Bovine Tuberculosis in Colombia; Findings from Histopathological, Microbiological and Molecular Tests

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Abstract

Tuberculosis is a zoonotic infectious disease, caused by bacteria of the tuberculosis complex. *Mycobacterium bovis* causes tuberculosis in water buffaloes (*Bubalus bubalis*), and it can also infect other domestic animals, wild fauna, and humans. The diagnosis of bovine tuberculosis (bTB) through intradermal injections is challenging, and to understand the behaviour of other diagnostic tests is crucial. The aim of this research is to analyse three diagnostic tests against bTB in water buffaloes with positive test DPP. Different diagnostic tests were tested in 50 buffaloes diagnosed with bTB Cervical Comparative Test, from the Colombian lower tropic. Lesions compatible with bTB in 26 buffaloes with a positive DPP ; Four out of 2 samples of *Mycobacterium bovis* in DPP-positive buffaloes were isolated and confirmed positively in tissues using PCR-HRM, three buffaloes showed granulomatous lesions in histological analyses with positive microbiological isolation; 17 DPP-positive buffaloes had a positive PCR-HRM test and nine of these buffaloes showed no histological findings compatible with bTB, leading to purely molecular diagnosis. Evidence in histological, microbiological, and molecular findings in DPP is positive for the water buffaloes. None of the complementary tests showed 100% concordance with the intradermal results obtained with the Cervical Comparative Test for bTB.

Introduction

Bovine tuberculosis is a globally distributed chronic contagious disease caused by the etiological agent *Mycobacterium bovis*, which can affect livestock, wild animals, and even humans ¹⁻³. Tuberculosis is an infectious disease with high zoonotic potential, with a high occupational risk and it is considered the biggest hindrance for the production of meat and dairy products, representing significant economic losses in several systems of production ^{4,5}. The possibility of human infection with *M. bovis* cannot be ignored, as it has been described in developed countries that 10 to 20% of human tuberculosis cases are caused by *M. bovis* ^{6,7}. Studies carried out by the Colombian National Institute of Health ("Instituto Nacional de Salud – INS") in 2011 identified the circulation of *M. bovis* in five Colombian departments, as well as a positive case of human tuberculosis caused by *M. bovis* in 2012 ⁸.

Colombia counts with a "National program of control and eradication of bTB, and certification of bTB-free lands" coordinated by the Colombian Agriculture and Livestock Institute ("Instituto Colombiano Agropecuario – ICA"). Currently, it is not clearly known the scale of the disease attributed to *M. bovis*. It is estimated that the prevalence of bTB is below 1% and that the disease is delimited according to the data, to areas located in the Antioquia, Boyacá, Cundinamarca, Cesar, Magdalena and Guajira departments, with 36 farms in sanitation during 2011. Nevertheless, the presence of this bacterium has not been verified in the rest of the country ⁹.

Several factors have limited an effective diagnosis of bTB in the buffalo population, including the lack of data about the prevalence and incidence of the disease. In 2019, there was a report about a focus of tuberculosis in buffaloes, a situation that broke the paradigm about the resistance of these animals to tuberculosis. Moreover, the role of non-typical mycobacteria in the epidemiology of tuberculosis in buffaloes is still poorly studied.

bTB can be diagnosed using direct or indirect methods, including the detection and identification of the etiological agent ^{4,10,11}. Tuberculin, histopathological and bacteriological tests are commonly used for the diagnosis of tuberculosis in bovine and buffalo populations. Tuberculosis in water buffaloes (*Bubalus bubalis*) has been traditionally diagnosed through the cervical comparative tuberculin test. However, the lack of specific parameters for tuberculin tests in buffaloes difficult the correct analysis of the test, given that it uses classification criteria targeted for bovines and not for buffaloes.

The absence of clinical signs in buffaloes, the difficulty of isolation of *M. bovis* from living animals, and the low antibody titers during the initial phase of the infection, limit the effectiveness of the clinical, serological, and bacteriological diagnosis. Alternative diagnostic methods have been proposed, such as serological tests via ELISA and molecular diagnostic tests using polymerase chain reaction (PCR) with different variants ^{12,13}.

The diagnosis of tuberculosis represents a big challenge for the early detection of the disease in infected animals. The preclinical stages of tuberculosis can be diagnosed in living animals using techniques measuring cellular immunity, such as the intradermal injection of tuberculin, the IFN- γ test, and the lymphocyte transformation test, besides some serologic tests that evaluate the humoral immune response, such as ELISA, microbiological cultures and molecular tests. The relation between sensitivity and specificity of each test references the key for the efficiency and accuracy of these diagnostic tests, which indicates the likelihood of false positives and false negatives results for bTB. None of the currently available tests for the diagnosis of bTB allows an exact determination of the state of the infection in the species ⁹. Thus, the development of new diagnostic tests for chronic infectious diseases, such as tuberculosis, can contribute to performing faster and more reliable diagnosis ^{12,13}.

Other direct methods of diagnosis include Ziehl-Neelsen staining ¹⁴, that allows the differentiation of acid-fast microorganisms such as mycobacteria, and histopathology that determines microscopically the presence of granulomatous lesions compatible with tuberculosis and the infiltration of different types of mononuclear cells ^{15,16}. Furthermore, the IFN- γ test measures the release of this cytokine from lymphocytes in whole blood in animals with suspected tuberculosis diagnosed with bovine and avian DPP ¹⁷, and with the microbiological culture, it is possible to isolate *M. bovis* from tissue biopsies coming from culled infected animals ^{14,18}. The PCR test as a molecular diagnostic approach can provide fast and reliable results as it can specifically, detect the presence of *M. bovis* through the detection of its genomic material ¹⁹⁻²¹. There exist different variants of real-time PCR, including the high-resolution melting PCR (PCR-HRM), which allows identifying nucleotide differences in the amplicon that can lead

to variations in melting curves, thus leading to the simultaneous identification and diagnosis of different mycobacteria in a single reaction. The importance of this methodology lies in its sensitivity and specificity, and it does not require posterior analysis for the identification such as Restriction fragment length polymorphism (RFLP) or sequencing, thus being cheaper and quicker.

Even though that bTB in Colombia is a disease of official control and mandatory statement, currently it is now clearly known the magnitude of the disease caused by *M. bovis*. Moreover, the diagnostic tests established by the current legislation generate uncertainty regarding the sensitivity and specificity of the results. The aim of this research was to explore histopathological, microbiological, and molecular diagnoses in culled water buffaloes that had a positive diagnosis of bTB through DPP.

Methods

Data collection

Samples were obtained from water buffaloes (*Bubalis bubalis*) of different breeds coming from the lower tropic of the municipalities of Planeta Rica and Ayapel, in the Colombian department of Córdoba. These buffaloes were positive for the comparative cervical test with DPP according to the National Program of Bovine Tuberculosis from the Colombian Agriculture and Livestock Institute ("Programa Nacional de Tuberculosis bovina, Instituto Colombiano Agropecuario – ICA"). Fifty young and adult buffaloes of both sexes destined for meat and milk production were recruited. All buffaloes were sacrificed according to the current sanitary regulations in Colombia²⁵, all buffaloes were culled according to the legal regulations of official controlled culling in Colombia. During the process, the animals were treated in accordance with the mandatory animal welfare standards of the beneficiary plant. These buffaloes were sacrificed using the stunning method using a captive bolt gun, generating a neurological blockage before cutting the carotid artery and jugular vein. Individual samples of one cm² from secondary mediastinal and retropharyngeal lymph nodes, as well as lung parenchyma, for further histopathologic, microbiologic, and molecular tests were collected. For microbiological culture, fragments from secondary lymph nodes and lung were combined, whereas for molecular diagnosis, samples for lung parenchyma and lymph nodes were analyzed. Histopathology samples were kept in buffered formalin and stored at -80°C.

Histopathologic diagnosis

Analysis of haematoxylin/eosin staining in the lung parenchyma (50) and mediastinal lymph nodes (50) were done for each buffalo. The tissues were evaluated by two independent pathologists through an identification protocol and classified with a score from 1 to 4, where scores of 1 and 2 represented lesions not compatible with bTB, and scores of 3 and 4 lesions were identified as lesions compatible with bTB (Arbitrary reference score). Two slides from each buffalo were taken in a biosafety level 3 facility for Ziehl-Neelsen stainings using a standardized protocol³⁰. The reading of the slides was performed in duplicate by different pathologists using an Eclipse E200 microscope (Nikon, Tokyo, Japan).

Microbiological culture

The microbiological isolation was performed in controlled conditions in a biosafety level 3 laboratory. We cultured combined lung and lymph node samples in Middlebrook 7H11 agar (Becton Dickinson®) for eight weeks. The samples were decontaminated using 4% NaOH and neutralized with a pH 7.0 buffer. Cultures were checked each week for a total period of two months. Positive samples were evaluated using a phenotypical identification protocol for mycobacteria, as well as a biochemical characterization via catalase and nitrites. Additionally, antibiotic sensitivity tests were carried out for *Mycobacterium bovis* against Pyrazinamide (PZA), Isoniazid (INH) and Rifampicin (RIF).

DNA extraction and PCR-HRM analysis

Nucleic acid extraction from lymph nodes and lungs were performed following manufacturer instructions (GeneJET™ Genomic DNA Purification Kit, Thermo Fisher Scientific, Waltham, MA, USA). The concentration and purity of the genomic material was measured using a Nanodrop® ND1000 (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the DNA was verified using a 1% agarose gel electrophoresis (Agarose I, Amresco®, Ohio, USA) with 1.8% GelRed nucleic acid stain (Biotium®, Fremont, CA, USA), ran at 110V for 50 minutes. Gels were analyzed in the BioRad Universal Hood II Gel Doc System (Laboratory Segregate, Milan, Italy). DNA samples were then aliquoted and stored at -80°C.

The PCRU-HRM assays were performed using the Type-it HRM PCR Kit (Qiagen), using a set of previously described primers for 16S rRNA: F3_Avj CTG GCT CAG GAC GAA CG and B3_Avj GCC CAT CCC ACA CGT CCG C³¹. The PCR reaction consisted of 1X HRM master mix (HotStarTaq Plus DNA polymerase, HRM PCR buffer with EvaGreen dye, Q-solution and dNTPs), 0.4µM of each primer, 10 – 50 ng/µl of DNA, and RNase-free water. All the PCR assays were done in a LightCycler® 96 real time PCR system (Roche diagnostics, Roche Molecular Systems, Inc Vedbaek, Denmark) using the following thermal profile: 1 cycle at 95°C for 600 seconds; 45 cycles consisting of a denaturing step at 95°C for 10 seconds followed by an annealing-extension step at 62°C for 30 seconds; temperature ramp from 80°C to 94°C with a temperature increment of 0.1°C each 2 seconds.

Data analysis

Frequency tables were performed using Excel® with the collected data, whereas the molecular biology results were analyzed with the software LightCycler® 96 real time PCR system version 1.05.

Legal and ethical considerations

This research was performed thanks to the authorization of ICA, Colombian Institute for Surveillance of Drugs and Food (“Instituto de Vigilancia de Medicamentos y Alimentos de Colombia – INVIMA”), the Quality direction and General Management of Medellín Livestock Centre, and the farms proprietaries of the sacrificed buffaloes unionized in the Colombian Association of Buffalo Breeders (Asociación Colombiana de Criaderos de Búfalos – ASOBÚFALOS). This project was approved by act number 14 of June 2015 from the Institutional Committee for Care and Use of Animals of Universidad CES (CICUA) in Medellín, Colombia. The CICUA committee assigned the code 99 to the research, and demanded the continuous reporting of adverse events, failures in the protocols described in the methodology, or failures in the application of good practices in the exercise of scientific activity. None of the above situations were reported. All procedures were carried out in accordance with current biosafety regulations for work with mycobacteria authorized by the ICA.

Results

Histopathology examination

Fifty-two percent (26 out of 50 buffalos) of the DPP-positive buffaloes showed lesions suggestive of tuberculosis. Nine buffaloes had grade-4 histological lesions, characterized by granulomas in lungs, liver, and secondary lymph nodes with the presence of giant multinucleated cells, besides interstitial pneumonia and lymphocytic pleuritis. Seventeen buffaloes had grade-3 lesions, with the presence of lymphohistiocytic fibrosing pleuritis, paracortical lymphoid hyperplasia, acute interstitial pneumonia, SLS (emphysema), active chronic lymphadenitis, histiocytic lymphadenitis, and lymphocytic pleuritis. Nevertheless, 48% (24 out of 50) of buffaloes with positive DPP did not show lesions compatible with tuberculosis, i.e. they showed grade 1 and 2 lesions. The main histological findings in these negative buffaloes were acute and chronic interstitial pneumonia, lymphoma, chronic lymphadenitis, hyperplastic lymphadenitis, depletion, non-suppurative periportal hepatitis, and reticulo-histiocytic hyperplasia (Figure 1).

Ziehl-Neelsen staining

The Ziehl-Neelsen analyses revealed the presence of acid-fast bacilli in 21 and 16 lung and secondary lymph node samples, respectively. Eleven buffaloes with histological findings compatible with bTB had the presence of acid-fast bacilli, whereas 18 buffaloes without histological findings compatible with bTB had the presence of these microorganisms in Ziehl-Neelsen analysis. Nonetheless, 15 buffaloes with histological findings compatible with bTB did not have evidence of acid-fast bacilli, as well as six animals without such histological findings (Table 1).

Bacterial isolation and antibiotic sensitivity tests

From the 50 cultured samples, four lung and lymph node samples showed positive signs of microbiological growth. The samples from positive cultures were identified with biochemical tests and the sensitivity to antibiotics tested. The bacteriological isolates showed negative results for nitrites and catalase. Two samples from buffaloes with a positive culture had also the presence of acid-fast bacilli in Ziehl-Neelsen stainings, whereas three buffaloes with a histopathologic diagnosis of tuberculosis had positive cultures. Lastly, one buffalo with positive microbiological isolation did not show histological lesions compatible with bTB. Regarding the sensitivity tests, 100% of the isolations were resistant *in vitro* towards PZA, but susceptible towards INH and RIF (Table 2).

PCR-HRM molecular diagnosis

We analyzed 50 DNA samples obtained from bovine tissues positive for DPP. The PCR-HRM test was positive for bTB and paratuberculosis (PTB) (Figure 4). Seventeen samples showed positive results with the test and were typed as bTB by *M. bovis*. From these positive samples, 9 did not have any histological features compatible with bTB, which clearly shows that not all animals develop lesions, or that the appearance of such lesions depends in the time of evolution of the disease.

Two DPP-positive buffalos with histologic granulomatous lesions that had an *M. bovis* isolation showed a positive result by PCR-HRM in secondary lymph nodes and lung tissue. Nine samples of buffaloes positive for DPP without compatible histological findings and with an absence of acid-fast bacilli in Ziehl-Neelsen stainings showed positive results via PCR-HRM (Table 1).

Lastly, the PCR-HRM test allowed to differentiate DPP-positive buffaloes infected with *M. bovis* or *M. avium subspecies paratuberculosis* (Data not showed).

Discussion

Tuberculosis is an infectious disease that can affect all vertebrate animals⁴. It causes great economic losses in animal production, and it is cataloged as a serious zoonosis by the World Health Organization, representing a hindrance to the commercial exchange of animal products

between countries. Buffaloes are a species susceptible to bTB, and cases of this disease have been reported in Italy, Brazil, India, and Australia. This research demonstrates the presence of *Mycobacterium bovis* in buffaloes within production systems in Colombia.

The results of this research in buffaloes with a positive DPP test and without apparent clinical signs, with no alterations in their hemograms and chemical profiles (Data not showed), but with the presence of granulomatous lesions is consequent with the descriptions of chronic disease with absence of clinical signs in the firsts stages of the disease. Moreover, this constitutes an important occupational hazard for the people working with these animals, such as veterinaries, rural workers, ranchers, meat processing plant personnel, among others ^{1,8}.

A previous study performed in Brazil in 2005 with water buffaloes from different regions of the Pará state ⁴ was aimed to compare the sensitivity and specificity of different diagnostic methods for tuberculosis in buffaloes. The DPP test was compared with diagnostic procedures such as histopathologic tests and bacteriological isolation, showing that 4.65% of the tested buffaloes had positive DPP results, 2.98% had inconclusive results, and 92.36% presented negative results. This data is consequent with our results, as the proportion of buffaloes with a positive DPP test was 3.07% in the tested ranches included in our study. However, these results contrast with a study carried out in water buffaloes from Pakistan ¹³, where the general proportion of animals with a positive DPP test was 24.7%, indicated a lower concordance with the results found in our study.

Studies performed using diagnostic tests for tuberculosis in species such as buffaloes pretend to contribute to policies of control and eradication of the disease by sanitary authorities ¹³. Nevertheless, recent findings have shown that the applicability of the DPP test in buffaloes does not hold the same levels of sensitivity and specificity as in bovines. Our research describes different diagnostic methods used for the diagnosis of bTB in animals with a positive DPP test. Only 4% of the DPP-positive animals could not be verified with any of the other methods used. However, and according to Seva *et al.* ²², non-specific reactions can greatly interfere with the interpretation of the results of tuberculin tests in areas with low tuberculosis prevalence, which can hide outbreaks of the disease or erroneously diagnose healthy animals when these tests are not interpreted correctly. These authors attributed these reactions to *M. avium subsp. paratuberculosis* and other pathogenic saprophyte or facultative mycobacteria.

This research proved how the histopathologic diagnosis confirmed 52% of the samples. Moreover, the PCR-HRM test could verify 34% of the samples, suggesting a lower sensitivity than the aforementioned technique. The PCR-HRM method allowed to identify some samples positive for *M. avium subsp. paratuberculosis* in these buffaloes. The Ziehl-Neelsen staining showed a variable pattern in DPP-positive buffaloes.

The low sensitivity of the PCR-HRM can be associated with a decreased bacterial load of *M. bovis* in tissues at the time of sampling, with the selection of the tissue for DNA extraction, or with the degradation of the genetic material in some of the samples. However, the PCR-HRM, by being a molecular diagnostic technique that allows the detection of mutations in specific regions of the amplicon that can lead to the identification of species, represents a diagnostic method relatively simple and quick for the identification of different mycobacteria species ²³. The use of molecular tests to confirm DPP-positive buffaloes ¹⁹ has demonstrated a good sensitivity, as previous studies with buffaloes have established a sensitivity of 76% by comparing a nested PCR molecular approach with the use of the intradermal cervical comparative test, data that contrast with our results. The use of PCR-HRM in our research allowed the discrimination of positive samples for *M. avium subsp. paratuberculosis* in buffaloes with a positive cervical comparative test against *M. bovis* (Data not showed).

Other studies have employed molecular methods to diagnose tuberculosis in buffaloes and bovines using DNA samples from tissues ^{19,24}. A study using PCR tests against IS1081 using DNA from bovine lymph nodes showed a higher proportion of positive results compared to the proportion of bacteriological cultures using tissue samples ²⁴. These results are consequent with our findings, in which we found 34% of the samples positive for PCR-HRM and 8% with positive bacteriological isolation. These results highlight the relevance of using molecular techniques such as real-time PCR with HRM analysis to improve the diagnosis in tissues with granulomatous lesions, given that the detection of lesions compatible with tuberculosis caused by *M. bovis* in post-mortem animals can be confused with lesions caused by *Nocardia sp.*, *Corynebacterium sp.*, and some parasites, which can also cause granulomas ²⁵. The inability to isolate mycobacteria in 84.84% of the samples from animals with a reactive tuberculin test can be associated with a decreased bacterial load of *M. bovis* in lymph nodes at the time of sampling, or with difficulties inherent to the isolation method for these etiological agents.

The isolation of *Mycobacterium spp.* in clinical samples coming from tissues was managed. Using microbiological cultures, we isolated mycobacteria from clinical samples obtained from buffaloes in sacrifice positive for DPP.

These isolations will be considered in further studies in order to perform a comparative genomic analysis according to the specific geographic region where buffaloes inhabit in Colombia, as it has been shown that there are genetic variations in the molecular typification of *M. bovis* related to the geographical origin of the samples ²⁶. The behavior in the antimicrobial sensitivity tests showed that all the bacteria isolated were resistant to PZA, which is a characteristic of *M. bovis* and has been previously documented ²⁷. All the isolated strains in this study were susceptible to INH and RIF, which indicates an important finding given the description of *M. bovis* strains resistant to these antibiotics ²⁸.

This study demonstrated that 52% of the sacrificed buffaloes positive for DPP had histopathological lesions compatible with bTB (Figure 1, 2, 3), thus proving the usefulness of the histopathologic diagnosis for tuberculosis. Recent studies have shown that the histopathologic diagnosis can be paired with other complementary tests for the diagnosis of tuberculosis in both humans and animals ^{28,29}.

In our study, the results from the microbiological culture and PCR-HRM confirmed the diagnosis of tuberculosis in buffaloes with an absence of histological lesions compatible with tuberculosis, representing a complementary aid for the conventional diagnosis. The evidence about negative results in histopathology, which were confirmed by other methods can be explained due to the extraction of the tissue fragments for the analysis and disease progression.

Lastly, it is important to mention that international sanitary authorities recognize the DPP test as the method for the diagnosis of bTB *in vivo*. This hypersensitivity test has proven to be effective in programs of sanitary control, but when this test is compared with other diagnostic approaches, it shows limitations in sensitivity and specificity, highlighting the requirement for more studies in the buffalo species.

Declarations

Authors contributions

PCC: sampling and processing, article redaction. DIB: sampling for histopathology lectures. JRB: writing and article redaction. RVJ: molecular diagnosis, article writing. EMR: molecular diagnosis, article writing. JBG: follow-up of cases, sampling, article writing. RRG: sampling, molecular diagnosis, Ziehl-Neelsen reading, microbiological isolations, article writing.

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Conflict of interests The authors declare that no conflict of interest exists related to the publication of this research.

Availability of data and materials

Patient information are not public due to the confidential nature of the collected personal data, but are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

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Tables

Table 1. Results of the diagnostic tests with buffaloes positive for DPP.

	LN	Lung	Grade 4 HPSS§	Grade 3 HPSS§	Grade 2 HPSS§	Grade 1 HPSS§
Number of DPP-positive buffaloes (n)	50 (100%)	50 (100%)	9 (100%)	17(100%)	22(100%)	2(100%)
ZN Acid-fast present	16 (32%)	21 (42%)	5 (55.55%)	6 (35.29%)	16 (72.72%)	2 (100%)
ZN Acid-fast absent	34 (68%)	29 (58%)	4 (44.45%)	11 (64.71%)	6 (27.27%)	0
Positive microbiological culture	4 (8%)	4 (8%)*	3 (33.33%)	0	1(4.54%)	0
Negative microbiological culture	46 (92%)	46 (92%)*	6 (66.66%)	17(100%)	21(95.45%)	2(100%)
Positive HRM	17 (34)	17 (34)	2 (22.22%)	6(35.29%)	7(31.81%)	2(100%)
Negative HRM	33 (66)	33 (66)	7 (77.77%)	11(64.70%)	15 (68.18%)	0

*Samples cultured LN+ lung

§ Histopathology Score for bTB. Grades 1 and 2: histologic lesions not compatible with bTB. Grades 3 and 4: histologic lesions compatible with bTB.

Table 2. Results of the diagnostic tests, antibiotic sensitivity, and basic biochemical tests from buffaloes with positive DPP-test with positive microbiological culture.

Identification	Diagnostic tests				Antibiotic sensitivity tests				Biochemical tests	
	Ziehl-Neelsen	Pathology Score	HRM	Culture*	PZA pirazinamida	INH Isoniazida	RIF rifampicina	2 TCH Hidracide tiofeno-2-carboxílico	Nitrites	Catalase
DPP+35	Acid-fast present	2	+	+	Resistant	Susceptible	Susceptible	Susceptible	Negative	Negative
DPP+37	Acid-fast present	4	-	+	Resistant	Susceptible	Susceptible	Susceptible	Negative	Negative
DPP+45	Acid-fast absent	4	-	+	Resistant	Susceptible	Susceptible	Susceptible	Negative	Negative
DPP+43	Acid-fast absent	4	+	+	Resistant	Susceptible	Susceptible	Susceptible	Negative	Negative

* Cultured in Middlebrook 7H11, decontaminating with NaOH4% and neutralizing with a pH 7 buffer.

Figures

Figure 1. Macroscopic lesions of DPP-positive water buffalo. A. Retropharyngeal lymph node granuloma lesion. B. Granuloma lesion in the mediastinal lymph node. C. granulomatous lesion in lung tissue. D. granulomatous lesion in the liver.

Figure 1

See image above for figure legend.

Figure 2. Histologic lesions not compatible with tuberculosis in water buffaloes positive for DPP. A. Lung tissue with chronic interstitial pneumonia. B. Secondary lymph node with reticulo-histiocytic hyperplasia. C. Lung with active chronic interstitial pneumonia. D. Secondary lymph node with hyperplastic lymphadenitis. 10X magnification.

Figure 2

See image above for figure legend.

Figure 3. Histologic lesions compatible with tuberculosis in water buffaloes positive for DPP. A lung tissue with granulomatous lesion ,lymphocytic pleuritis and interstitial pneumonia, B lymph node with lymphoid hyperplasia. C. lung with granulomatous pneumonia. D. secondary lymph node with granulomatous lymph adenitis. Magnification A, B 10X. C, D 40X.

Figure 3

See image above for figure legend.

Figur 4. QPCR HRM results for mycobacterial positive controls. A melting curve for 1. *Mycobacterium tuberculosis*, 2. *Mycobacterium bovis*, 3. *Mycobacterium avium sub species paratuberculosis*. B fluorescence curve for positive mycobacterial controls.

Figure 4

See image above for figure legend.