

A cross-sectional study on prevalence of bovine tuberculosis in Indian and crossbred cattle in Gangetic delta region of West Bengal, India

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Abstract

Aim: The aim of this study was to investigate the incidence of bovine tuberculosis (BTB), an old chronic disease having zoonotic potential, covering four districts in Gangetic delta region of West Bengal, India, and to find the prevalence in organized as well as backyard herds and variation in relation to their age, sex, and breeds.

Methods: The incidence of BTB in exotic and indigenous breeds of cattle (n=173) of various age groups was investigated employing tuberculin (single intradermal tuberculin and comparative cervical tuberculin) tests and gamma interferon assay. Further, milk samples (n=96) from milching animals and antemortem (n=519) samples (nasal swab, buccal swab, and aspirates from pre-scapular lymph nodes) were also screened employing bacteriological and molecular techniques.

Results: In total, 36 (25.4%) animals from organized and one (3.2%) from backyard farming sector were found positive to BTB. Polymerase chain reaction (PCR) of milk samples based on 16S rRNA amplified the 1030 bp band in four samples indicating them belonging to genus *Mycobacterium*. Species-specific primers used to differentiate between *Mycobacterium bovis* and *M. tuberculosis* confirmed the presence of *M. bovis*. 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 (p<0.05) among cows/heifers (25.8%) compared to bulls/bullocks (7.3%). Although BTB-positive cattle were detected in all the age groups, no statistical difference (p=0.779) was found among them.

Conclusion: The findings indicate a higher prevalence of BTB in exotic crossbred animals in Gangetic delta and variation in breed susceptibility, thereby suggesting an urgent review of the present policy on adopting national crossbreeding program and implementation of "One Health" approach.

Keywords: bovine tuberculosis, cattle, India, prevalence, West Bengal.

Introduction

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is a highly prevalent infectious disease of cattle, buffaloes, and many wild species worldwide [1,2]. Due to its chronic and progressive nature, this disease causes a significant economic impact accounting 10-25% loss in productive efficiency in dairy cattle, often resulting in high morbidity in animals. The World Health Organization has also classified BTB as one of the seven neglected zoonotic diseases having a potential to infect human beings, either by consuming raw milk, meat, and their products [3] or inhaling infective droplets or direct contact with infected animals [4]. Being zoonotic in

nature, this disease also poses a significant public health problem, particularly in immunocompromised persons [5]. People living in rural areas are at higher risk as they usually share the same microenvironment and dwelling premises with animals. Although a direct correlation between *M. bovis* infection in cattle and the disease in human population has been well documented in developed countries, very little information is available from developing countries [6]. As per the latest reports available, 149,000 new human cases of zoonotic tuberculosis were detected globally in 2015, of which 13,400 deaths were due to *M. bovis* [4]. However, the above data may not truly represent the epidemiological status of human tuberculosis caused by *M. bovis* in India due to lack of effective disease surveillance, non-availability of more sensitive and specific diagnostic test, and effective reporting systems.

The most effective strategy for the control of BTB requires identification and removal of the infected animal from the herd. BTB is usually diagnosed based on delayed hypersensitivity reactions using various tuberculin tests such as single intradermal test (SIT),

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comparative intradermal, short thermal, and stormont tests. However, no single test can diagnose BTB at all stages of infection, as these tests have limitations on sensitivity and specificity [7]. To overcome this problem, animals are tested individually using comparative cervical tuberculin test (CTT) and gamma interferon (γ -IFN) assay alongside SIT to increase diagnostic sensitivity [8].

Keeping this in view, a cross-sectional study was conducted to find the most suitable antemortem tests out of tuberculin tests (SIT and CTT) and γ -IFN assay for screening of BTB in individual cattle and the variation in prevalence rate in relation to age, breed, and sex, covering four districts in Gangetic delta region of West Bengal.

Materials and Methods

Ethical approval

During this study, all animal handling procedures were performed following approved guidelines as laid down by the Institute Animal Ethics Committee.

Study population

A cross-sectional study was carried out to find the prevalence of BTB in cattle covering four districts, viz., Burdwan, Hooghly, Nadia, and South 24 Parganas in Gangetic delta region of West Bengal state.

A total of 173 (41 male and 132 female) cattle of different breeds in three age groups (1-5, 6-10 and 11-15 years) were selected for the study purpose. Other than non-descript animals (ND), the crossbred animals were Jersey cross (JC), Holstein-Friesian cross (HFC), Red Sindhi cross (RSC), Gir cross (GC), Sahiwal cross (SC), Jersey-Tharparkar cross (JTH), and Sahiwal-Tharparkar cross (STH). Of the 173 animals, 142 were from five organized farms and 31 were from four backyard farming sectors.

In vivo single and comparative CTTs

The SIT and CTT tests were conducted in animals (n=173) following the protocol of the World Organization of Animal Health [9]. The tuberculin protein purified derivative (PPD) kits were procured from Prionics, the Netherlands. After measurement of the skin-fold thickness, bovine PPD tuberculin (0.1 ml containing 3000 IU/dose) and avian PPD (0.1 ml containing 2500 IU/dose) were injected intradermally in the center of the middle-third region in both sides of the neck avoiding the fine superficial capillaries. The skin-fold thickness of each injection site was re-measured after 72 h (± 6 h) by vernier calipers to record the increment of skin-fold thickness, if any. The test results of the intradermal tuberculin tests were interpreted as per the OIE standards [9].

In vitro γ -IFN test

The release of γ -IFN cytokine in the blood of the cattle was determined by γ -IFN assay using commercially available bovine γ -IFN ELISA kit (BOVIGAM® assay) (Prionics, Switzerland) following the protocol of Wood and Jones [10]. Briefly, heparinized whole

blood collected from the individual animal was stimulated with bovine and avian PPD antigens along with phosphate-buffered saline (PBS, pH 7.2) as control and incubated at 37°C for 18-24 h. The γ -IFN present in the plasma supernatants was quantified in a sandwich ELISA following manufacturer's instructions. The blood plasma samples with stimulation by bovine PPD and having an OD value (≥ 0.100) above that of avian PPD and nil antigen (PBS) indicated the presence of *M. bovis* infection.

The tuberculin tests (SIT and CTT) and γ -IFN assay in cattle were done simultaneously to ensure the reliability of the results as described elsewhere [11]. Based on the test results, the cattle positive to either SIT and γ -IFN assay or CTT and γ -IFN assay was interpreted as positive to BTB.

Collection and processing of samples

Antemortem samples (n=519) such as nasal swab, buccal swab, and aspirates from pre-scapular lymph node(s) were collected from all the animals (n=173). Besides, milk samples (n=96) were also collected from all the quarters of the udder of milching cattle. The samples were brought to the laboratory under cold chain for screening through bacteriological and molecular techniques.

Cultural isolation of *Mycobacterium* spp.

For cultural isolation of *Mycobacterium* spp., the antemortem samples were processed and decontaminated both by Petroff's sodium hydroxide method [12] and sulfuric acid method [13]. The processed samples were centrifuged at 1300x g for 15 min, and the sediments were suspended in 3 ml sterile physiological saline solution for inoculation. Before use, the slants were flooded with 2 ml sterile physiological saline solution containing 5000 IU of penicillin and 1 mg of cycloheximide and allowed to stand for 10 min till excess fluid was poured off. All the samples were inoculated into Lowenstein-Jensen (LJ) with 0.5% (w/v) sodium pyruvate (LJ-P) and LJ with 2% glycerol (LJ-G) slants in duplicates. The slants were incubated at 37°C in an upright position up to 8 weeks and routinely observed for the growth of mycobacteria.

Ziehl-Neelsen (ZN) staining

For primary screening of the cultures grown on LJ media, ZN staining method was performed [14]. The smears were stained with carbol fuchsin and counter stained with 0.1% methylene blue and finally washed with water, dried, and observed under oil immersion (100X) lens.

Genomic DNA extraction

HiPurA™ multi-sample DNA purification kit (HiMedia, India) was used for the extraction of genomic DNA from antemortem samples, cultural isolates (about 5×10^6 cells) as well as reference strains of mycobacteria (*M. bovis* AN5 and *Mycobacterium tuberculosis* H37Rv) in LJ media as per manufacturer's instructions. The genomic DNA was preserved

in 100 µl of elution buffer and stored at -80°C until further use.

Identification and molecular typing of *Mycobacterium* spp.

The identification of *Mycobacterium* spp. in cultural isolates as well as tissue, swab, and milk samples was done by PCR following the protocol [15]. Further, amplification of a region of difference 9 (RD9) and 500 bp fragments were also done to differentiate the presence of *M. tuberculosis* and *M. bovis* using the primers and thermal cycling conditions following the protocol of Das *et al.* [16]. The reference strains (*M. bovis* AN5 and *M. tuberculosis* H37Rv) were also run along with the samples for confirmation. A detailed account of the oligonucleotide primers used and PCR conditions maintained for confirmation of *Mycobacterium* spp. and *M. tuberculosis* complex is presented in Table-1 [15,16]. Amplified products were visualized and photographed under gel documentation system after electrophoresis in 1.5% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) under 100 V for 1 h for bands of DNA of appropriate size.

Statistical analysis

Statistical analysis (descriptive and qualitative) was done using the Statistical Package for Social Statistics program version 20. Correlation coefficient, Chi-square, and Fisher's exact tests were used to establish relationships between the variables. The results were then presented using tables, besides narrative descriptions.

Results

All the antemortem samples (n=519), namely, nasal swab, buccal swab, and aspirates from pre-scapular lymph nodes, collected from 173 cattle from organized and backyard farming system, tested negative for BTB by ZN staining as well as cultural isolation. However, of 96 milk samples, sediments from three were found positive by ZN staining, whereas two milk samples, processed by Petroff's sodium hydroxide method, were found positive for characteristic dysgonic, non-pigmented, and off-white mycobacterial colonies on LJ-P. The cultural isolates checked by ZN staining were also found positive.

DNA isolated from tissue samples as well as reference strains of mycobacteria (*M. bovis* AN5, *M. tuberculosis* H37Rv) was amplified by PCR based on 16S rRNA, 500 bp fragment, and RD9 region. All antemortem samples including nasal swab, buccal swab, and aspirates from pre-scapular lymph nodes tested negative for BTB. The processing of milk samples (n=96) by PCR amplified the 1030 bp band in 4 milk samples, indicating that they belong to genus *Mycobacterium*. Further, the samples tested for species-level identification using specific primers yielded a 500 bp fragment (Figure-1) and 206 bp product (Figure-2), while none were positive for 333 bp for RD9 region, thereby confirming these as *M. bovis*.

The screening of cattle by SIT, CTT, and γ -IFN assay for the diagnosis of BTB is presented in Table-2. Of 173 cattle tested by both SIT and CTT on an individual basis, 108 (62.4%) cattle tested positive. On the other hand, test results of 65 (37.6%) cattle were found as doubtful and negative by SIT in comparison to 87 (50.3%) positive and 86 (49.7%) under

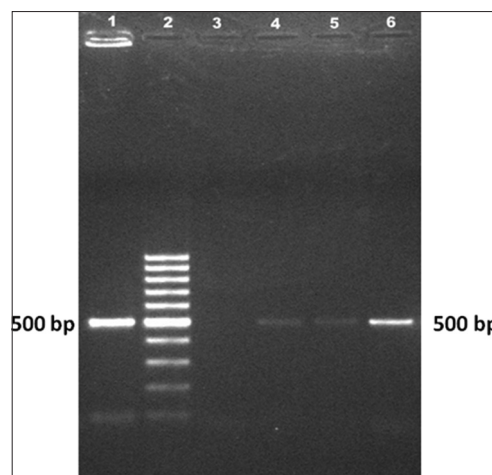


Figure-1: 1.5% agarose gel stained with ethidium bromide showing amplification of 500 bp fragment (*Mycobacterium bovis* specific) from positive milk samples indicating the presence of *M. bovis*. Lane 1: *M. bovis* AN5 (positive control); Lane 2. 100 bp Marker; Lane 3. Negative control; Lane 4. ND/1639; Lane 5. ND/1935; Lane 6. ND/1754.

Table-1: Details of primers and thermal cycling condition for identification of *Mycobacterium* species and *Mycobacterium tuberculosis* complex.

Genes	Primer name	Primer (5'-3') sequence	Amplicon size (bp)	PCR condition	References
16S rRNA	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	1030	94°C 5 min, 39× (94°C 30 s, 62°C 3 min, 75°C 3 min) 75°C 7 min	[15]
RD9	MYCGEN-R RD9F	TGCACACAGGCCACAAGGGA GTGTAGGTCAGCCCCATCC	333/206	95°C 5 min, 35× (95°C 1 min, 54°C 1 min, 72°C 1 min) 72°C 10 min	[16]
500 bp fragment	RD9I RD9R JBF JBR	CAATGTTTGTTCGCTGC GCTACCCTCGACCAAGTGTT TCGTCCGCTGATGCAAGTGC CGTCCGCTGACCTCAAGAAG	500		

inconclusive and negative category by CTT. The test results of γ -IFN assay conducted in samples from 173 cattle interpreted 41 (23.7%) animals positive and 132 (76.3%) as negative. The samples from cattle positive to γ -IFN assay were also positive to SIT, except four (4) interpreted as inconclusive. Based on the criteria of case definition, a total of 37 (21.4%) cattle were interpreted as true positive (Table-2), and of them, 36 (25.4%) were from organized farms and one (3.2%) from backyard farming system. The correlation between positive and negative results of

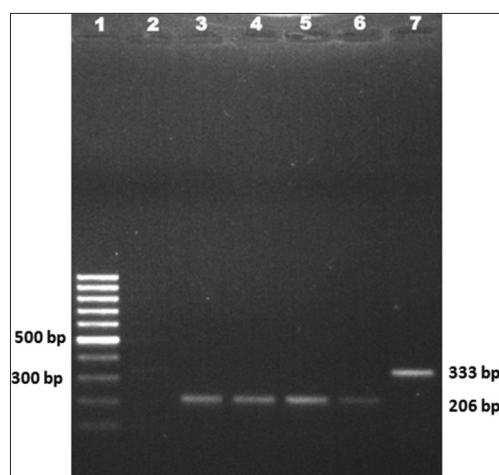


Figure-2: 1.5% agarose gel stained with ethidium bromide showing the amplification of RD9 region (206-bp). Lane 1: 100 bp DNA marker; Lane 2: Negative control; Lane 3: Positive control (*Mycobacterium bovis* AN5); Lane 4: ND/1639; Lane 5: ND/1935; Lane 6: ND/1754; Lane 7: Positive control (*Mycobacterium tuberculosis* H₃₇R_v).

Table-2: Results of single intradermal tuberculin test, comparative cervical tuberculin test, and gamma interferon assay for the diagnosis of bovine tuberculosis.

Tests	Final diagnosis		Total n=173 (%)	p value
	Negative n=136 (%)	Positive n=37 (%)		
Single intradermal tuberculin test				
Positive	72 (52.9)	36 (97.3)	108 (62.4)	<0.001*
Doubtful/negative	64 (47.1)	1 (2.7)	65 (37.6)	
Total	136 (78.6)	37 (21.4)#	173 (100)	
Comparative cervical tuberculin test				
Positive	53 (39.0)	34 (91.9)	87 (50.3)	<0.001*
Doubtful/negative	83 (61.1)	3 (8.1)	86 (49.7)	
Gamma interferon assay				
Positive	4 (2.9)	37 (100)	41 (23.7)	<0.001*
Negative	132 (97.1)	0	132 (76.3)	

#Bovine tuberculosis positive from organized farms 36 (25.4%) and 1 (3.2%) from backyard farm. *Highly significant ($p < 0.001$).

Table-3: Prevalence of bovine tuberculosis in different breeds of cattle in West Bengal.

Cattle breed	Final diagnosis		Total (%)	p value
	Negative (%)	Positive (%)		
Exotic cross (JC, HFC, and JTH)	51 (65.4)	27 (34.6)	78 (100)	<0.001*
Indian indigenous (SC, ND, GC, RSC, and STH)	85 (89.5)	10 (10.5)	95 (100)	
Total	136 (78.6)	37 (21.4)	173 (100)	

*Significant, Chi-square test. JC=Jersey cross, HFC=Holstein-Friesian cross, RSC=Red Sindhi cross, GC=Gir cross, SC=Sahiwal cross, JTH=Jersey-Tharparkar cross, STH=Sahiwal-Tharparkar cross

SIT, CTT, and γ -IFN assay was found to be highly significant ($p \leq 0.001$). The analysis of data on the prevalence of BTB in exotic cross and indigenous breeds of cattle is presented in Table-3.

In exotic cross (JC, HFC, and JTH), the prevalence of BTB (34.6%) was found to be significantly higher ($p < 0.001$) compared to indigenous cattle (SC, ND, GC, RSC, and STH) (10.5%). On further analysis of data on the prevalence of BTB in crossbred cattle (Table-4), it was found that JC (43.9%) and JTH (44.4%) were more susceptible compared to HFC (17.9%). It was further observed that the rate of BTB prevalence in organized farms ranged from 18 to 61% (data not shown).

Gender-wise distribution of animals and their variation with respect to the prevalence of BTB is presented in Table-5. On data analysis, it was found to be statistically significant ($p < 0.05$) with higher positivity among cows/heifers (25.8%) compared to bulls/bull-ocks (7.3%).

Further, the incidence of BTB in cattle of various age groups (1–5, 6–10, and 11–15 years) was studied and presented in Table-6. Although BTB-positive cattle were detected in all age groups, there was no significant statistical variation ($p = 0.779$) among them.

Discussion

The present study was carried out to find the prevalence of BTB using different tests in different cattle breeds (indigenous or native cattle and cross-breds) in organized and backyard farms covering four districts in Gangetic delta of West Bengal. In total, 37

Table-4: Prevalence of bovine tuberculosis in different exotic crossbred cattle in West Bengal.

Cattle breed	Final Diagnosis		Total (%)	p value
	Negative (%)	Positive (%)		
HFC	23 (82.1)	5 (17.9)	28 (100)	<0.001*
JC	23 (56.1)	18 (43.9)	41 (100)	
JTH	5 (55.6)	4 (44.4)	9 (100)	
Total	51 (65.4)	27 (34.6)	78 (100)	

*Significant, Chi-square test. HFC: Holstein-Friesian cross, JC: Jersey cross, JTH: Jersey-Tharparkar cross

Table-5: Gender-wise prevalence of bovine tuberculosis in cattle in West Bengal.

Gender	Final diagnosis		Total (%)	p value
	Negative (%)	Positive (%)		
Female	98 (74.2)	34 (25.8)	132 (100)	<0.012*
Male	38 (92.7)	3 (7.3)	41 (100)	
Total	136 (78.6)	37 (21.4)	173 (100)	

*Significant, Chi-square test

Table-6: Prevalence of bovine tuberculosis in cattle of different age groups.

Age in years	Final diagnosis		Total (%)	p value
	Negative (%)	Positive (%)		
1-5	64 (81.0)	15 (19.0)	79 (100)	0.779*
6-10	62 (76.5)	19 (23.5)	81 (100)	
11-15	10 (76.9)	3 (23.1)	13 (100)	
Total	136 (78.6)	37 (21.4)	173 (100)	

*Non-significant, Fisher's Exact test

animals were found positive to BTB, of which 36 were from organized farms and one from backyard farming system with a prevalence rate of 25.4% and 3.2%, respectively. In India, several workers have reported a high prevalence of BTB in organized farms. In a comparative study, Mukherjee [17] reported a prevalence of tuberculosis in two dairy herds at 15.76% (Northern India) and 0.65–1.85% (Western India), while Thakur *et al.* [18] recorded 14.31% (overall animal prevalence) and 16.67% (farm prevalence) in Himachal Pradesh. In southern states of India such as Tamil Nadu and Karnataka, the rate of prevalence was 34.58% and 30–35%, respectively [19]. Such a wide variation in prevalence of BTB could be due to breed type, screening tests used, management practices followed in the farming system, contact between animals of the same or different herds sharing common facilities, and grazing areas. [20].

The intradermal tuberculin test is recognized by both the OIE and the European Commission as a primary screening test for detection of tuberculosis in cattle [8]. However, confirmatory test for BTB is still a challenge since available diagnostic tools have limitations on sensitivity and specificity and no single test can diagnose BTB at all stages of infection [7]. In the present study, positivity of cattle to BTB using different screening tests (SIT, CTT, and γ -IFN assay) ranged from 23.7% to 62.4%. Various studies have been carried out to evaluate the sensitivity and specificity of tuberculin tests (SIT and CTT) in cattle using different antigens under various epidemiological

conditions [21]. Aranaz *et al.* [22] suggested that infection with *M. avium* subsp. *paratuberculosis* is regarded as interference factor in the diagnosis of bovine TB using PPDs. In general, the CTT test shows a higher specificity than the SIT test in detriment of the sensitivity, since it can distinguish animals infected with non-tuberculous mycobacteria or those vaccinated against paratuberculosis [21,22]. The variation in test results using SIT, CTT, and γ -IFN assays might be due to exposure of infected animals to other sensitizing mycobacteria [23] or prevalence of *M. avium* subsp. *paratuberculosis* as reported recently in the study area by this group of authors [24]. However, the sensitivity and specificity were found to be higher in γ -IFN assay than both the intradermal tests (SIT and CTT) which are in corroboration with the previous studies [25,26].

M. tuberculosis has been reported in cattle by several researchers in India [27]. In fact, RD9 region is present in *M. tuberculosis*, while this region is absent in *M. bovis* and *M. bovis* BCG strains [16]. PCR amplification of some RD can distinguish between *M. bovis* and *M. tuberculosis* [28]. Besides, 500 bp fragment has also been reported to be *M. bovis* specific, located at 3' end of the putative gene called RvD1-Rv2031c [29]. Therefore, in this study, it was presumed that either *M. tuberculosis* or *M. bovis* would be present in milk and other antemortem samples. Of 96 milk samples, two were positive for cultural isolation, whereas sediments from four milk samples were confirmed by 206 bp and 500 bp fragments in PCR assay for the presence of *M. bovis*. However, the reference strain

of *M. tuberculosis* H37Rv yielded a product of 333 bp which showed the presence of RD9 region. From this, it is concluded that screening of raw milk samples simultaneously by PCR and cultural isolation can be performed to confirm BTB in cows.

The prevalence of BTB was significantly higher in organized farms with more exotic or crossbred animals (JC, HFC, and JTC) compared to indigenous breeds. The findings on breed susceptibility to BTB corroborates with the observations reported earlier by different researchers. Sharma *et al.* [30] concluded that Indian breeds were less affected by BTB than pure European breeds such as Jersey, Holstein, Friesians, and Brown–Swiss. Ameni *et al.* [1] demonstrated the breed level difference of cattle in susceptibility to BTB and indicated that *Bos indicus* cattle are more resistant than *B. taurus*. Thakur *et al.* [18] also reported higher positivity in JC and HFC breeds in Himachal Pradesh, India. The host genetics and its variation could be the reason for susceptibility to BTB in European breeds as reported by researchers [31].

The role of gender, if any, on incidences of BTB in cattle population was studied. A significant difference ($p=0.012$) in incidences of BTB between genders was noticed in female animals (25.8%) showing higher prevalence compared to male (7.3%). Higher positivity in a female may be due to their maintenance in the same herd for several years as also reported earlier [32]. On the other hand, the introduction of mechanization in agriculture may have lowered the incidences of BTB in bullocks in eastern India, as farmers no longer rear them for years.

The risk of tuberculosis is highly age-dependent in human beings. However, limited data are available on age-dependent risks in cattle. Brooks-Pollock *et al.* [33] reported age-specific incidences with highest rates of infection in cattle aged between 12 and 36 months. Thakur *et al.* [18] recorded higher incidence of BTB in cattle above 6 years age, whereas Moiane *et al.* [34] recorded that animals older than 4 years are more likely to be infected and vulnerable to BTB compared to young ones. In the present study, although incidences of BTB in cattle of various age groups (1–5, 6–10, and 11–15 years) varied, there was no statistically significant difference among them.

The key issues that prevent to understand the true burden of zoonotic BTB in an animal population are the absence of systematic surveillance and lack of standard laboratory techniques in diagnosing *M. bovis* infection. From this study, it can be deduced that prevalence of BTB is mostly restricted to organized farms with more incidences in female and crossbred European cattle breeds in Gangetic delta region of West Bengal. Based on the sensitivity, SIT is recommended for screening of herds, whereas for individual cattle, both CTT and γ -IFN assays may be considered as methods of choice to maximize the detection of TB-infected animals, as they exhibit

higher sensitivity, specificity, and accuracy. However, for confirmatory diagnosis, a battery of tests including CTT, γ -IFN assay, and screening of milk by PCR is recommended. As incidences of BTB are high in crossbred exotic cattle, highly productive indigenous germplasm may be considered for crossbreeding program. The detection of live zoonotic mycobacteria in milk is a cause of concern from a public health point of view. To overcome this problem, pasteurization of milk followed by boiling is strongly recommended before human consumption. To minimize the burden of BTB in cattle and its further spread to the human population, an effective national program involving “One Health” approach is required to combat this re-emerging disease.

Authors' Contributions

RD and PD conceived and designed the study. RD, AC and SB carried out sampling and laboratory analysis. PKN and SuB analyzed and interpreted the data. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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