Bovine Tuberculosis Testing in Colombia: Comparative Histopathological, Microbiological, and Molecular Biology Findings

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Abstract: *Introduction*: Bovine tuberculosis (bTB) is a zoonotic infectious disease present in Colombia, caused by *Mycobacterium bovis,* and causes tuberculosis in water buffalo (*Bubalus bubalis*). Diagnosis of bovine tuberculosis through the intradermal test is difficult; evaluating and understanding the behavior of other diagnostic tests is necessary.

Objective: To describe the behavior and results of different diagnostic methods for bovine tuberculosis in water buffalo positive for the Purifed Proteic Derivate (DPP) intradermal test.

Methodology: In water buffaloes positive for comparative cervical tuberculin test, different diagnostic methods were applied, described, and compared: Ziehl-Neelsen staining, microbiological culture, histopathological analysis, and PCR-HRM.

Results: Histopathological tests showed that 26 water buffalo positive for DPP (52%) had histological lesions compatible with bovine tuberculosis. 37% of the evaluated samples from tuberculin-positive Buffalo's lungs and secondary lymph nodes showed acid-alcohol-resistant bacillus with Ziehl-Neelsen staining. Four samples of *Mycobacterium bovis* from tuberculin-positive buffalo were isolated and identified, with two of these isolates confirmed from tissues with PCR-HRM, and three buffalo with microbiological isolates presented granulomatous lesions through histological analysis. Seventeen tuberculin-positive buffalo (34%) tested positive for real-time PCR HRM, and nine of these buffalo did not have histological lesions compatible with bTB and were confirmed with the molecular test.

Conclusion: Our results provide positive evidence of histological findings, microbiological isolation, and molecular diagnosis of tuberculin-positive water buffalo in the lowlands of Colombia. None of the complementary tests performed showed 100% concordance with the comparative cervical tuberculin test results for bTB.

Keywords: Microbiological isolation, *Bubalus bubalis*, DPP, Histopathology, *Mycobacterium bovis*, PCR HRM.

1. INTRODUCTION

Bovine tuberculosis is a chronic contagious disease with a global distribution caused by the etiological agent *Mycobacterium bovis*. This disease can affect livestock, wildlife, and even humans [1]. Tuberculosis is an infectious disease with significant zoonotic potential and high occupational risk. It is considered a major obstacle in meat and dairy production, leading to substantial economic losses in many production systems [2]. The possibility of human infection with *M. bovis* cannot be ignored. In developed countries, it has been recognized that 10% to 20% of human tuberculosis cases are caused by *M. bovis*, emphasizing the need for early diagnosis to control and limit its spread [3].

Studies conducted by the Colombian National Institute of Health (INS) in 2011 identified the circulation of *M. bovis* in cattle from five Colombian departments and confirmed a case of human tuberculosis caused by this species. However, the same institution did not find any cases of this disease in humans exposed to livestock production systems with cases of bovine tuberculosis [4]. *M. bovis* is an intracellular pathogen responsible for severe cases of pulmonary and extrapulmonary granulomas. The bacterium has pathogenic capabilities that can alter respiratory epithelial cells, leading to the formation of a chronic immune response affecting respiratory function. Additionally, the simple epithelial lining of the pulmonary alveolus is relatively susceptible to various virulence factors of the bacterium [5].

While Colombia has a national program for the control and eradication of bovine tuberculosis, along

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with the certification of disease-free farms managed by the Colombian Agricultural Institute (ICA) [6], the true extent of the disease attributed to *M. bovis* is not well understood. It is estimated that the prevalence of bovine tuberculosis is less than 1% and is mainly confined, as demonstrated, to specific areas in the departments of Antioquia, Boyacá, Cundinamarca, Cesar, Magdalena, and Guajira, where there are sanitation herds and local disease control programs in place.

Several factors have limited the effective diagnosis of tuberculosis in the buffalo population, including the lack of data on disease prevalence and incidence. Bovine tuberculosis can be diagnosed using direct and indirect methods, including the detection and identification of the etiological agent. Tuberculin tests, histopathological examinations, and bacteriological tests are commonly used for diagnosing tuberculosis in cattle and buffalo populations [7]. Tuberculosis in water buffalo (*Bubalus bubalis*) has traditionally been diagnosed using the comparative cervical tuberculin test [8]. However, the lack of specific parameters for the tuberculin test in buffalo makes accurate analysis challenging, as it uses classification criteria designed for cattle and not specifically for buffalo. In the absence of clinical signs in buffalo, the difficulty of isolating *M. bovis* from live animals, and low antibody titers during the early phase of infection, the effectiveness of clinical, bacteriological, and serological diagnostic tests is limited. Alternative diagnostic methods have been proposed, including serological ELISA tests and molecular diagnostic tests using polymerase chain reaction (PCR) [9].

Diagnosing tuberculosis presents a significant challenge for early detection in infected animals. Preclinical stages of tuberculosis can be diagnosed in live animals using tests that measure cell-mediated immunity, such as the intradermal injection of tuberculin, the IFN-γ test [10], and lymphocyte transformation tests. Additionally, some serological methods evaluate humoral immune responses, such as ELISA tests [11], microbiological culture methods, and molecular tests [9]. The key to the efficiency and accuracy of these diagnostic methods lies in the balance between sensitivity and specificity. This balance determines the occurrence of false positives and false negatives for bovine tuberculosis. None of the available methods for diagnosing tuberculosis in cattle allows for an exact determination of the infection status of the species [7]. Developing new diagnostic methods for chronic diseases like tuberculosis can contribute to faster and more reliable diagnoses [12].

Other direct diagnostic methods include Ziehl-Neelsen staining [13], which differentiates acid-fast microorganisms like mycobacteria and histopathology, which microscopically determines the presence of granulomatous lesions consistent with tuberculosis [14], the IFN-γ test, which measures the release of interferon-gamma from lymphocytes in the blood of suspected tuberculosis animals diagnosed with bovine and avian DPP [15], microbiological culture, which is a test that can isolate *M. bovis* from tissue biopsies of infected slaughtered animals [16], and polymerase chain reaction (PCR), which is a molecular diagnostic test that provides rapid and reliable results by directly detecting the presence of *M. bovis* through the detection of its genomic material [17]. There are variations of real-time PCR, including High-Resolution Melting (HRM) analysis, which is a post-amplification DNA analysis method that identifies nucleotide variations represented in melting curve analysis. This method allows for the simultaneous diagnosis and identification of mycobacterial species with differences in a genomic region in a single reaction. The importance of this method lies in its sensitivity, specificity, and the fact that it does not require further identification analyses such as Restriction Fragment Length Polymorphism (RFLP) or sequencing, making it both cost-effective and fast.

Bovine tuberculosis in Colombia is a disease subject to official control and mandatory reporting. The extent of the disease attributed to *M. bovis* is not well understood. Additionally, the diagnostic tests established by current legislation create uncertainty regarding the specificity and sensitivity of the results. This research aimed to describe the results of histopathological, microbiological, and molecular diagnostic findings in water Buffalo that were slaughtered with positive results for bovine tuberculosis using the intradermal DPP bovine method.

2. MATERIAL AND METHODS

2.1. Study Population

Samples were obtained from water buffaloes (Bubalis bubalis) of different breeds from the lowlands of the municipalities of Planeta Rica and Ayapel, Córdoba department, Colombia. These buffaloes tested positive for the comparative cervical test with

DPP conducted by the National Bovine Tuberculosis Program of the Colombian Agricultural Institute (ICA). A total of 50 young and adult buffaloes of both sexes, intended for meat and milk production, were examined. All buffaloes were slaughtered in accordance with the current sanitary regulations in Colombia [18].

2.2. Sample Collection

Individual samples of 1 cm^2 were obtained from mediastinal and retropharyngeal secondary lymph nodes, as well as lung parenchyma, for histopathological, microbiological, and molecular examinations. For microbiological culture, secondary lymph nodes and lung tissue fragments were combined. For molecular diagnosis, samples were taken from lung parenchyma and lymph nodes. Samples for histopathology were stored in buffered formalin and frozen at -80°C.

2.3. Histopathological Examination

Hematoxylin/Eosin staining was performed for lung parenchyma [50] and mediastinal lymph node [50] samples from each sampled buffalo. Histological slides were reviewed by two independent pathologists. The tissues were examined using an identification protocol and classified on a scale of 1 to 4; 1 and 2 represented lesions not compatible with bovine tuberculosis, while 3 and 4 represented lesions compatible with bovine tuberculosis (arbitrary reference score). Two slides were prepared for each individual under biosafety level 3 conditions for Ziehl-Neelsen staining, carried out according to a revised protocol [13]. Slide readings were performed in duplicate by different pathologists using an Eclipse E200 microscope from Nikon (Tokyo, Japan).

2.4. Microbiological Culture

Microbiological isolation was carried out under controlled biosafety level 3 conditions. Combined samples of lymph nodes and lungs were cultured on Middlebrook 7H11 medium (Becton Dickinson®) for 8 weeks. The samples were decontaminated using 4% NaOH and neutralized with a pH 7 buffer. Cultures were examined every week for a period of two months. Positive samples were evaluated using a phenotypic identification protocol for mycobacteria and biochemical identification of catalase and nitrites. Additionally, sensitivity tests were performed for *Mycobacterium bovis*, including pyrazinamide (PZA), isoniazid (INH), and rifampicin (RIF).

2.5. DNA Extraction and PCR-HRM Analysis

Nucleic acids were extracted from lymph nodes and lung tissues following the manufacturer's instructions using the GeneJET™ Genomic DNA Purification Kit [Thermo Fisher Scientific, Waltham, MA, USA]. Concentration and purity were determined using a Nanodrop® ND1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The samples were stored at -80°C and aliquoted for DNA integrity verification using agarose gel electrophoresis (1.8% agarose I Amresco® Ohio, USA) with Red Nucleic Acid Gel Stain (Biotium® Fremont, CA, USA) for 50 minutes at 110 V. Gel images were captured using a Biorad Universal Hood II Gel Doc System (Laboratory Segrate, Milan, Italy).

Real-time PCR HRM assays were performed using the Type-it HRM PCR Kit (Qiagen). The primers used in these assays were previously described [19], F3_Avj CTG GCT CAG GAC GAA CG and B3_Avj GCC CAT CCC ACA CGT CCG C for 16S rRNA. Each reaction contained 1X HRM master mix (HotStarTaq Plus DNA polymerase, HRM PCR buffer with EvaGreen dye, Q-solution, and dNTPs), 0.4 µM of each primer, 2 µl [10-50 ng/µl] of sample DNA, and RNase-free water. All assays were performed using a LightCycler® 96 real-time PCR system (Roche diagnostic, Roche Molecular Systems, Inc Vedbaek, Denmark) with the following thermal profile: 95°C for 600 seconds for 1 cycle; denaturation at 95°C for 10 seconds and annealing-extension at 62°C for 30 seconds for 45 cycles. Temperature ramping from 80°C to 94°C with a temperature increment of 0.1°C every 2 seconds.

2.6. Data Analysis

Frequency tables were created in Excel® with the obtained data, and molecular biology results were analyzed using Light Cycler® 96 real-time PCR system software version 1.05.The diagnostic test results were analyzed using the statistical software Stata, which has serial number 30120589707 and is licensed for CES Poblado, Universidad CES. The chi-square test was performed to establish independence in the diagnostic tests with a confidence level of 95%.

2.7. Ethical and Legal Considerations

This research was conducted with the authorization of the Colombian Agricultural Institute (ICA), the Colombian Institute for the Surveillance of Drugs and Food (INVIMA), the Quality Directorate, and the

General Management of the Central Livestock Market of Medellín, as well as the buffalo owners who provided the animals, affiliated with the Colombian Association of Buffalo Breeders (ASOBUFALOS). The project was approved by Resolution No. 14 of June 2015 of the Institutional Committee for the Care and Use of Animals of the University CES CICUA.

3. RESULTS

3.1. Histopathological Examination

Of the total buffalo that tested positive with the DPP test, 52% (26/50) presented histological lesions suggestive of tuberculosis. Nine buffalo had histological grade 4 lesions, characterized by granulomas in the lungs, liver, and secondary lymph nodes with multinucleated giant cells, interstitial pneumonia, and lymphocytic pleuritis. Seventeen buffalo had grade 3 lesions characterized by lymphohistiocytic fibrosing pleuritis, lymphoid hyperplasia (paracortical), acute interstitial pneumonia, lymphoid hyperplasia, active chronic interstitial pneumonia, hyperplastic histiocytic lymphadenitis, SLS (emphysema), active chronic lymphadenitis, histiocytic lymphadenitis, and lymphocytic pleuritis.

However, 48% (24/50) of the buffalo that tested positive with the DPP test did not have lesions compatible with tuberculosis (they had grade 1 and 2 lesions). The main histological lesions found in these buffalo, which were negative based on histopathological findings, included chronic and acute interstitial pneumonia, lymphoma, chronic lymphadenitis, hyperplastic lymphadenitis, depletion, non-suppurative periportal hepatitis, and reticulon-histiocytic hyperplasia (Figures **1**, **2**).

3.2. Ziehl-Neelsen Staining

The Ziehl-Neelsen staining revealed the presence of acid-fast bacillus (AFB) in 21 lung samples and 16

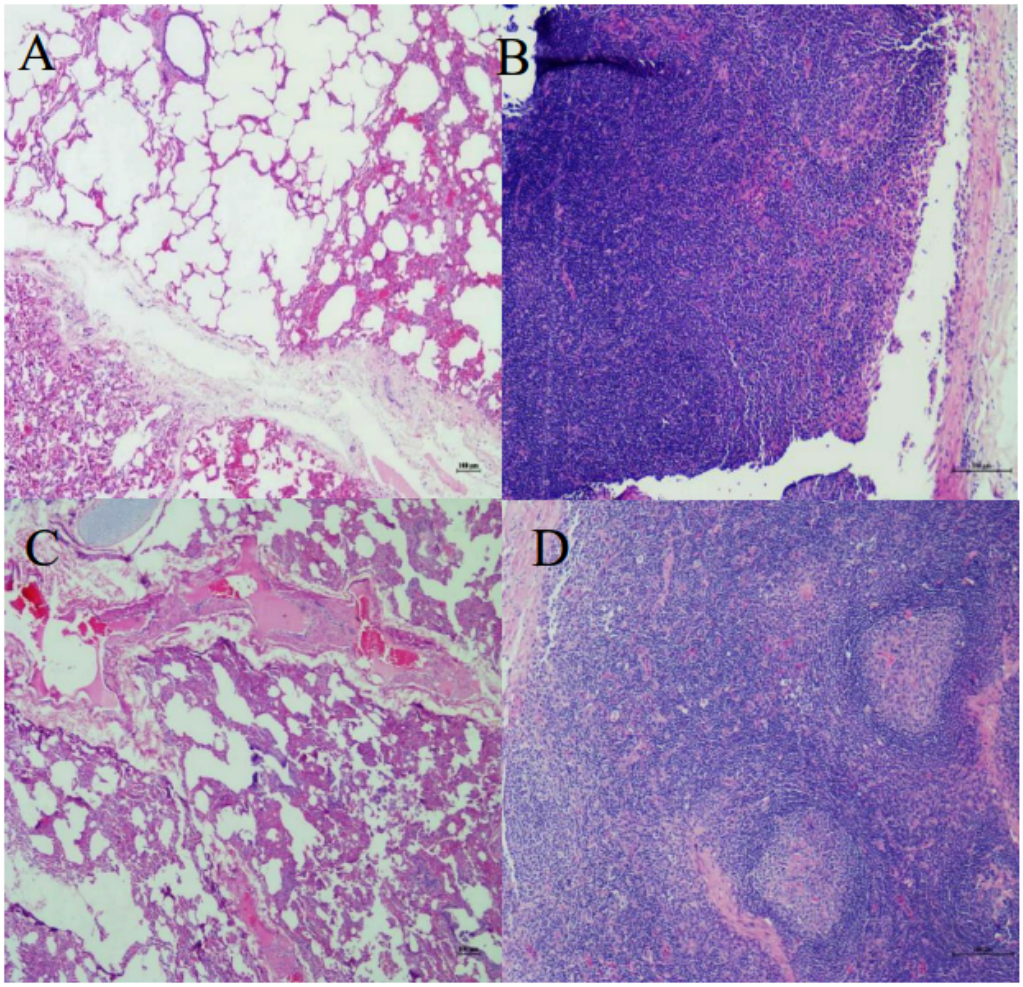

Figure 1: Histological lesions in tuberculin-positive water buffalo not compatible with tuberculosis. **A**. Lung tissue with chronic interstitial pneumonia. **B**. Secondary lymph node with reticulo-histiocytic hyperplasia. **C**. Lung with active chronic interstitial pneumonia. **D**. Secondary lymph node with hyperplastic lymphadenitis. 10X

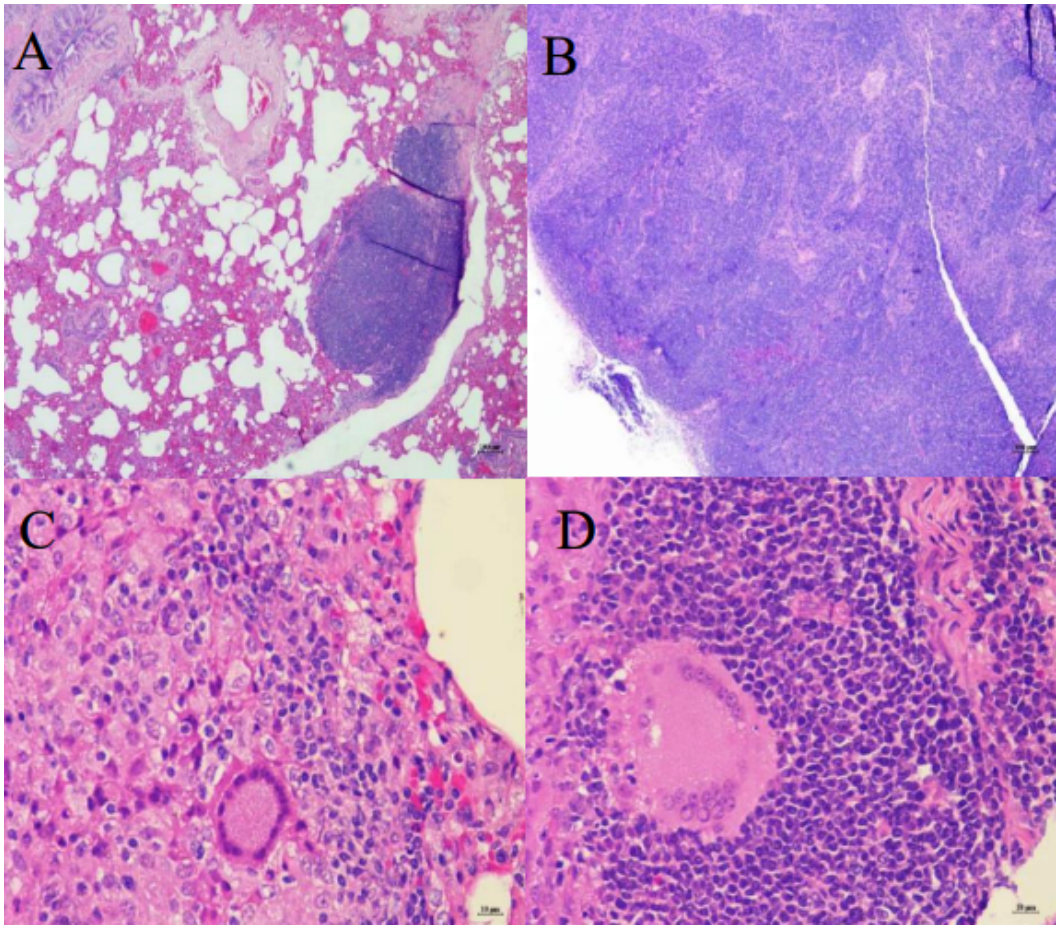

Figure 2: Histological lesions in tuberculin-positive water buffaloes compatible with tuberculosis. **A**. Lung tissue with lymphocytic pleuritis and interstitial pneumonia. **B**. Lymph node with lymphoid hyperplasia. **C**. Lung with granulomatous pneumonia. **D**. Secondary lymph node with granulomatous lymphadenitis. Magnifications **A**, **B** 10X. **C**, **D** 40X

secondary lymph node samples. Among the buffalo with histological lesions compatible with bovine tuberculosis (Figure **3**), 11 had AFB. In contrast, 18 buffalo with histological lesions compatible with bovine tuberculosis did not show acid-fast bacillus in the Ziehl-Neelsen test. Of the 15 buffalo with histological lesions compatible with bovine tuberculosis who did not show evidence of AFB in the Ziehl-Neelsen test, 6 buffalo without histological lesions compatible with tuberculosis also did not have acid-fast bacillus present (Table **1**).

3.3. Bacterial Isolation and Sensitivity Testing

Out of the 50 cultured samples, 4 lung and lymph node samples tested positive in microbiological cultures. The positively cultured samples were identified through biochemical tests, and antibiotic sensitivity was determined. The isolated cultures showed negative results for nitrites and catalase. Two buffalo samples that tested positive in microbiological cultures also exhibited the presence of AFB in Ziehl-Neelsen staining. Three buffalo with positive cultures had a histopathological diagnosis compatible with bovine tuberculosis. Finally, one buffalo with microbiological isolation did not present histological lesions compatible with bovine tuberculosis. Regarding sensitivity testing, 100% of the isolated cultures exhibited an *in vitro* resistance pattern to PZA but sensitivity to INH and RIF (Table **2**).

3.4. Molecular Diagnosis PCR-HRM

A total of 50 DNA samples obtained from buffalo tissues that tested positive in the DPP were analyzed. The PCR-HRM test was positive for bovine tuberculosis (bTB) and paratuberculosis (PTB) (Figure **4**). Seventeen samples analyzed by PCR-HRM tested positive and were characterized as bTB caused by *M. bovis*. Out of these, 9 out of 17 samples from buffalo that tested positive by PCR-HRM did not present histological lesions compatible with bovine tuberculosis. This clearly demonstrates that not all of them develop compatible lesions or that these histopathological lesions depend on the duration of the

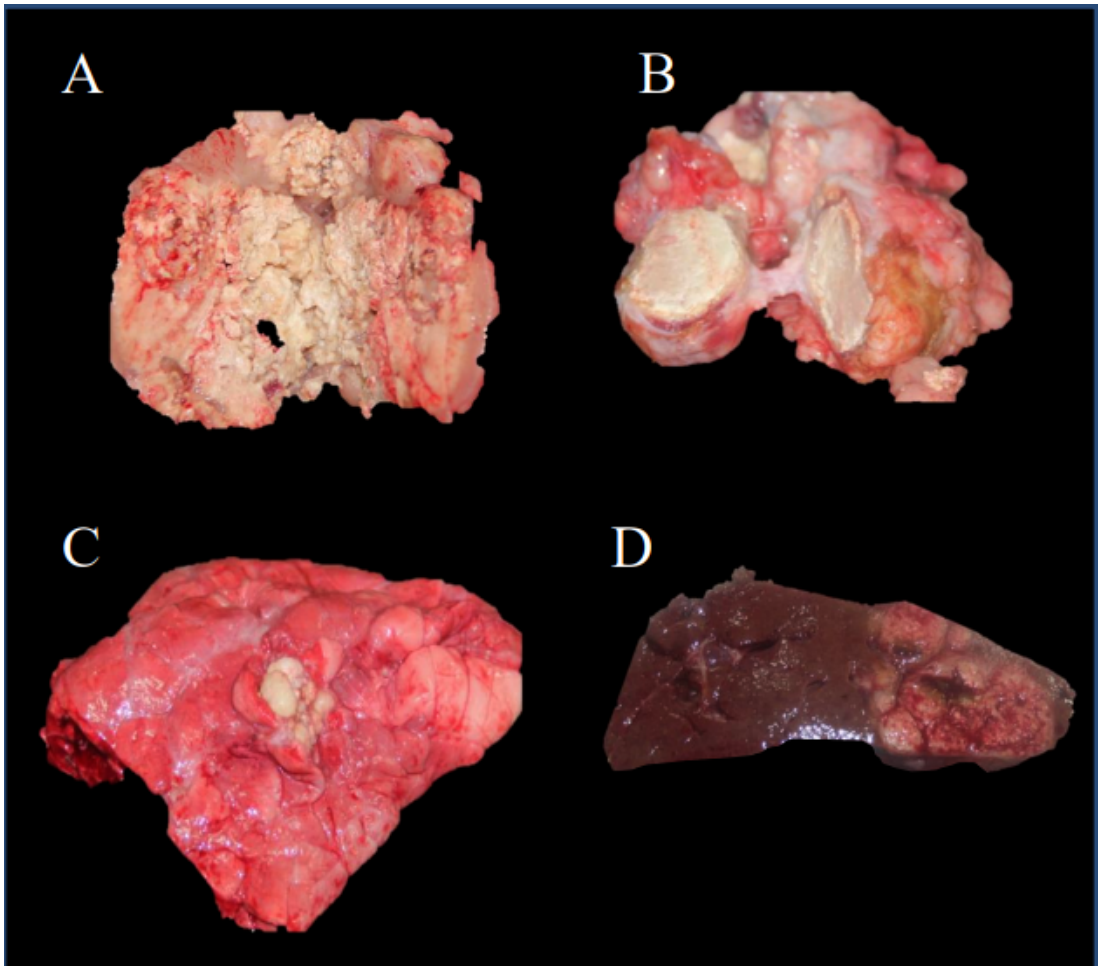

Figure 3: Macroscopic lesions in tuberculin-positive water buffaloes. **A**. Granuloma lesion in retropharyngeal lymph node. **B**. Granuloma lesion in a mediastinal lymph node. **C**. Granulomatous lesion in lung tissue. **D**. Granulomatous lesion in the liver.

Table 1: Diagnostic Test Results for Tuberculin-Positive Buffaloes

	LN*	Lung	Score 4 SHP†	Score 3 SHP†	Score 2 SHP†	Score 1 SHP†	P-value**
Number of positive buffaloes to tuberculin [n]	50[100%]	50 [100%]	9 [100%]	17 [100%]	22 [100%]	2 [100%]	
ZN BAAR present	16 [32%]	21 [42%]	5 [55.55%]	6 [35.29%]	16 [72.72%]	2 [100%]	0,0710
ZN BAAR Absent	34[68%]	29 [58%]	4 [44.45%]	11 [64.71%]	6 [27.27%]	0	
Microbiological culture+	4 [8%]	4 [8%]*	3 [33.33%]	0	1 [4.54%]	0	0,0198
Microbiological culture -	46 [92%]	46 [92%]*	6 [66.66%]	17 [100%]	21 [95.45%]	2 [100%]	
HRM +	17 [34]	17 [34]	2 [22.22%]	6 [35.29%]	7 [31.81%]	2 [100%]	0,2125
HRM -	33 [66]	33 [66]	7 [77.77%]	11 [64.70%]	15 [68.18%]	0	

* LN [mediastinal lymph node].
† Histopathology score for bTB ranging from 1 to 4 grades. Grades 1 and 2 are not compatible with histopathological lesions associated with bTB. Grades 3 and 4 are compatible with histopathological lesions associated with bTB.
** The results represent the comparison between the tests and the pathology score; values were considered significant at P≤0,05.

infection. Two buffalo that tested positive in the DPP with granulomatous histological lesions where *M. bovis* was isolated also tested positive in the PCR-HRM test using clinical samples from secondary lymph nodes and lung tissue. Nine samples from buffalo that tested positive in the DPP without histological lesions compatible with bovine tuberculosis and with no presence of AFB in Ziehl-Neelsen staining yielded positive results in the PCR-HRM test (Table **1**). Finally, the PCR-HRM test allowed differentiation between

Table 2: Results of Diagnostic Tests, Antibiotic Sensitivity, and Basic Biochemical Tests for Tuberculin-Positive Buffaloes with Positive Microbiological Cultures

	Diagnosticstests	Antibioticsensitivitytests		Biochemicaltests						
Identification	Ziehl-Neelsen	Pathology score	HRM	Cultivation result*	PZA pirazinamida	INH Isoniazida	RIF rifampicina	2 TCH Hidracida del ácido tiofeno-2-carboxílico	Nitritos	Catalasa
DPP+35	BAAR present	Score2 2/22 [9,09%]	1/17 [5,88%]	1/50 [2%]	resistent	Susceptible	Susceptible	Susceptible	negative	negative
DPP+37	BAAR present	Score 4 4/9 [44,44%]	1/33 [3,03%]	1/50 [2%]	resistent	Susceptible	Susceptible	Susceptible	negative	negative
DPP+45	BAAR Absent	Score 4 4/9 [44,44%]	1/33 [3,03%]	1/50 [2%]	resistent	Susceptible	Susceptible	Susceptible	negative	negative
DPP+43	BAAR Absent	Score 4 4/9 [44,44%]	1/17 [5,88%]	1/50 [2%]	resistent	Susceptible	Susceptible	Susceptible	negative	negative

*Cultured in Middlebrook 7H11, DecontaminatedNaoH al 4% and neutralizedwith Buffer pH 7.

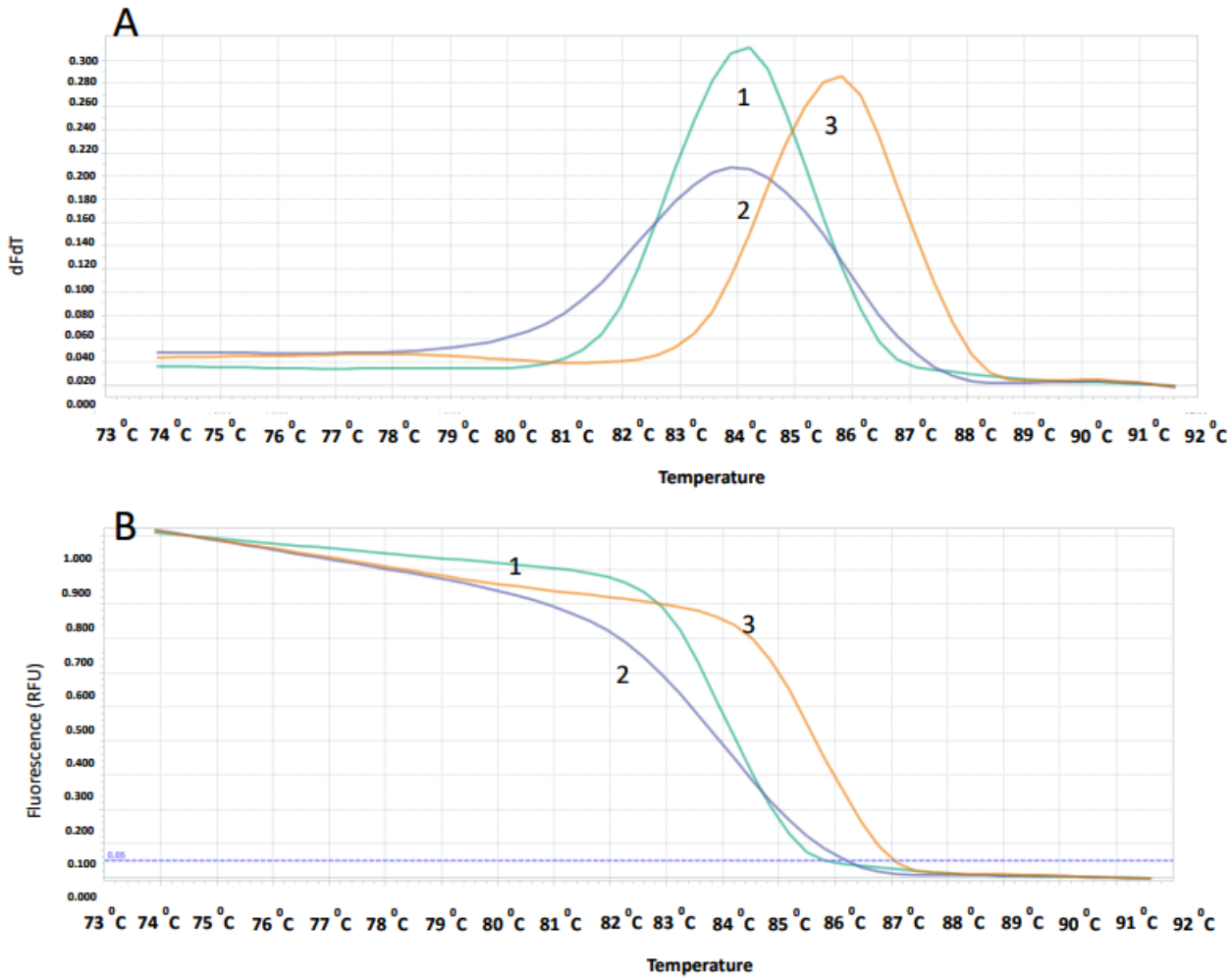

Figure 4: qPCR HRM results for positive controls of mycobacteria. **A** Melting curve for 1. Mycobacterium tuberculosis, 2. *Mycobacterium bovis*, 3. *Mycobacterium avium* subsp. paratuberculosis. **B** Fluorescence curve for positive controls of mycobacteria.

buffalo positive in the DPP infected with *M. bovis* and those infected with *M. avium* subspecies paratuberculosis (Data not shown)

4. DISCUSSION

Tuberculosis is a debilitating zoonotic infectious disease that can affect all vertebrate animals [20]. It causes significant economic losses in animal production. It is classified as a serious zoonosis by the World Health Organization (WHO) [21], posing a barrier to the international trade of animal products between different countries. Buffalo is a susceptible animal to Bovine Tuberculosis, which has been reported in this species in Italy [22], Brazil [7], and India [23]. This research provides evidence of the presence of *Mycobacterium bovis* in buffaloes in production systems in Colombia.

The results of this research on buffaloes positive in the DPP test without apparent clinical signs, no alterations in blood profiles, and serochemical profiles (unpublished data) but with the presence of granulomatous lesions are consistent with the descriptions of chronic disease with absent clinical signs in the early stages of the disease. Additionally, this represents a significant occupational risk for those working with these animals, such as veterinarians, cattle ranchers, rural workers, personnel in slaughterhouses, among others [24].

According to research conducted in Brazil in 2015 with water buffaloes in different regions of the state of Pará, Brazil [7], the aim was to compare the sensitivity and specificity of different diagnostic methods for tuberculosis in buffaloes. The DPP test was compared with diagnostic methods such as histopathological examination and bacterial isolation. It was observed that 4.65% of the sampled buffaloes tested positive in the DPP test, 2.98% had inconclusive results, and 92.36% had negative results. These results are consistent with our research, where we found a proportion of 3.07% of buffaloes testing positive in the DPP test in the selected buffalo farms for this study. These proportions of DPP-positive buffaloes contrast with research on water buffaloes in Pakistan [17], where an overall proportion of animals testing positive in the DPP test was 24.7%, showing less agreement with the findings in our study.

Research conducted on tuberculosis diagnostic methods in species like buffaloes aims to contribute to disease control and eradication policies implemented by health authorities [17]. However, the applicability of the DPP test in buffaloes has shown, in recent research, that these intradermal injection tests exhibit varying levels of sensitivity and susceptibility in diagnostic tests for cattle. Our research describes different diagnostic methods employed for diagnosing bovine tuberculosis in animals that tested positive for the intradermal reaction test. However, no concordance was found with the results of the DPP test. Only 4% of the positive DPP diagnoses could not be verified using any of the methods employed. Nevertheless, in accordance with Seva *et al*. [15], nonspecific reactions can significantly interfere with the interpretation of tuberculin test results in areas with low tuberculosis prevalence, which can obscure disease outbreaks or misdiagnose healthy animals when test results are not properly interpreted. These authors attributed these reactions to *M. avium* subsp. paratuberculosis and other pathogenic saprophytic or facultative mycobacteria.

This research demonstrated that histopathological diagnosis confirmed 52% of the samples. Additionally, molecular diagnosis with PCR-HRM could confirm 34%, showing lower sensitivity than the former methodology. PCR-HRM allowed the identification of some samples positive for *M. avium* subspecies paratuberculosis in these buffaloes. The Ziehl-Neelsen test presented variable results in buffaloes that were positive for DPP.

The PCR-HRM test had low sensitivity, and this result may be associated with a decrease in the load of *M. bovis* in the tissues at the time of sampling, tissue selection for DNA extraction, or the degradation of genetic material in some samples. However, the PCR-HRM (*High-resolution melting analysis*) technique is a molecular diagnostic technique based on real-time PCR that allows the detection of mutations in specific amplified regions. These mutations represent specific changes that allow the identification of different mycobacterial species. This relatively simple and fast diagnostic method enables the identification of mycobacterial species [25, 26]. The use of molecular tests to confirm DPP-positive buffaloes [27] has been shown to have good sensitivity. In research conducted with buffaloes, a sensitivity of 76% was established when comparing molecular PCR tests with animals subjected to the comparative cervical intradermal test, data that contrasts with our results. PCR-HRM in this research allowed the discrimination of samples positive

for *M. avium* subspecies paratuberculosis in buffaloes positive for the comparative cervical test for *M. bovis* (unpublished data).

Other research studies have employed molecular methods to diagnose tuberculosis in buffaloes and cattle using DNA from tissue samples [27, 28]. One study obtained results from molecular PCR tests for IS1081 DNA from bovine lymph nodes, yielding more positive results than microbiological cultures of tissue samples [28]. These results are consistent with our findings, in which we obtained 34% of positive samples by PCR-HRM and 8% of positive results from microbiological cultures. These results underscore the importance of using real-time PCR with high-resolution melting (HRM) curve analysis to enhance the diagnosis of tissues with granulomatous lesions. This is because the detection of lesions compatible with *M. bovis*-induced tuberculosis in post-mortem animals can be confused with lesions caused by *Nocardia* spp, *Corynebacterium* spp, and some parasites, among other microorganisms that cause granulomas.

The inability to isolate mycobacteria in 84.84% of the samples from animals that reacted to tuberculin may be associated with a decrease in the load of *M. bovis* in the lymph nodes at the time of sampling or with inherent difficulties in the method used to isolate these etiological agents.

Our research successfully isolated *Mycobacterium bovis* from clinical samples obtained from slaughtered DPP-positive buffaloes. Future research will consider these isolates to conduct comparative genomic analysis based on the specific geographic region where some buffaloes inhabit Colombia. It has been shown that genetic variations in the molecular typing of *M. bovis* are related to the geographical origin of the samples [29]. The behavior of all the bacteria isolated in the antimicrobial resistance tests for pyrazinamide (PZA) showed resistance, characteristic of *M. bovis* isolates and has been documented previously [30, 31]. All the cultures isolated in this research showed sensitivity to isoniazid (INH) and rifampicin, which is important considering that resistant strains of *M. bovis* have been reported [32].

This research demonstrated that 52% of the slaughtered buffaloes that tested positive for tuberculin skin test presented histological lesions compatible with bovine tuberculosis (Figure **1**, **2**). The utility of histopathological diagnosis in tuberculosis has been tested. Recent research has shown that histopathological diagnosis can be accompanied by other complementary tests for diagnosing tuberculosis in humans and animals [32, 33]. In this study, microbiological culture and PCR-HRM tests confirmed tuberculosis diagnoses in buffaloes that did not present histological lesions compatible with tuberculosis, becoming a complementary aid to conventional diagnosis. The evidence of negative results by histopathology confirmed by other methods may be due to the collection of tissue fragments for analysis and the progression of the disease.

Finally, it is important to mention that the tuberculin skin test is recognized by international health authorities as a method for the *in vivo* diagnosis of bovine tuberculosis. This hypersensitivity test has proven to be effective in health control programs, but it is recognized that the test has limitations in sensitivity and specificity compared to other diagnostic methods. Further research is needed on the buffalo species.

CONCLUSION

Other direct diagnostic methods demonstrated the presence of *M. bovis* in buffaloes positive for the tuberculin test. The complementary tests used in this research did not confirm a low number of samples from buffaloes positive for the comparative intradermal test. The diagnostic methods presented in this research did not show concordance with the comparative cervical test. The *M. bovis* cultures isolated in water buffaloes in this research demonstrated *in vitro* sensitivity to Isoniazid and Rifampicin.

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CONFLICT OF INTEREST

The undersigned authors declare that there are no conflicts of interest related to the publication of this research.

AUTHOR CONTRIBUTIONS

PPC: Sample collection and processing, article writing.

DIB: Sample collection, histopathological analysis.

JRB: Article writing and editing.

RVJ: Molecular diagnosis, article writing.

EMR: Molecular diagnosis, article writing.

JBG: Case follow-up, sample collection, article writing.

RRG: Sample collection, molecular diagnosis, Zielh-Neelsen reading, microbiological culture, article writing.

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