were collected, properly mixed and placed upright position into the ice box samples and transported to the laboratory aseptically for further serological and molecular work. Blood samples for DNA extraction were stored at 4^{o}C till further processing. Sera samples were separated by centrifugation at 3000 rpm for 5 min. (Hermle labortechnik, USA) and transferred into 2 ml sterile cryovials and stored at -20^{o}C till further use. For interferon gamma release assay blood was collected in heparinized vacutainers and kept at room temperature during transportation. After incubation plasma was recovered and stored at -20°C till further processing.

3.1.3 Chemicals and reagents

All the chemicals used in this study were of molecular biology grade and procured from Thermos fisher Scientific (USA), Promega (USA), Bio stone (U.K), and Hi-media (India).

3.1.4 Glassware

The glassware was obtained from Borosil (India). All the glassware was sterilized in a hot air oven at 160°C for one hour before its use.

3.1.5 Plasticware

All the plasticwares including centrifuge tubes, microcentrifuge tubes, micro tips and PCR tubes (flat cap) used for the molecular study were DNAse/RNAse free (Axygen, USA).

3.2 METHODS

3.2.1 Single Comparative Intradermal Tuberculin Test (SCITT) for diagnosis of bovine tuberculosis

Single comparative intradermal tuberculin test was performed as per OIE (2009). For SCITT bovine and avian PPD were injected simultaneously in the middle third part of the neck. Firstly, the area at the site of injection was clipped

and cleaned with an antiseptic solution. The distance between avian and bovine PPD was kept between 12-15cm. Thereafter thickness of the skin fold was measured with a digital Vernier calliper. 0.1 ml each of avian PPD (2500 IU) (Thermo Fisher-Prionics) and bovine PPD (3000 IU) (Thermo Fisher-Prionics) were injected in each site with multi-dose intradermal tuberculin injector (NJ, Phillips) obliquely into the deeper layer of skin. After 72 hrs of injection skin fold thickness was re-measured. The interpretation of comparative intradermal test was done as per OIE (2009) guidelines as follows:

1. Positive reactor > 4 mm greater compare to avian PPD.
2. Negative reactor does not exceed 1 mm or no reaction to the bovine antigen.
3. Inconclusive reaction < 4 mm though reaction to PPD-B and PPD-A exceeded 1mm.

3.2.2 Interferon gamma release assay (IGRA) for diagnosis of bovine tuberculosis

The sample preparation for interferon gamma release assay was performed as per following procedure

1. Blood collection and aliquoting: Five (5) ml of blood was collected from each animal into blood collection tube containing heparin as anticoagulant. Blood samples were transported to laboratory at room temperature (22 ±3°C) and put into culture within 30 hrs of collection. Blood samples were mixed by gently inverting tubes 10 times prior to dispensing. Three 1.5 ml aliquots of heparinised blood from each animal were dispensed into wells of 24 wells tissue culture plate using sterile pipettes.

2. Stimulation antigens addition and incubation: One hundred (100) µl of PBS (Nil antigen control), buffer I (Bovine PPD, 0.3 mg/ml) and buffer II (Avian PPD,0.3 mg/ml) were added using aseptic technique to the appropriate 3 wells containing the blood previously dispensed. Antigens were mixed thoroughly into the aliquoted blood by using a microplate shaker set for 1 minute on high speed.

Tissue culture plate containing blood and antigens were incubated for 16-24 hrs at 37°C in humidified incubator.

3. Plasma collection and storage: After incubation, 24-well plates were centrifuged at 500 g for 10 minutes at room temperature. Five hundred (500) µl of plasma was removed from the supernatant to separate storage tubes. Each microtube was sealed with an appropriate cap before storage. For longer periods, samples were stored at -20°C up to use. Samples was allowed to equilibrate to room temperature prior to testing by ELISA. Each tube was carefully vortexed several times immediately prior to assay for IFN-gamma release assay.

4. Interferon gamma release assay: ELISA protocol: Interferon gamma release assay by ELISA was carried out according to manufacturer's protocol AsurDX^{TM} *Mycobacterium bovis* INF -gamma kit (Biostone animal health, LLC).

1. Anti-IFN-gamma Ab-coated Plate and all reagent components were brought at room temperature for at least an hour.

2. Fifty (50) µl of diluent solution was added to each well of the plate strips (including the sample wells and control wells).

3. Fifty (50) µl of plasma samples were added per sample well in duplicates.

4. Fifty (50) µl of bovine IFN-gamma Positive Control was added to two control wells.

5. Fifty 50 µl of bovine IFN-gamma Negative Control was added to two control wells.

6. The solution was mixed in the wells by gently rocking the plate manually for 1 minute.

7. Plate was covered with foil and incubated for 60 minutes at room temperature. Direct sunlight and air vents during incubation was avoided.

8. The solution in the wells was discarded.

9. The plate was washed by adding 250 µl of 1X wash solution to each well of plate. Wash solution was discarded and tap dry on paper towels. This process was repeated 5 times. Next step was immediately performed before drying of plate.

10. Fifty (50) µL of anti-INF-gamma Ab-biotin conjugate was added to each well of plate, then 50 µl of streptavidin-HRP conjugate was added to each well of the plate. The solution was mixed in well by gently tracking the plate manually for 1 minute.

11. Plate was covered with foil and incubated for 60 minutes at room temperature.

12. The plate was washed by adding 250 µl of 1X wash solution to each well of the plate. The wash solution was discarded and tap dry on paper towels. This process was repeated total 5 times.

13. One hundred (100) µl of TMB Substrate was added to each well of the plate. Plate was covered with foil and incubated for 15 minutes at room temperature.

14. One hundred (100) µl of stop solution was added to each well. Plate was read on an ELISA plate reader (BIO Tek instruments, Inc USA) at 450 mm wavelength.

15. The optical density (OD) of the wells was measured at 450 nm within 15 minutes after colour development has been stopped.

Result and interpretation were done as per manufacturers guidelines:

A. The mean OD of the Bovine IFN-gamma Negative Control must be <0.2
B. The mean OD of the Positive Control must be >0.5
C. Plasma samples

Positive Sample: OD Bovine PPD-PBS antigen > 0.2 and OD Bovine PPD-Avian PPD > 0.2

Negative Sample: OD Bovine PPD-PBS antigen <0.2 or OD Bovine PPD-Avian PPD <0.2

3.2.3 Isolation of DNA from blood samples

The genomic DNA from blood sample was isolated by using the Hi-media DNA Purification kit as per the manufacturer's protocol.

1. Two hundred (200) µl of blood samples were taken into 2 ml microcentrifuge tube.
2. Twenty (20) µl of proteinase k (20mg /ml) was added and mixed by vortexing for 10-15 seconds to ensure thorough mixing.
3. Two hundred (200) µl of lysis solution was added into blood samples and mixed by vortexing to obtain a homogenous mixture and then incubated at 55°C for 10 minutes.
4. Two hundred (200) µl of ethanol was added to above lysate for its binding with the spin column. These lysates were then transferred into HI- elute miniprep spin column (capped) and centrifuged at 10,000 rpm for 1 minute. The flow-through liquid was discarded.
5. Five hundred (500) µl of diluted prewash solution was added to the column and centrifuged at 10,000 rpm for one minute and flow-through liquid was discarded.
6. Five hundred (500) µl of wash solution were added and centrifuged at 15,000 rpm for 3 minutes and flow through liquid was discarded. Then the empty column was centrifuged once again to remove ethanol properly.
7. The column was then placed in a new 2 ml collection tube. Thereafter, 100 µl of elution buffer were directly added to the spin column and incubated at room temperature for one minute. Then it was centrifuged at 10,000 rpm for 1 minute and finally stored at-20°C till further use.

3.2.4 Conventional Polymerase Chain Reaction (PCR) for diagnosis of bovine tuberculosis

Insertion sequence IS6110 was used for genus identification of *Mycobacterium tuberculosis* complex and 12.7 kb fragment primers-based PCR for the species differentiation. The sequence of primers and amplicon size is given in table 3.2. The PCR was carried out using reaction mixture table 3.3. in thermal cycler (Eppendorf Vapoprotect, USA) using following cyclic conditions in table 3.4. and 3.5.

Table 3.2: Primer sequence used for conventional PCR

Sr. No	Gene/ region targeted	Primer sequence 5′--3′	Size	References
1.	IS6110	F:GACCACGACCGAAGAATCCGCTG R:CGGACAGGCCGAGTTTGGTCATC	445bp	Thakur *et al.*, (2016)
2.	12.7 kb	F: CACCCCGATGATCTTCTGTT R1: GCCAGTTTGCATTGCTATT R2: GACCCGCTGATCAAAGGTAT	823bp	

Table 3.3: PCR reaction mixture for conventional PCR

Ingredients	**Volume (µl)**
2x Go taq Green Master mix (Promega)	12.5 µl
Forward primer (10 pm)	1.0 µl
Reverse primer (10 pm)	1.0 µl
Nuclease free water	8.0 µl
DNA	2.5 µl
Total	**25 µl**

Table 3.4: Cycling condition for IS 6110 gene of *Mycobacterium tuberculosis* complex

Step I	Initial denaturation	94°C for 5 min.	1 cycle
Step II	Denaturation	94°C for 30 sec.	}30 cycle
	Annealing	56°C for 30 sec.	
	Extension	72°C for 30 sec.	
Step III	Final extension	72°C for 10 min	1 cycle
Step IV	Hold	4 °C	

Table 3.5: Cycling condition for 12.7 kb fragment of *Mycobacterium bovis* and *Mycobacterium tuberculosis*

Step I	Initial denaturation	94°C for 5 min.	1 cycle
Step II	Denaturation	94°C for 45 sec.	}30 cycle
	Annealing	59°C for 45sec.	
	Extension	72°C for 1 min.	
Step III	Final extension	72°C for 10 min	1 cycle
Step IV	Hold	4 °C	

3.2.5 Agarose gel electrophoresis

Ten (10) µl of PCR product was mixed with 1 µl of 6X loading dye (Promega, USA) and loaded into 1.5% agarose gel in 0.5X Tris-borate-EDTA buffer containing ethidium bromide (0.5 µg /ml) along with standard molecular size marker 100 bp DNA ladder (Promega, USA). The gel was electrophoresed in a horizontal gel electrophoresis system at 85 volt, 100 milliamperage current for 45 min (Genaxy, India). After 45 min, agarose gel was observed under ultraviolet transilluminator and photographed in gel documentation system (Syngene, USA).

3.2.6 Real-time PCR for diagnosis of bovine tuberculosis

Real-time PCR for detection of *Mycobacterium tuberculosis* complex was done according to Sanchez-Carvajal *et al.,* (2021). Real-time PCR for species differentiation was done according to Halse *et al.,* (2011). The primer and probe sequences used for real-time is given in table 3.6. The reaction mixture used for real-time is given in table 3.7. The cycling condition used for real-time PCR for *Mycobacterium tuberculosis* complex is given in table 3.8. The cycling condition used for real-time PCR for *Mycobacterium tuberculosis* and *Mycobacterium bovis* is given in table 3.9. Real-time PCR was carried out in Roche light cycler 96 (Switzerland).

Table 3.6: Primers and probe sequences used for real-time PCR

Sr. No	Gene/region targeted	Primers and probe sequence (5′--3′)	References
1	IS6110	F: GGTAGCAGACCTCACCTATGTGT R: AGGCGTCGGTGACAAAGG Probe: **FAM** CACGTAGGCGAACCC **BHQ13**	Sanchez-Carvajal *et al.,* (2021)
2.	RD1	F: CCCTTTCTCGTGTTTATACGTTTGA R: GCCATATCGTCCGGAGCTT Probe: **FAM** CACTCTGAGAGGTTGTCA **BHQ13**	Halse *et al.,* (2011)
3	RD4	F: CCACGACTATGACTAGGACAGCAA R: AAGAACTATCAATCGGGCAAGATC Probe: **FAM** ACCAGTGAGGAAACC **BHQ13**	

Table 3.7: Reaction mixture for real-time PCR

Ingredients	**Volume (µl)**
Go Taq qPCR Master mix (Promega)	5 µl
Forward primer (10pm)	0.5 µl
Reverse primer (10pm)	0.5 µl
Probe (10pm)	0.5 µl
Nuclease free water	2.5 µl
DNA	1 µl
Total	**10 µl**

Table 3.8: Cycling condition for real-time PCR for *Mycobacterium tuberculosis* complex

Stages	PCR condition	Temperature and Time	Cycle
Stage I	Preincubation	95°C for 5 min	1
Two step amplification			
Stage II	Incubation	95°C for 15 sec	} 40 cycle
	Annealing	60°C for 30 sec	

Table 3.9: Cycling condition for real-time PCR for RD1 and RD4 region differentiating *M. bovis* from *M. tuberculosis*

Stages	PCR condition	Temperature and Time	Cycle
Stage I	Preincubation	95°C for 10 min.	1
Two step amplification			
Stage II	Incubation	95°C for 15 sec	} 40 cycle
	Annealing	60°C for 35 sec	

3.2.7 Standardization of peptide based In-house ELISA for diagnosis of bovine tuberculosis

In present study, peptides viz. Pep A, Pep Q and Dap E provided by GeNext Genomics, Nagpur were used to perform Indirect ELISA. The Indirect ELISA was standardized for each of antigens for cattle with checkerboard analysis as per following protocol.

3.2.7.1 Protocol for Indirect ELISA

1. Polyvinyl microtiter plates (Nunc, Denmark) were coated with 100 µl/well of antigen-coated buffer at concentrations of 50 ng and 100 ng per well and incubated at 4°C for 8 h or overnight.

2. The coated plates were then washed three times with PBS-T (PBS containing 0.05% Tween- 20), pH 7.2 to remove unadsorbed antigen.

3. The unsaturated sites on the plate were blocked by adding 2% bovine serum albumin (BSA) prepared in PBS (phosphate-buffered saline) at 200 µl/well and incubated at 4°C for 8 h or overnight.

4. The plates were then washed thrice with PBS-T.

5. The hyper-immune serum, as well as healthy serum, was diluted in range of 1:100 to 1:800 and each dilution was added @ 100 µl/well and plates were further incubated at 37°C for 1 hr.

6. The plates were then washed three times with PBS-T and supplemented with anti-IgG conjugate HRPO (Sigma Aldrich, USA) in PBS at 1:5000,

1:10000 and 1:20000 at a ratio of 100 µl/well. respective wells and incubated at 37°C for 1 h. Plates were then washed three times with PBS-T.

7. Finally, 1 mg/ml O-phenylenediamine dihydrochloride (OPD) solution (Sigma Aldrich, US) in substrate buffer mixed with 1.2 µl 30% H2O2 per 10 ml 100 µl substrate buffer per well was added.

8. The plates were then incubated in the dark for 15 min, then 2N sulfuric acid was added at a concentration of 100 µl/well to stop the reaction, and color growth was measured at 450 nm using an ELISA reader (BIO Tek instruments, Inc USA).

9. Optical Density (OD) values of the sample showing positive to negative (P/N) ratio of 2 when compared with healthy serum will be considered standardized for that particular antigen, serum and conjugate concentration.

Thus, indirect ELISA using Pep A, Pep Q and Dap E using positive/ negative sera and their respective anti-species conjugate was standardized. The concentration of peptide antigen and dilution of primary and secondary antibody used for indirect ELISA is given in table 3.10.

Table 3.10: In house indirect ELISA standardised in cattle

Antigen	Concentration	Primary antibody(serum)	Secondary Antibody
Pep A	50ng/well	1:400	1:10000
Pep Q	50ng/well	1:400	1:10000
Dap E	50ng/well	1:400	1:10000

3.2.8 Pathology of bovine tuberculosis

At Akola, one cow under study was died. The smear from nasal secretion was prepared, and a detailed post-mortem examination was carried out. The representative tissues of lungs, peritoneum, mediastinal lymph node, heart, spleen, liver, kidney and intestine were collected in 10% neutral buffered formalin,

processed for routine paraffin embedded section and stained with H&E as per Luna (1968).

RESULTS AND DISCUSSION

Bovine tuberculosis is a chronic bacterial disease caused by *Mycobacterium bovis*, a member of the group *Mycobacterium tuberculosis* complex (MTC) that has a wide host range. It predominantly affects cattle; other animals may become infected. The prevalence of bovine tuberculosis in cattle is not exactly known in central India. No single test is available that can accurately diagnose bovine tuberculosis; combinations of tests are required for better diagnosis. Therefore, the present study aimed to assess the prevalence using conventional skin tests and molecular diagnostic and serology from Maharashtra, Madhya Pradesh, and Chhattisgarh state.

4.1 Single Comparative Intradermal Tuberculin Test (SCITT) for diagnosis of bovine tuberculosis

In the present study, single comparative intradermal tuberculin test was carried out for 418 animals, and the result is given in table 4.1. After 72 hrs of inoculation, swelling of > 4mm developed at the injection site of bovine PPD, was considered positive (plate 1). The overall prevalence found by the single comparative intradermal tuberculin test was 1.67% (n=7).

Table 4.1: Prevalence of bovine tuberculosis by Single Comparative Intradermal Tuberculin Test

(P- Positive, **I**- inconclusive, **N**- Negative, **A+ve** - only PPD A positive**)**

Sr. No.	Place		No. of Animals	P	I	N	A +ve
1	Maharashtra	Pune	42	0	0	42	0
		Solapur	47	0	0	47	1
		Ahmednagar	47	0	0	47	1
		Wadsa	32	1	0	32	1
		Jalgaon	24	0	0	24	0
		Akola	38	2	3	37	1
2	Chhattisgarh	Durg	50	0	0	50	0
3	Madhya Pradesh	Chhindawara	138	4	6	134	0
Total			418	7	9	413	4
Percentage (%)				1.67	2.1	98.8	0.95

The findings of the present study are in accordance with the findings of Gumi *et al*., (2012), who found 2% prevalence in cattle from Somali, South Ethiopia. Ameni *et al.,* (2013) reported a prevalence of 1.8% in Central Ethiopia. Ghebremariam *et al.,* (2018) reported low prevalence (1.2%) in individual animals and 3.2% herd prevalence of bovine tuberculosis in four regions of Eritrea. Trangadia *et al*., (2013) found a prevalence between 0.59% to 2.39% in three different farms from Gujarat and Utter Pradesh state. Mukherjee (2006) found a prevalence between 0.65% and 15.76% in three different farms in northern India. Sharma *et al*., (2011) observed a prevalence range from 1.53% to 13.91% during different years of study. In the metanalysis study, a prevalence of 2.7% and 6.3% were reported from Maharashtra and Madhya Pradesh, respectively (Srinivasan *et al.,* 2018). Ameni *et al.,* (2007) found that 4.8% of animals reacted to both PPD. 8.7% of animals reacted to only bovine PPD, and 1.3% of animals reacted to only PPD A. Noorrahim *et al.,* (2015) found 4.33% (13/300) animals positive for bovine TB, and 61.54% (8/13) were positive to avian tuberculin.

SCITT constitutes a good indication of mycobacterial exposure; even this test does not always discriminate between cattle with tuberculosis and those exposed to non-pathogenic organisms (Whelan *et al.,* 2003). Low prevalence in the present study may be due to climatic conditions, lower cattle density, and housing of animals in an open area in the extensive cattle production system in general. In the present study, no. of indigenous (zebu) cattle (n=318) were considered relatively resistant to bovine tuberculosis compared to exotic breeds. Several hosts related factors like malnutrition, recent infection with *M. bovis*, co-infection with non-tuberculous mycobacteria, infestation with gastrointestinal parasites, and generalized tuberculosis can decrease reactivity to the SCITT (Ameni *et al.,* 2000; Flynn *et al*., 2007) and be ruled out to have influenced our study outcome. Emaciated animals have a lower proportion of lymphocytes; likewise, animals become anergic, so they have a lower response to tuberculin. Animals in good health respond better to tuberculin than animals in poor health (Ameni *et al.,* 2007).

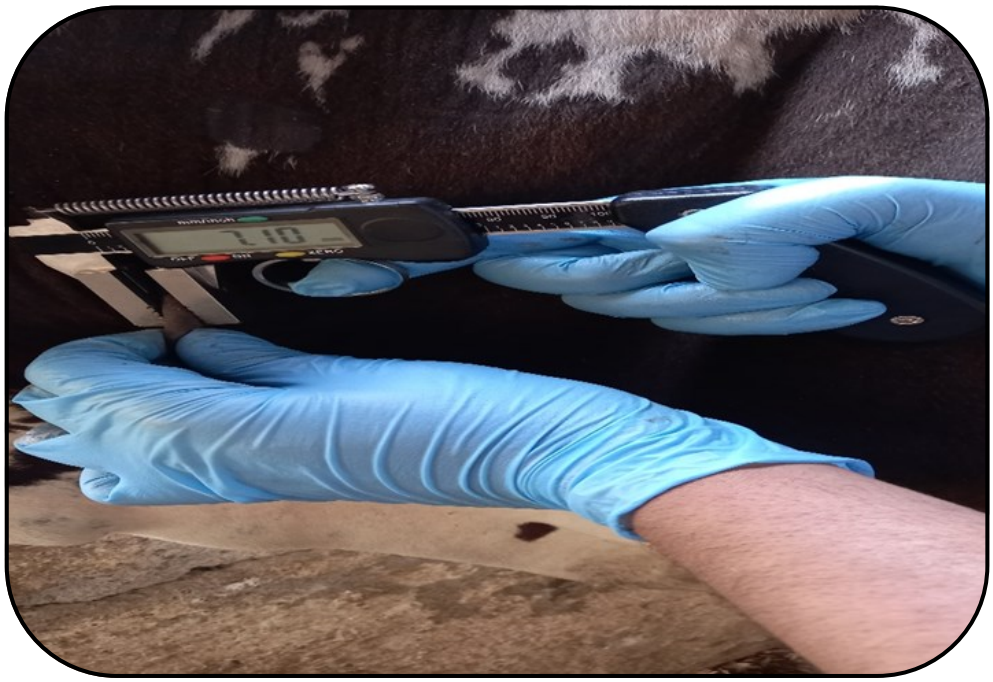

Plate 1: Cattle showing increase in thickness at bovine PPD injection site after 72 hrs

Some reports show higher positivity using a comparative intradermal tuberculin test. Brahma *et al.,* (2019) found 19.80 % of animals positive from Panjab, Barua *et al.,* (2017) found 16.58% of animals positive from Meghalaya, and Sharma *et al.,* (2019) found a prevalence 34.0% in organized government dairy farms using SCITT. is a difficult task to compare the result of different authors because of environmental factors, immune status, the prevalence of disease in different geographical areas, and the nature of tuberculin that different authors used (Boukary *et al.,* 2011).

Due to false negative response to the skin test, many animals remain infected within the herd, which lead to further progression of the disease as there is no test and slaughter policy available in India. So, diagnosis of bovine tuberculosis with other ancillary tests along with tuberculin test is necessary.

4.2 Interferon Gamma Release Assays (IGRA) for diagnosis of bovine tuberculosis

In the present study an interferon gamma release assay was carried out for 90 samples. [Akola (n= 38), Wadasa (n=32) and Chindawara (n=20)]. Out of these 18 (20%) animals showed a positive response to interferon gamma (plate 2).

Interferon gamma has good sensitivity and specificity, although the test has some constraints. The test should be carried out within 30 hrs after blood collection. Due to the high cost of the interferon gamma release assay kit, only 90 samples were processed in the present study. The finding of present study is in accordance with previous studies, which reported a prevalence of 22.22% (Neeraja *et al.,* 2014) and 19.37% (Raffo *et al.,* 2018) in cattle.

Interferon gamma release assay is an ante mortem test for the identification of latent mycobacterial infection. This test is based upon the principle that the release of interferon gamma by T helper cell after stimulation with PPD *in vitro*. BCG-vaccinated animals did not respond to gamma interferon, so can be easily differentiated from infected from vaccinated animals (DIVA) (Vordermeier *et al.,* 2011).

Gormley *et al.,* (2013) reported 10.83% positivity by interferon gamma release assay. Filia *et al.,* (2016) reported (0.82%) prevalence. Katale *et al.,* (2017) reported (2%) of animals were positive. Mareledwane *et al.,* (2022) reported only 4.4% prevalence which by interferon gamma release assay. These are at lower side as compared to the present study. Some reports are showing high prevalence using interferon-gamma release assay. Thakur *et al.,* (2010) reported (78.26%) prevalence. Lopes *et.al.,* (2012) reported 29/102 (28.4%) animal positives which are slightly higher side as compared to the present study.

4.3 Conventional PCR for diagnosis of bovine tuberculosis

In the present study, a total of 418 samples of blood were collected from 3 states of India, viz Maharashtra, Madhya Pradesh, and Chhattisgarh. The result of conventional PCR for diagnosing bovine tuberculosis is given in table 4.2. Total 17/418 samples were positive for M*ycobacterium tuberculosis* complex (plate 3). All these *Mycobacterium tuberculosis* complex positive samples were further subjected to 12.7 kb PCR which showed an 823bp band specific for *Mycobacterium bovis* (plate 4). A total of 7/38 (18.5%) from the Akola region, 3/32 (9.3%) from Wadasa, and 5/138 (3.62%) from Chindawara were positive for *Mycobacterium bovis.*

Table 4.2: Result of conventional PCR for diagnosis of bovine tuberculosis

Sr. No.	Place		No. of samples	IS 6110 (MTC)	*M. bovis*
1	Maharashtra	Pune	42	0	0
		Solapur	47	0	0
		Ahmednagar	47	0	0
		Wadasa	32	4	3
		Jalgaon	24	0	0
		Akola	38	7	7
2	Chhattisgarh	Durg	50	0	0
3	Madhya Pradesh	Chhindawara	138	6	5
Total			418	17	15
Percentage (%)				4.06	3.58

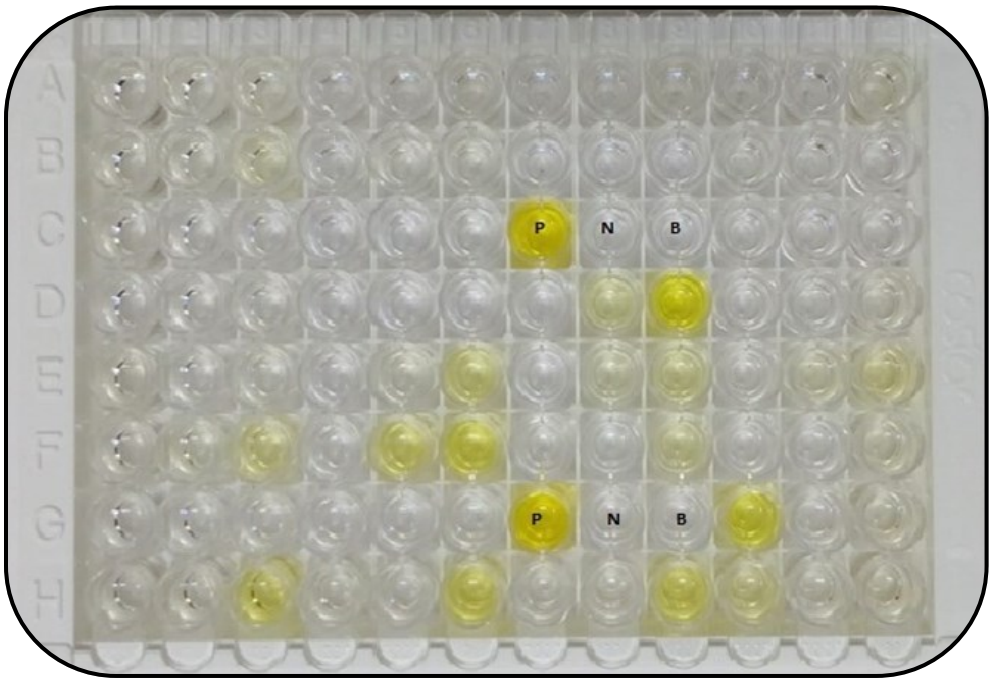

Plate 2: showing interferon gamma release assay for serum samples of cattle

P- Positive control
N- Negative Control
B – Blank

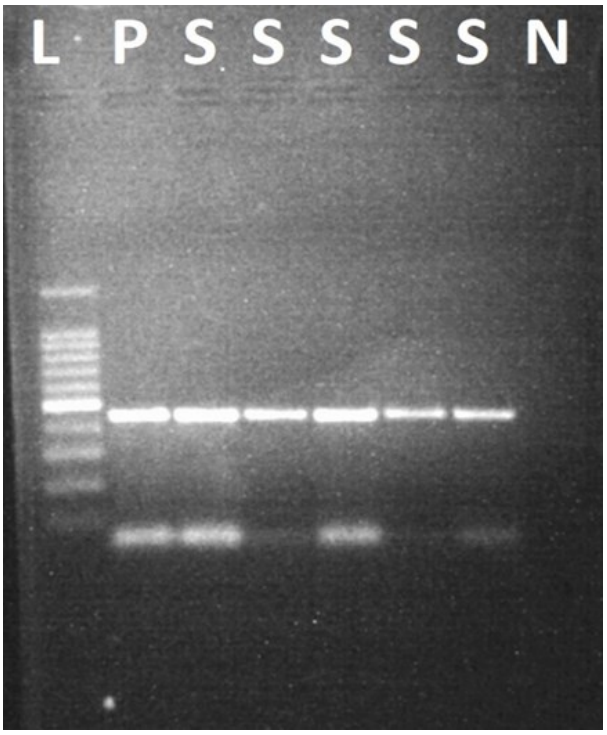

Plate 3: Gel electrophoresis of IS6110 gene (445bp) of *Mycobacterium tuberculosis* complex

L- Ladder (100bp); S- Field samples; N- Negative control

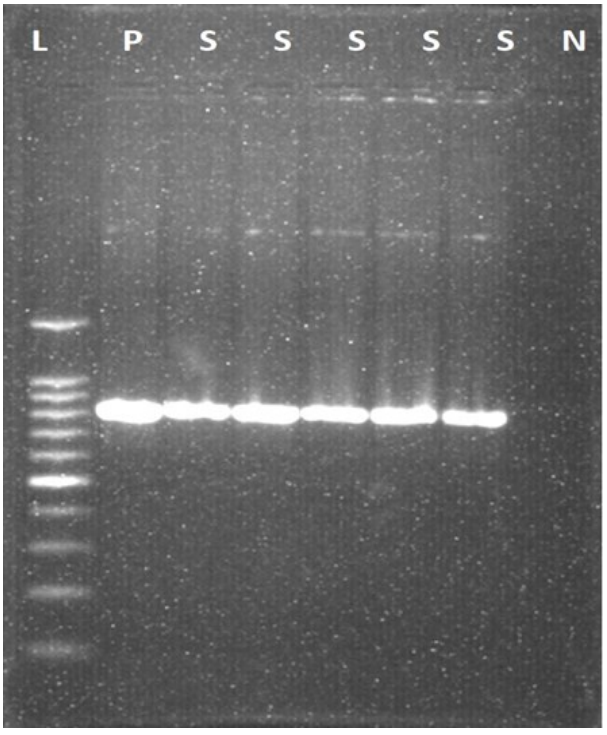

Plate 4: Gel electrophoresis of 12.7 Kb region of *Mycobacterium bovis* (823bp)

L- Ladder (100bp); S- Field samples; N- Negative control

In the present study overall prevalence using conventional PCR was 3.58% (15/418) for *Mycobacterium bovi*s. Many researchers found positive PCR results for bovine tuberculosis by using various clinical samples viz blood, nasal swab, milk, and suprascapular lymph node. The finding of the present study is in accordance with the previous study, which reported 3.3% (Filia *et al.,* 2016), 3.4% (Basit *et al.,* 2015), and 3.99% (Kapalamula *et al.,* 2023).

Bovine tuberculosis is present in some endemic regions. There is region-to-region variation in positivity from the bovine population and this could be the reason for the low prevalence of disease in the present study.

4.4 Real-time PCR for diagnosis of bovine tuberculosis

A total of 418 blood samples were subjected to real-time PCR. *Mycobacterium tuberculosis* complex (MTC) was confirmed by targeting IS6110. Out of the total 418 samples screened 24 (5.74%) were positive for *Mycobacterium tuberculosis* complex (MTC). The result is given in table 4.3. The cycle threshold (Ct) value for these positive samples ranged from 28.3 to 35.1 (plate 5). These 24 positive samples were further tested for *Mycobacterium bovis* and *Mycobacterium tuberculosis* by targeting Region of divergence 1 (RD1) and Region of divergence 4 (RD4), respectively. A total 22/24 (5.26%) samples were positive [Akola (n=12), Madhya Pradesh (n=6), and Wadasa (n=4)] for *Mycobacterium bovis*. Two samples were neither positive for *M. bovis* nor for *M. tb*. The Ct value of *M. bovis* samples ranged from 26.8 to 33.95 for RD1 (plate 6).

The finding of the present study was correlated with the finding of Cezar *et al.,* (2016), who reported 16/401 (4%) blood samples positive by real-time PCR. Bezerra *et al*., (2015) reported 4.79 % prevalence of *M. bovis* from Brazil. Ortu *et al.,* (2006) reported 5.6% prevalence using real-time PCR.

Many researchers have used real-time PCR to diagnose bovine tuberculosis to determine sensitivity and specificity. They mainly used post-mortem tissue and nasal swabs of acid-fast positive animals. Moreover, some scientists used real-time PCR for prevalence studies using clinical samples from

intradermal and other tuberculosis test-positive animals. But in the present study, blood samples from cattle were collected irrespective of skin test-positive animals. Random sampling nature from comparatively large geographical areas might be the reason for low prevalence in this study. Sampling time also influences the result of PCR as bacteremia and time of dissemination of *M. bovis* in the blood stream may be transient. There are studies showing that most of the Tuberculin Sensitivity Test and Interferon Gamma Release Assay reactor animals failed to be detected by PCR in blood samples (Maggioli *et al.,* 2016; Brahma *et al.,* 2019).

Table 4.3: Result of a real-time PCR assay for diagnosis of bovine tuberculosis

Sr.no.	Place		No. of samples	MTC	RD1 *M. bovis*	RD4 *M. tb*
1	Maharashtra	Pune	42	0	0	0
		Solapur	47	0	0	0
		Ahmednagar	47	0	0	0
		Wadasa	32	4	4	0
		Jalgaon	24	0	0	0
		Akola	38	13	12	0
2	Chhattisgarh	Durg	50	0	0	0
3	Madhya Pradesh	Chhindawara	138	7	6	0
Total			418	24	22	0
Percentage (%)				5.74	5.26	0

The finding of the present study was correlated with the finding of Cezar *et al.,* (2016), who reported 16/401 (4%) blood samples positive by real-time PCR. Bezerra *et al*., (2015) reported 4.79 % prevalence of *M. bovis* from Brazil. Ortu *et al.,* (2006) reported 5.6% prevalence using real-time PCR.

Many researchers have used real-time PCR to diagnose bovine tuberculosis to determine sensitivity and specificity. They mainly used post-mortem tissue and nasal swabs of acid-fast positive animals. Moreover, some scientists used real-time PCR for prevalence studies using clinical samples from intradermal and other tuberculosis test-positive animals. But in the present study, blood samples from cattle were collected irrespective of skin test-positive

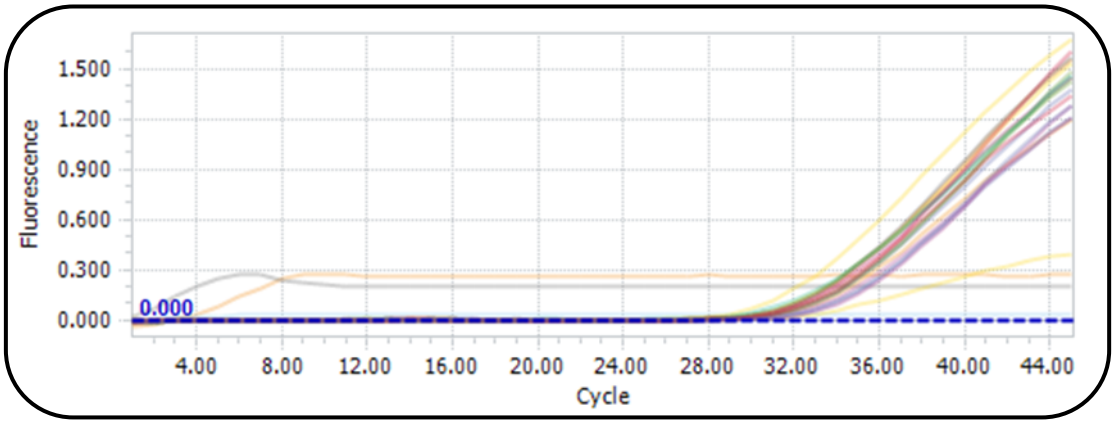

Plate 5: Absolute quantification curves of real-time PCR for *Mycobacterium tuberculosis* complex

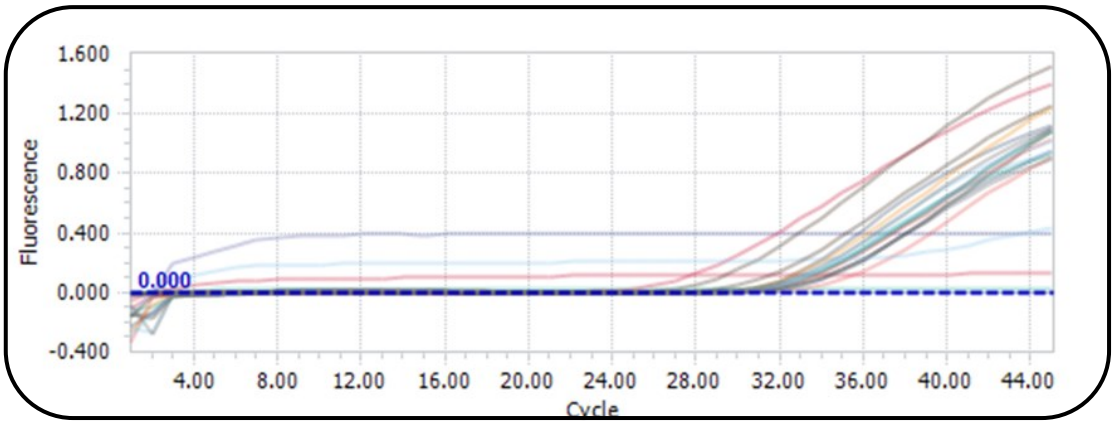

Plate 6: Absolute quantification curves of real-time PCR for RD1 of *Mycobcterium bovis*

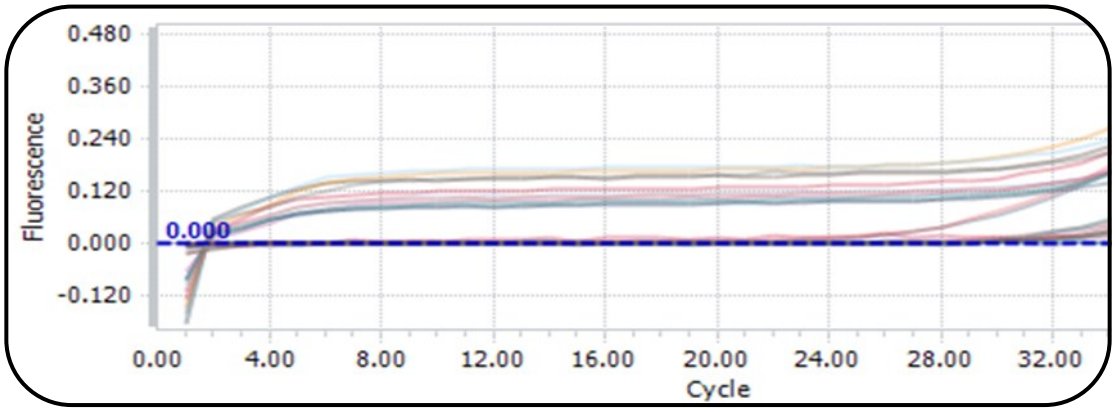

Plate 7: Absolute quantification curves of real -time PCR for RD4 of *Mycobacterium bovis*

animals. Random sampling nature from comparatively large geographical areas might be the reason for low prevalence in this study. Sampling time also influences the result of PCR as bacteremia and time of dissemination of *M. bovis* in the bloodstream may be transient. There are studies showing that most of the Tuberculin Sensitivity Test and Interferon Gamma Release Assay reactor animals failed to be detected by PCR in blood samples (Maggioli *et al.*, 2016; Brahma *et al.*, 2019).

4.5 Peptide based In-house ELISA for diagnosis of bovine tuberculosis

In-house ELISA is a diagnostic tool incorporated in the present study which is based upon humoral immunity repose (IgG antibody). Peptide antigens used in the present study were PEP A, PEP Q, and DAP E.

A total of 418 samples were subjected to indirect in-house ELISA. The result of peptide-based ELISA is given in table 4.4. Total 15/418 (3.58%) serum samples were positive by PEP A (plate 8) and 18/418 (4.3%) by PEPQ and DAP E, respectively (plate 9 and plate 10). The overall prevalence was found (4.3%) by (considering the serum sample positive for any two peptides from the three mentioned above) using indirect ELISA.

Table 4.4: Result of in-house peptide-based indirect ELISA

Sr. No.	Place		No. of samples	PEP A	PEP Q	DAP E
1	Maharashtra	Pune	42	0	0	0
		Solapur	47	0	0	0
		Ahmednagar	47	0	0	0
		Wadasa	32	1	1	1
		Jalgaon	24	0	1	1
		Akola	38	10	11	11
2	Chhattisgarh	Durg	50	0	0	0
3	Madhya Pradesh	Chhindawara	138	4	5	5
Total			418	15	18	18
Percentage (%)				3.58	4.3	4.3

Sonekar (2021) carried out indirect ELISA for sample collected in and around the Nagpur region using PEP A, PEP Q and DAP E. Author found an

overall seroprevalence of 16.61% from the total 331 cattle and buffalo tested. This finding was higher side as compared to the present study. Muasya *et al*., (2019) reported 3.99% (11/276) prevalence using MPB70 and MPB 83 recombinant peptides as antigens. Infantes Lorenzo *et al.,* (2017) reported 4% prevalence using P22 multiprotein complex; these studies are comparable with the present study.

4.6 Statistical analysis for a risk factor in animals for bovine tuberculosis

a. Sex of animal

The null hypothesis (H_0) $_{is}$ TB infection is independent of sex of the animal.

Table 4.5: Chi-square test for sex-independent positivity

sex	Observed frequency		Expected frequency		Chi-square value	CV value	P value
	TB positive	TB negative	TB positive	TB negative			
Male	3	50	2.78	50.21	0.020724	3.841	0.885533
Female	19	346	19.21	345.78			

(N=418 cattle)

Based upon X^2 value as a value less than critical value we fail to reject the hypothesis, thus indicating that sex and tuberculosis development are unrelated.

b. Age of animal

The null hypothesis (H_0) is TB infection is independent of age of the animal**.**

Table 4.6: Chi-square test for age-independent TB positivity

Age	Observed frequency		Expected frequency		Chi-square value	CV value	P value
	TB positive	TB negative	TB positive	TB negative			
4-6yrs	6	342	18.315	329.68	52.197	3.841	5.01E-13
6-8yrs	16	54	3.684	66.315			

(N=418 cattle)

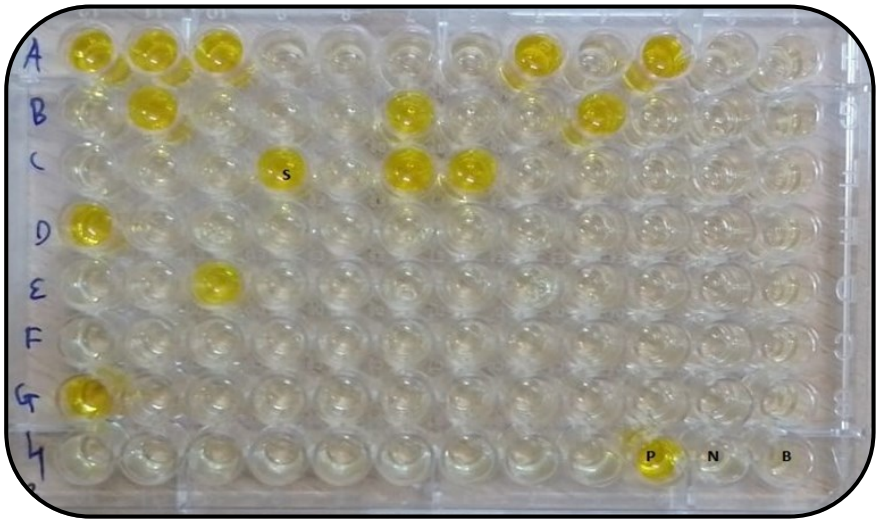

Plate 8: In -house indirect ELISA for PEP A

P- Positive control N – Negative control B- Blank S- Sample

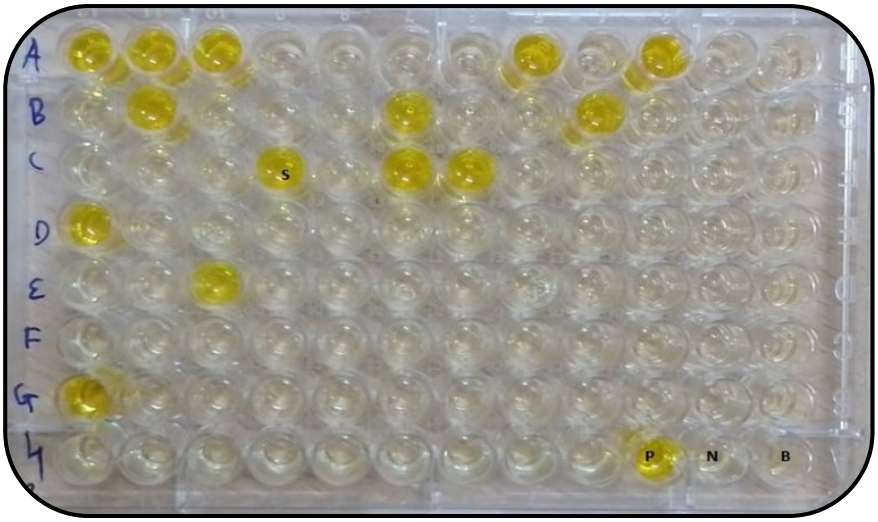

Plate 9: In -house indirect ELISA for PEP Q

P- Positive control N – Negative control B- Blank S- Sample

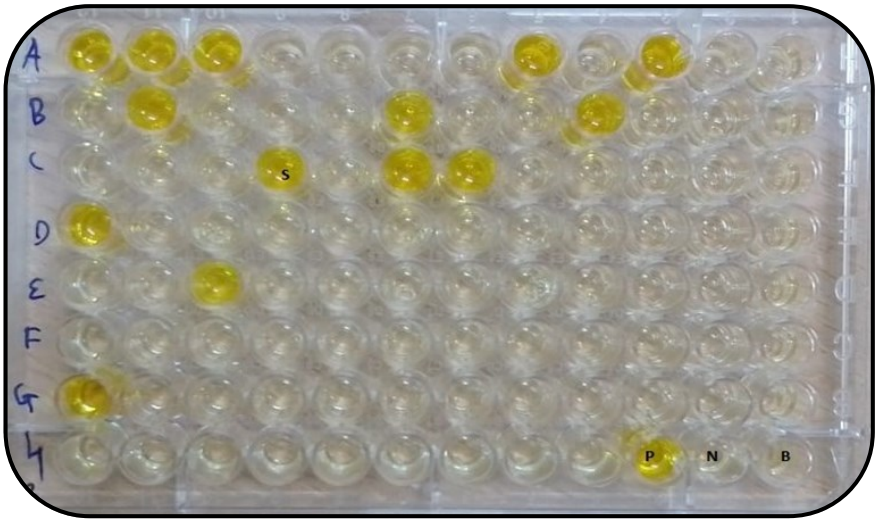

Plate 10: In -house indirect ELISA for DAP E

P- Positive control N – Negative control B- Blank S- Sample

Based upon X^2 value, as they are more than critical value null hypothesis is rejected, so bovine tuberculosis is more likely to present in old animals than younger animals

c. Body condition of animals

The null hypothesis (H_0) is TB infection is independent of the body condition of the animal.

Table 4.7: Chi-square test for body condition independent TB positivity

Body condition	Observed frequency		Expected frequency		Chi-square value	CV value	P value
	TB positive	TB negative	TB positive	TB negative			
apparently healthy	12	190	10.63	191.3	0.5284	5.991	0.827374784
slightly emaciated	9	182	10.05	180.94			
emaciated	1	24	1.31	23.68			

(N=418 cattle)

As X^2 is less than critical value we fail to reject the null hypothesis indicating that bovine tuberculosis may be present in both apparently healthy and emaciated animal

4.7 Pathology of bovine tuberculosis

Smear from nasal secretion revealed numerous pinkish acid-fast bacteria with Ziehl-Neelsen (ZN) staining (plate 11). Gross examination of lungs revealed multiple discrete small nodules (plate 12) and multiple nodules on the peritoneum (plate 13). Microscopic examination of the lung sections revealed multiple granulomas consisting of a central area of caseous necrosis surrounded by numerous macrophages, epithelioid cells, lymphocytes, fewer plasma cells, and multinucleated giant cells (Langhans giant cells), calcification and fibrosis (plate 14-16).

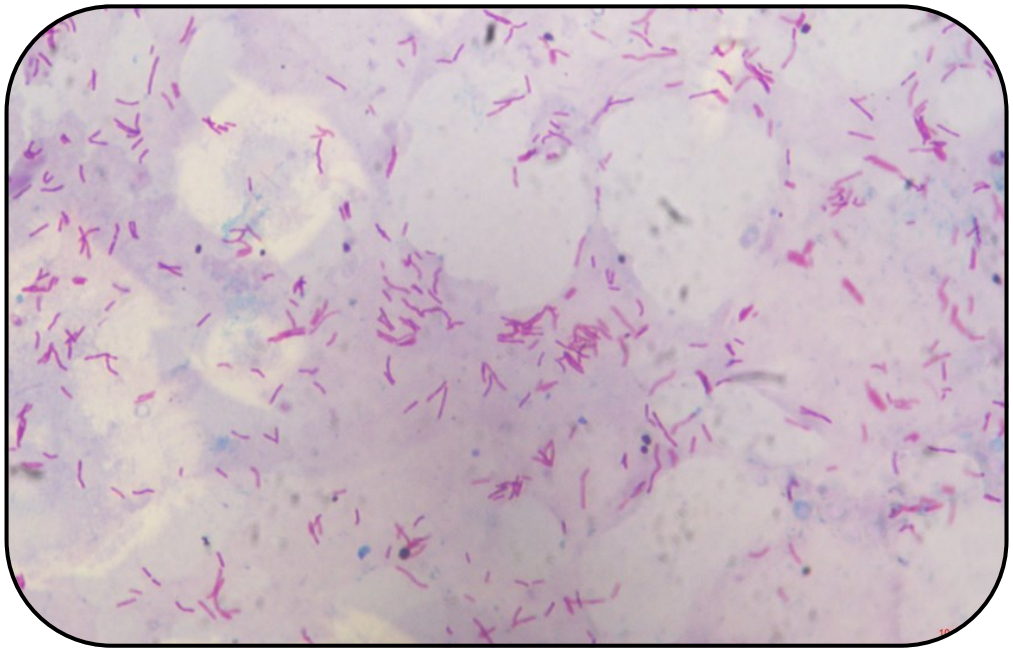

Plate 11: Impression smear of nasal secretion showing numerous acid fast bacteria of *Mycobacterium bovis* (Ziehl–Neelsen stain, 100x)

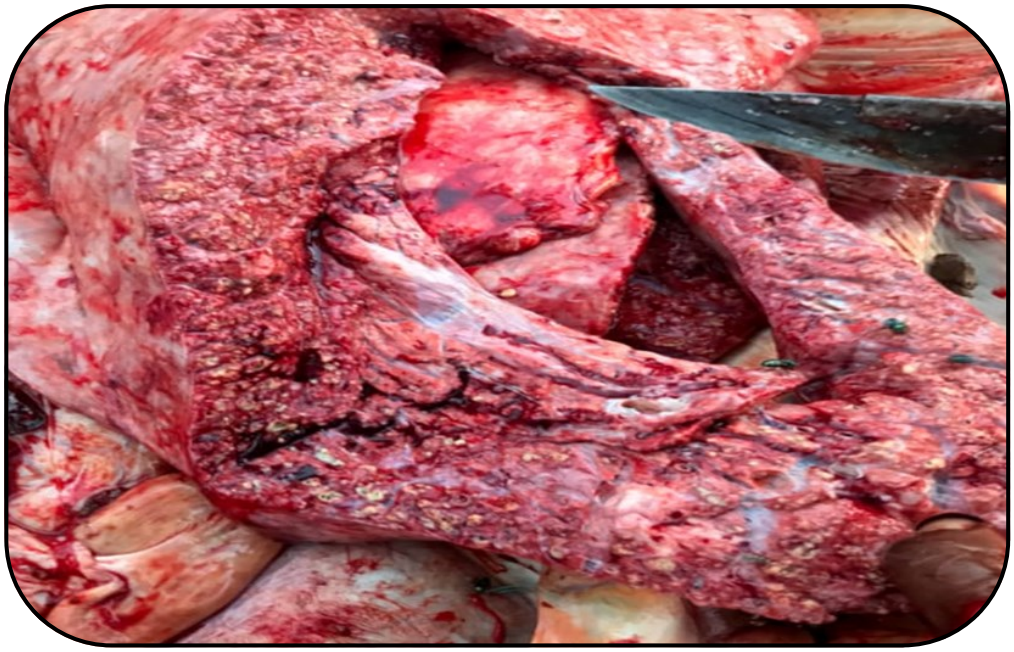

Plate 12: Cut surface of lungs showing multiple discrete small tubercles

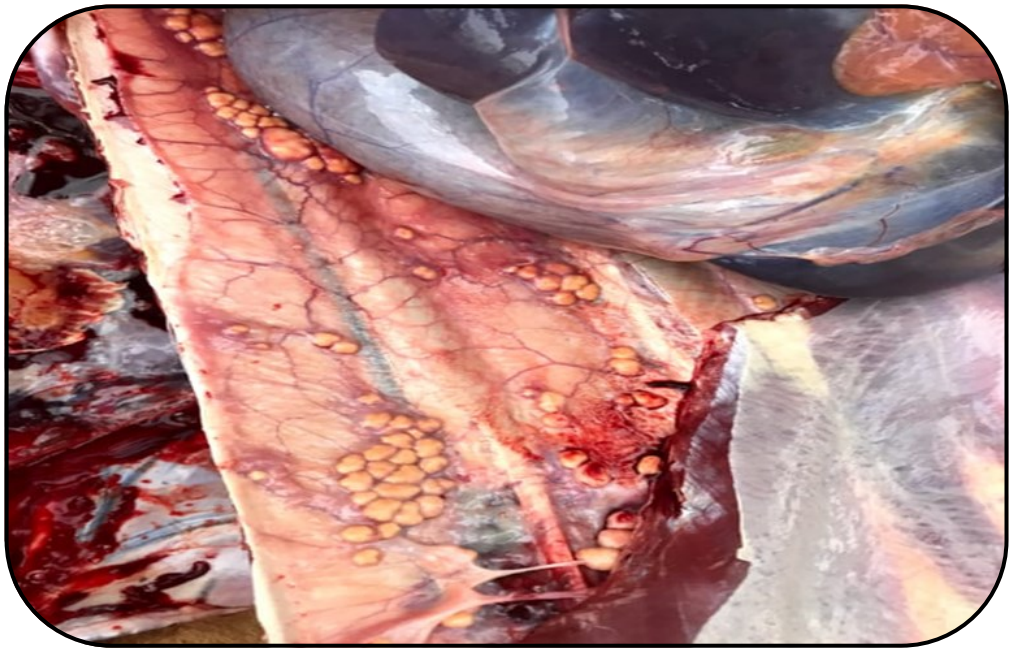

Plate 13: Multiple nodules on peritoneum of cow

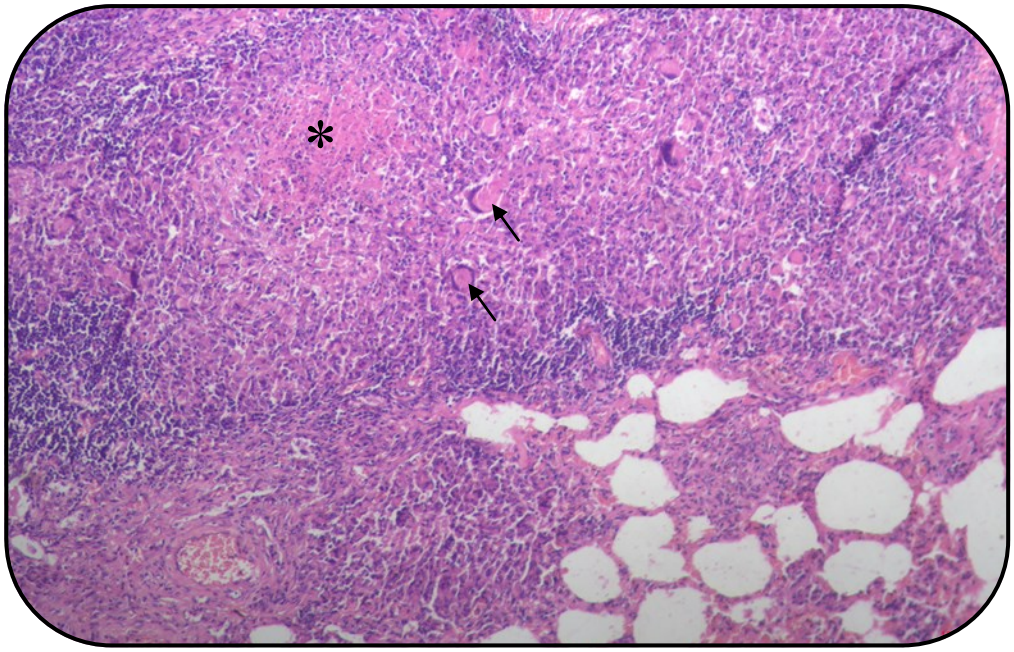

Plate14: Section of lungs showing central area of caseous necrosis (asterisk) surrounded by macrophages, lymphocytes, epithelioid cells and giant cells (arrows) H&E

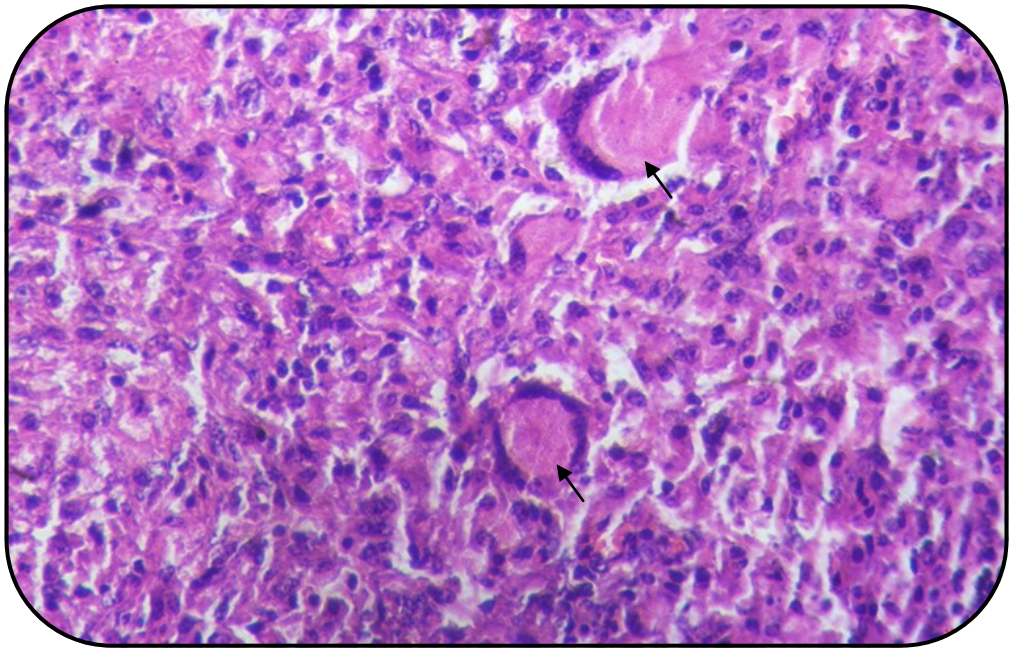

Plate 15: Section of lungs showing multinucleated Langhans giant cells (arrow) cells and epithelioid cells H&E 40x

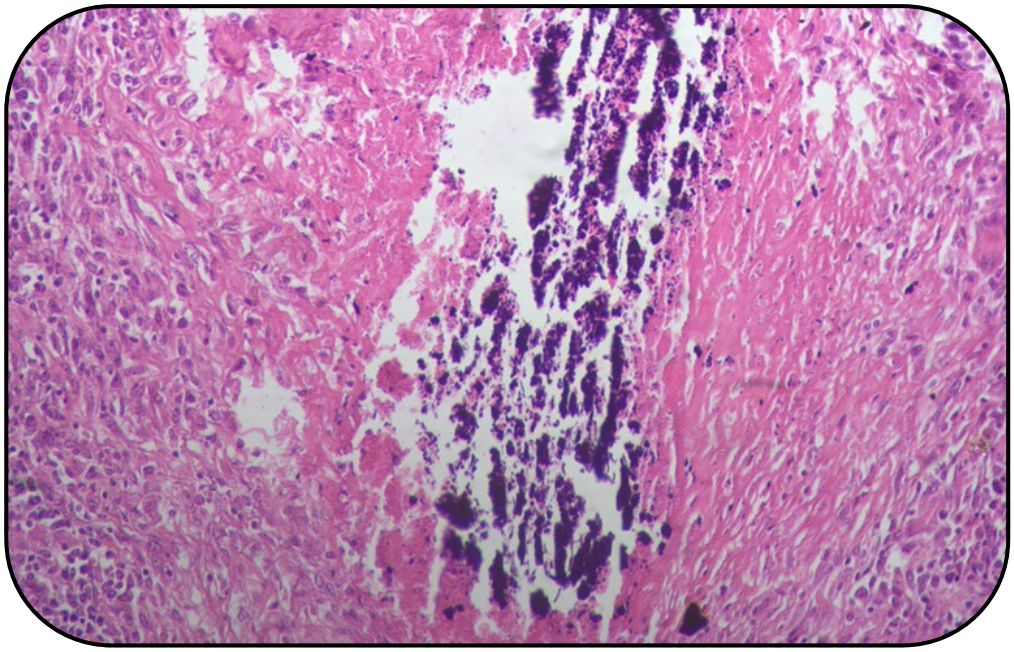

Plate16: Section of lungs showing central calcification at in the caseous necrotic area H&E 10x

SUMMARY AND CONCLUSIONS

Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis* leading to a loss in productivity and signifies a crucial public health risk.

In the present study, 418 blood and serum samples of cattle were screened from Maharashtra, Madhya Pradesh, and Chhattisgarh state. Samples were collected by random sampling by targeting animals with emaciated body conditions. Before collecting blood samples, animals were screened for bovine tuberculosis by conventional test (Single comparative intradermal tuberculin test). Blood and serum samples were collected irrespective of the either positive, negative, or inconclusive tuberculin test result. Interferon gamma release assay was carried out for 90 cattle from Chhindawara (M.P), Akola, and Wadsa (Maharashtra). All blood samples were screened by conventional PCR targeting IS6110 for genus identification and 12.7kb region for species differentiation. Real-time PCR was carried out by targeting IS6110 for genus identification and RD1 and RD4 region for species differentiation. Sera samples were screened by in-house indirect ELISA by using synthetic peptide as antigen viz PEP A, PEP Q, DAP E for presence or absence of IgG antibody against *Mycobacteria*.

In the Single comparative Intradermal tuberculin test, 1.67% positivity was observed. A total 18/90 (20%) animals showed a positive response to the interferon-gamma release assay. Prevalence by conventional PCR was 4.06% for the genus *Mycobacterium tuberculosis* Complex (MTC) and 3.58% for *Mycobacterium bovis.* By real-time PCR, 5.74 % of samples were positive for genus MTC, and 5.26 were positive for *Mycobacterium bovis*. Out of a total of 418 samples, 3.58%, 4.3%, 4.3% of serum samples turn positive by PEP A, PEP Q, and DAP E, respectively. Grossly, the lungs revealed multiple discrete nodules and granulomatous inflammation with characteristic Langhans giant cells were present microscopically.

Following conclusions are drawn from the present study

1. Bovine tuberculosis is prevalent in Maharashtra, Chhattisgarh, and Madhya Pradesh states.
2. Confirmatory diagnosis of bovine TB can be done in the early stage in live animals with multiple approaches like skin test followed by Interferon gamma release assay and a molecular technique like PCR.
3. Peptide antigen ELISA based upon humoral immune response is also an effective additional tool and must be incorporated with other tests to diagnose bovine tuberculosis.

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APPENDIX I

Reagents for agarose gel electrophoresis:

I) Tris borate EDTA buffer (TBE)

A) Preparation of 5X TBE (100 ml)

Tris base	:	5.4 gm
Boric acid	:	2.75 gm
0.5 MEDTA (pH 8)	:	2 ml
Distil Water	:	100 ml

B) Preparation of 0.5X TBE (500 ml)

0.5X TBE	:	50 ml
Distil Water	:	450 ml

II) Preparation of Ethidium bromide Solution

Ethidium bromide	:	10 mg
Distil water	:	1 ml

APPENDIX II

Reagents for indirect ELISA

1) Phosphate Buffer Solution (PBS) (For Scrum and Conjugate Dilution)

Disodium hydrogen phosphate (unhydrous) ($Na:HPO_4$)	:	1.16 gm
Potassium dihydrogen phosphate (KH:PO.)	:	0.2 gm
Potassium chloride (KCI)	:	0.2 gm
Sodium chloride (NaCI)	:	8.0 gm
Distilled water	:	1000 ml
pH	:	7.2±0.2

Autoclaved

2) Carbonate buffer (For Antigen dilution)

Sodium bicarbonate ($NaHCO_3$)	:	2.93 gm
Sodium carbonate (Na_2CO_3)	:	1.5 gm
Distilled water	:	1000 ml
pH	:	9.2 to 9.6

Autoclaved

3) Washing Buffer (PBS-T)

Tween-20	:	0.5 ml
Phosphate buffer solution (PBS)	:	1000 ml

4) Substrate buffer (For OPD dilution)

Tri-sodium citrate	:	7.634 gm
Citric acid	:	4.943 gm
Distilled water	:	1000 ml

5) Blocking buffer

Bovine serum albumin	:	20 gm
Phosphate buffer saline	:	10000 ml

6) O-phenylene dia nine dihydrochloride (OPD)

O-phenylene diamine dihydrochloride (OPD)	:	1.0 g
Hydrogen peroxide (H_2O_2)	:	120 µl
Substrate buffer	:	1000 ml

7) Stopping reagent

2N Hydrogen sulphate (H_2SO_4)	:	54.4 ml
Distilled water	:	945.6 ml

VITA

The author Dr. Bankar Aniket Sarjerao was born on 9th October 1996 in Loni Vyankanath, Ahmednagar, Maharashtra state. He has completed his Secondary School Certificate Examination in the year 2012 from Shree Chhatrapati Shivaji Vidyalaya Belwandi BK, Shrigonda and Higher Secondary School Certificate Examination in the year 2014 from the Maharaja Jivajirao Shinde College Shrigonda, Ahmednagar.

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During Graduation and Post-graduation, the author had actively participated in various co-curricular activities and had attended various national and international conferences and workshops.

Thesis Abstract

a)	Title of thesis	:	**PREVALENCE AND MOLECULAR DIAGNOSIS OF BOVINE TUBERCULOSIS**
b)	Full Name of Student	:	**BANKAR ANIKET SARJERAO**
c)	Name & Address of Advisor/ Guide	:	**Dr. M. P. Kaore** Assistant Professor Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur Master of Veterinary Science
d)	Degree to be awarded	:	**MASTER OF VETERINARY SCIENCE**
e)	Year of award of degree	:	**2023**
f)	Major subject	:	**Veterinary Pathology**
g)	Total number of pages in the thesis	:	**40**
h)	Number of words in the thesis abstract	:	**242**
i)	Signature of student		
j)	Signature, Name & Address of forwarding authority	:	

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ABSTRACT

Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis* leading to a loss in productivity and signifies a crucial

public health risk. The present study was conducted to know the prevalence of bovine tuberculosis in central India in cattle. Four hundred eighteen cattle from Maharashtra, Madhya Pradesh, and Chhattisgarh were screened by a single comparative intradermal tuberculin test (SCITT) and molecular technique like conventional and real-time PCR. Interferon gamma release assay was carried out for 90 cattle from nearby places (Chhindawara, Akola, and Wadsa) from Nagpur. In-house indirect ELISA was carried out by using synthetic peptide as antigen viz PEP A, PEP Q and DAP E for the presence or absence of IgG antibody Against *Mycobacteria*.

By Single Comparative Intradermal Tuberculin Test, 1.67% prevalence was found. A total of 18/90 (20%) animals showed a positive response to the interferon-gamma release assay. Prevalence by PCR was found 4.06% positive for the genus *Mycobacterium tuberculosis* complex (MTC), and 3.58% of samples were found positive for *Mycobacterium bovis.* Using real-time PCR, 5.74 % of samples were found positive for genus MTC, and 5.26% were positive for *M. bovis*. Out of 418 samples, 3.58%, 4.3%, 4.3% of serum samples turn positive by PEP A, PEP Q, and DAP E, respectively. Diagnosis of bovine tuberculosis can be made early in live animals with multiple approaches like skin tests followed by Interferon-gamma release assay and a molecular technique like PCR and peptide based indirect ELISA.

प्रबंध सारांश

अ.	प्रबंधाचे शिर्षक	:	गोवंशीय क्षयरोगाचे प्राबल्य आणि आण्विक निदान
ब	विद्यार्थ्यांचे पुर्ण नाव	:	बनकर अनिकेत सर्जेराव
क.	मार्गदर्शकाचे नाव आणि पत्ता	:	डॉ. मे. पु. कावरे सहाय्यक प्राध्यापक, पशुवैद्यकीय विकृतीशास्त्र विभाग, नागपूर पशुवैद्यकीय महाविद्यालय, नागपूर
ड.	प्रदान करण्यात येणारी पदवी	:	स्नातकोत्तर पदवी (एम. व्ही. एस. सी.)
इ.	पदवी प्रदान करण्याचे वर्ष	:	२०२३
फ.	मुख्य विषय	:	पशुवैद्यकीय विकृतीशास्त्र
ग.	प्रबंधातील एकूण पृष्ठे	:	४०
ह.	सारांशातील एकूण शब्द	:	२०२
ई.	विद्यार्थ्यांची सही	:	
ज.	अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नांव आणि पत्ता	:	

सहयोगी अधिष्ठाता
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सारांश

सदर शोधप्रबंध हा भारतातील मध्यवर्ती ठिकाणातील गोवंशीय क्षयरोगाची व्याप्ती जाणून घेण्याकरिता करण्यात आला. गोवंशीय क्षयरोग हा दीर्घकालीन राहणारा संसर्गजन्य रोग असून तो मायकोबॅक्टरियम बोविस या जिवाणूंमुळे होत

असून त्यामुळे जनावरांपासून होणाऱ्या उत्पादनामध्ये प्रचंड घट होते आणि तो जनावरांपासून माणसाला होणाऱ्या आजारांमध्ये मोडला जातो. गोवंशीय क्षयरोगाचे पूर्णपणे निदान करणारी एकही चाचणी आतापर्यंत अस्तित्वात नाही. या शोधप्रबंधामध्ये ४१८ गाईगुरांची चाचणी करण्यात आली. त्यामध्ये प्रामुख्याने महाराष्ट्र मध्यप्रदेश व छत्तीसगडमधील मधील जनावरांचा समावेश होता. चाचण्यांमध्ये प्रामुख्याने अंतत्वाचेतील तुलनात्मक ट्यूबरक्यूलिन चाचणी, गामा इन्टर्फेरोन चाचणी पीसीआर, रिअल टाइम पीसीआर, व अप्रत्यक्ष एलीसा चाचणी या चाचण्यांचा समावेश होता. गामा इन्टर्फेरोन, चाचणी ही नागपूर वरून जवळ असणाऱ्या ठिकाणावरून एकूण ९० जनावरांच्या रक्तजलावर करण्यात आली. त्यामध्ये प्रामुख्याने वडसा अकोला व छिंदवाडा या ठिकाणांचा समावेश होता.

अंतत्वचेतील तुलनात्मक ट्यूबरक्यूलिन चाचणीला १.६७ टक्के गोवंशिय जनावरांनी उत्तम प्रतिसाद दर्शवला. इन्टर्फेरोन गामा चाचणी मध्ये १८ टक्के सकारात्मकता आढळली. पीसीआर चाचणीव्दारे एकूण ३.५८ टक्के जनावरांमध्ये सकारात्मकता आढळली. तसेच रिअल टाइम पीसीआर चाचणीव्दारे सर्वात जास्त ५.६ टक्के जनावरे बाधित असल्याचे निदर्शनास आले. एलिसा तपासणी मध्ये प्रतीपिंडाच्या पडताळणीसाठी PEP A, PEP Q, आणि DAP E या प्रतिजनांचा समावेश करून त्याप्रती गोवंशीय जनावरांच्या नमुन्यात अनुक्रमे ३.५८ टक्के ,४.३ टक्के ४.३ टक्के सकारात्मकता निदर्शनात आली. सदर अभ्यासावरुन गोवंशीय क्षयरोगाच्या अचुक निदानासाठी पारंपरिक चाचण्यासोबत आण्विक निदान करणे गरजेचे असल्याचे समजून येते.