MYCOBACTERIUM BOVIS AND THE POTENTIAL RISK TO HUMAN HEALTH IN
AMAZONAS STATE, BRAZIL

By

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ABSTRACT

MYCOBACTERIUM BOVIS AND THE POTENTIAL RISK TO HUMAN HEALTH IN
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We performed the first epidemiological study of the *Mycobacterium bovis* (*M. bovis*) in Amazonas state, Brazil. The goal of this project was, through a One Health Approach, to clarify the burden of *M. bovis* in Amazonas State. To achieve the goal our study addressed four fundamental questions regarding the epidemiology of bovine Tuberculosis (bTB): 1) What is the *M. bovis* prevalence and profile in cattle and buffalo in Amazonas State?; 2) What are the major risk factors for animal-to-animal bTB transmission in the area?; 3) How can the detection of animals (cattle and buffalo) and herds infect by *M. bovis* be improved?; and 4) Does consumption of raw milk from cattle and buffalo represents a risk for zoonotic Tuberculosis (zTB) to human population in Amazonas state?

The study used a two-phases cross-sectional design. During the first phase, from July 2016 to February 2018, a total of 214 animals (91 buffalo and 123 cattle) that were considered bTB suspects had tissues collected for laboratory confirmation using culture, polymerase chain reaction (PCR), spoligotyping, and whole genome sequencing (WGS). During the second phase, from February 2019 to August 2019, a total of 250 samples of raw milk were collected, 91 milk samples from cattle and 159 from buffalo, for analysis in pools using shotgun metagenomic sequencing (SMS).

Our results demonstrated that Amazonas state presented the highest bTB prevalence ever reported, both in cattle (3%) and buffalo (11.8%), in Brazil. Additionally,

M. bovis presents a distinct genetic profile in the state when compared with the rest of the country with surprising identification of ancient strains (Lb1) never described in South America. Species (*Bubalus bubalis*), herds size (>100 animals) and the presence of both species (buffalo and cattle) in the herd were the major risk factors for the infection by *M. bovis* in the state. Sole adoption of Tuberculin Skin Test (TST) protocols failed to detect aged animals and herds infected by the *M. bovis*, but the antibody assay was able to detect infected animals and herds missed by the Comparative Cervical Tuberculin test (CCT). *Mycobacterium tuberculosis complex* (MTC) species genetic material were identified in all pools of raw milk. Genetic material consistent with *M. bovis* were identified in seven pools of raw milk (1 cattle and 6 buffalo). Buffalo presented significantly higher *M bovis* infection prevalence than cattle and significantly a higher contamination (rate) in raw milk compared with raw milk from cattle.

The findings support the original hypothesis that *M. bovis* represents a potential risk to public health in the area. Combination of epidemiological, clinical, and laboratorial data are measures recommended to strengthen bTB programs in Amazonas and Brazil. The popular practice of consumption of raw milk and its derivatives in Amazonas state poses a high risk of transmitting *M. bovis* to humans. Therefore, education measures to increase awareness of zTB in key public and private stakeholders are necessary to nurture the engagement and collaboration needed to effectively address the challenge to end TB. Finally, we recommended further molecular studies to clarify the origins of *M. bovis* in the region, its possible spread to the local wildlife, and active surveillance for *M. bovis* in TB patients to assess the impact on human health in the region.

This Dissertation is dedicated to my father and mother, José and Walkiria, my life examples, and to my son and daughter, Pedro and Emmy, my life motivation.

"*I am hungry for solid things and eager to live only the essentials. Personally, I think that there comes a time in people's lives, when the only duty is to fight fiercely to introduce, at the time of each day, the maximum of "eternity".*"
João Guimaraes Rosa

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KEY TO ABBREVIATIONS

ADAF	Amazonas State Animal Health Agency
AFB	Acid-Fast Bacilli
BCG	Bacille Calmette-Guérin
bTB	Bovine Tuberculosis
CC	Clonal Complexes
CCT	Comparative Cervical Tuberculin
CFT	Caudal Fold Tuberculin
CFT10	Culture Filtrate Protein 10
CMI	Cell-Mediated Immune response
DR	Directed Repeated
ELISA IDEXX™	Enzyme Linked Immunosorbent IDEXX Laboratories
ELISA	Enzyme Linked Immunosorbent Assay
EPTB	Extra Pulmonary Tuberculosis
ESAT6	Early Secretory Antigenic Target Protein 6
GLMM	Generalized Linear Mixed Models
GTA	Guide for Animal Transportation
HDI	Human Development Index
LST	Lesions Suggestive of Tuberculosis
MAPA	Ministry of Agriculture, Livestock, and Supply, Brazil
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat
MS	Ministry of Health, Brazil

MTC	*Mycobacterium Tuberculosis Complex*
NTM	Non-Tuberculosis Mycobacteria
OH	One Health
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PNCEBT	Program for Control and Eradication of Bovine Brucellosis and Tuberculosis, Brazil
PNCTB	National Program for the End of Human Tuberculosis
PPD	Purified Protein Derivative
PPDa	Purified Protein Derivative avian
PPDb	Purified Protein Derivative bovine
SCT	Single Cervical Tuberculin
SDGs	United Nations Sustainable Development Goals
Se	Sensitivity
SMS	Shotgun Metagenomic Sequencing
SNPs	Single Nucleotide Polymorphisms
Sp	Specificity
TB	Tuberculosis
The Union	International Union against Tuberculosis and Lung Disease
TST	Tuberculin Skin Test
UK	United Kingdom
US	United States
WGS	Whole Genome Sequencing
WHO	World Health Organization

zTB   Zoonotic tuberculosis

CHAPTER I – INTRODUCTION

Tuberculosis (TB) due to *Mycobacterium bovis* (*M. bovis*) remains a major endemic infectious disease in livestock worldwide and a serious zoonosis [1]. In 2015, it was estimated that more than 50 million cattle were infected by *M. bovis* worldwide [2]. From July to December 2018, of the 188 countries and territories reporting their bovine tuberculosis (bTB) situation to the World Organization for Animal Health (OIE), 96 countries (51%) reported the presence of the disease [3]. In 2019, there were an estimated 140,000 new cases of zoonotic tuberculosis (zTB) and some 12,500 people died of the disease. Although this represents a small proportion of the total human TB disease burden, it's an important hamper to the goal of the World Health Organization (WHO) to end the TB epidemic by 2030 [4].

The economic burden caused by *M. bovis* is multifaced and multisectoral with consequences on livestock production, animal health, wildlife, and human health [5]. In livestock, it impacts negatively on profitability and can decimate years of genetic improvement towards desirable production traits causing global economic losses estimated around $3 billion annually, including those resulting from trade barriers [6]. It also impacts negatively on the welfare of affected farming families [7]. However, most of the time, cost assessments focus primarily on livestock production losses [8]. In the United States (US), bTB was completely eradicated in many herds at a cost of $450 million over 50 years using a "test and slaughter" strategy combined with meat inspection [9]. In England, the fight against bTB has cost almost £500m annually since 2004 and it is estimated that it will top £1 billion over the next decade [10]. In Argentina,

the annual economic losses due to bTB has been estimated to be $63 million [11]. To date, no study has been performed in Brazil to estimate productivity losses in terms of meat and milk and the economic costs due to *M. bovis* infection.

Since 2001, Brazil has the National Program for Control and Elimination of Bovine Tuberculosis (PNCEBT), which is based on "test and slaughter" and risk-zoning [12]. The diagnostic method adopted is the Tuberculin Skin Test (TST), with the intradermal injection of bovine tuberculin Purified Protein Derivative (PPD) [12]. The Caudal Fold Tuberculin test (CFT), the Single Cervical Tuberculin test (SCT), and the Comparative Cervical Tuberculin test (CCT) are the official tests of detection [12]. In contrast, with similar programs in other countries, PNCEBT has no mandatory confirmatory post-mortem tests, such as culture or molecular diagnosis [13]. Grounded in voluntary actions and no compensatory (indemnity) measures for the farmers, PNCEBT has made slow progress toward bTB eradication [13]. From 2005 to 2014, adopting the CCT, 14 epidemiological studies were conducted in 13 states [14]–[27]. The herd prevalence ranged from 0.5% to 11%, while individual animal prevalence was from 0.035% to 1.3%. In Amazonas state, prevalence of bTB is unknown, there are no initiatives toward bTB eradication, and control measures in cattle and buffalo are limited or absent in most of the municipalities. The state bTB economic burden is unknown, however considering the occurrences of carcass condemnations, due to lesions suggestive of bTB, in regional slaughterhouses it is estimated to be considerable.

Brazil is within the 30 highest human TB High Burden countries, accounting for 0.9% of cases worldwide [28]. In 2018, 4,490 deaths were due TB in Brazil and the mortality rate was 2.2 per 100,000 persons with an average annual reduction from 2.2

to 2.3 deaths per 100,000 persons since 2010 [29]. In 2019, 73,864 new cases of TB were identified in Brazil resulting in an incidence rate of 35.0 cases per 100,000 persons. Although there was a constant downward trend in the last decade, the TB incidence rate in the country increased in the years 2017 and 2018 compared to the previous period [29]. Moreover, incidence rates are not homogeneous between states, which requires the development of specific actions, considering the particularities of each location. In Amazonas state, the TB incidence rates have been rising in the last decade, reaching 74.1 cases per 100,000 persons in 2019 – the highest incidence in the country and mortality rate of 3.8 per 100,000 persons [29]. In addition, Manaus, the capital of Amazonas state, presents the highest incidence rate within the capitals with 104.7 per 100,000 persons and mortality rate of 4.7 per 100,000 persons [29]. In Latin America, it is expected a minimum 2% of the total pulmonary TB cases and 8% of extrapulmonary TB cases are caused by *M. bovis* [11]. Although zTB cases have never been reported in Amazonas [30], the major risk factors associated with zTB, such as occurrence of bTB, low rates of milk pasteurization, and high consumption of products from raw milk are daily routine in the state's municipalities. Therefore, a conservative figure expected would be an occurrence of 55 new cases of pulmonary TB and 34 new cases of extrapulmonary TB due to *M. bovis* in Amazonas in 2019.

The pursuit of understanding TB due to *M. bovis* separately in human and animals and without considering the environmental influence leads to an incomplete understanding of disease risks and, consequently, missed opportunities for the control and elimination of the disease. Besides in cattle and humans, *M. bovis* has a wide variety of hosts, including water buffalo (*Bubalus bubalis*), swine, sheep, camelids, and

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free-ranging and captive wildlife. Its ability to infect a such variety of species can be attributed to the different routes of transmission [31]. Knowledge of the TB determinant factor's and their interactions is important for the establishment of public policies and the planning of effective preventive and control measures for the disease. The One Health (OH) approach seems to be most adequate strategy to understand the dynamic of *M. bovis* for the control of the TB epidemic. The OH is a collaborative, multisectoral, and transdisciplinary approach, that recognizes the interconnection between people, animals, plants, and their shared environment, and the closer cooperation between human and animal health results in benefits that are not achieved through the two professions working independently [32], [33]. Cases of OH approaches include actions like the 2000-2003 study to understand the prevalence of Crimean-Congo Hemorrhagic Fever in Kazakhstan; the 2007 investigation that determined toxin from the blue-green algae was killing sea otters in Monterey Bay, California; the 2015 China's Stepwise Approach to Rabies Elimination (SARE) Workshop; and the 2017 Uzbekistan's initiative through multisectoral approach to prevent zoonotic diseases [34]. An OH approach is clearly warranted for TB [35].

CHAPTER II – LITERATURE REVIEW

THE NATURE AND EVOLUTION OF THE *MYCOBACTERIUM TUBERCULOSIS*

COMPLEX

Mycobacterium Tuberculosis Complex (MTC) is a highly successful clonal group of pathogens that cause TB disease in humans and animals [35, 40]. MTC members have evolved from a common ancestor and alignable regions of MTC genomes are over 99.95% identical, with horizontal gene transfer and large recombination events considered absent [38]. These pathogens have exclusively evolved through single nucleotide polymorphisms (SNPs), indels, deletions of up to 26 Kb, duplication of few paralogous genes families, and insertion sequences (IS), leading to a large variation of virulence and host tropism [38]. MTC includes *M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. mungi*, *M. orygis*, *M. pinnipedii*, *M. suricattae*, and *M. tuberculosis* [37], [39].

Genetic evidence indicates that the most common ancestor of MTC emerged some 40,000 years ago from its progenitor in East Africa, the region from where modern human populations disseminated around the same period [44, 46]. Molecular sequencing of the *M. bovis* genome challenged the natural epidemiological hypothesis that *M. tuberculosis* is a human-adapted variety of *M. bovis* that was acquired from cattle [41]. In fact, *M. tuberculosis* and *M. bovis* genomes sequencing, demonstrated that the old story should be reversed, as *M. tuberculosis* is more ancestral than *M. bovis* [41, 46]. Compared with *M. tuberculosis*, *M. bovis* has a reduced genome due to deletion of genetic information. This irreversible genetic event seems to be responsible for differences in pathogenicity and host range between the species [41, 42, 44, 45]. *M.*

tuberculosis comprises seven human-adapted lineages, on the other hand *M. bovis* has a broader host range and can infect and cause TB in multiple host species, including humans [39].

Originally, human TB was thought to be caused solely by *M. tuberculosis*, later a zoonotic form of the disease caused by *M. bovis* and acquired through contact with unpasteurized dairy products was uncovered [37], [44]. Correspondingly, bTB was believed to be caused only by *M. bovis* [42]. Currently the occurrence of interspecies disease by most members of the MTC is well known. Examples of these include: *M. africanum* (humans and cattle), *M. bovis* (cattle, water buffalo, swine, sheep, camelids, deer, antelopes, African buffalo, badgers, brushtail possums, wild boar, ferrets, hedgehogs, rodents, lagomorphs, rhinoceroses, primates, and humans), *M. caprae* (goats, cattle, wild boar, pigs, and humans), *M. microti* (rodents and humans), and *M. tuberculosis* (humans, cattle, primates, elephants, and buffalo) [30, 40]. Recent discoveries in molecular analysis and genomic sequence mapping have led to a new understanding of the pathogenesis, host range, evolution, and phenotypic differences within the MTC [40], [45].

PATHOGENY OF TB

In humans, TB is a pulmonary and systemic disease caused predominantly by *M. tuberculosis.* TB infection occurs when a susceptible person inhales droplet nucleus containing *M. tuberculosis* organisms [46]. The bacilli that reach the alveoli of the lungs are phagocytosed by alveolar macrophages and most of these bacilli are destroyed or inhibited [47]. A small number of bacilli can resist the bactericidal mechanisms induced by the macrophage and multiply within the phagosome, thus avoiding apoptosis [48].

Pathogenic mycobacteria induce macrophage necrosis allowing the release of bacilli to the extracellular environment, where they are phagocytosed by other macrophages [47], [48]. This cycle ends with the formation of granuloma, the hallmark structure of TB [46]–[48]. The granuloma is a compact aggregate of immune cells that segregate the infecting mycobacteria and ends the progressive infection [46]–[48]. Historically, the granuloma is regarded as a host-protective structure. However, recent studies have shown that *Mycobacterium* have a more active role in this process. It is now known that in fact *Mycobacteria* exploit granuloma formation for their proliferation and dissemination within the host [47], [48]. After this point, the bacilli enter in the state of latency, in which the bacillary forms can survive under the conditions of stress generated by the host [49].

M. tuberculosis mostly causes latent infection in mammals, whereas *M. bovis* mostly produces an acute infection [50]. In fact, the most usual manifestation of TB in adults is the reactivation of a pre-existing, chronic infection. On the other hand, there is controversy as to whether this process of passage from active form to latency occurs in cattle with bTB [50]. To date, some researchers propose the existence of latency in bTB [51], [52], although the evidence in the literature is not convincing [52]. In animal models, *M. bovis* has been more hostile than *M. tuberculosis* on experimental infection of cattle, goats, rabbit, and mice [53]–[55]. Moreover, transcriptome and proteome analysis comparing the *M. bovis* and *M. tuberculosis* have demonstrated the host cellular responses to both pathogens differ significantly [56]–[58].

The pathogenesis of the infection is a key component to understanding *M. bovis* epidemiology [59]. Most of the primary understanding of the pathogenesis of the

disease in cattle came from extrapolation of data from humans and laboratory animals [52], [59]. The severity of the disease and the nature of the symptoms depend on the host species susceptibility, invading genotype and on the prevalence of bTB [60]. The distribution of lesions in affected cattle, humans and laboratory animals demonstrate the preeminence of the respiratory system as doorway and primary site of infection [52], [53]. Pathogenesis studies strongly suggest that the route of transmission of bTB is largely via the respiratory system, requiring transmission via infectious aerosols. Therefore, bTB is mainly a respiratory infection and is believed to occur via "direct" aerosol transmission between animals in close contact [7]. Moreover, epidemiological modelling studies considering no latency period in bTB indicates that the disease basic reproductive ratio would range from 1.5 in a herd with 30 cattle up to 4.9 in a herd of 400 animals [61].

In naturally infected cattle, TB lesions are found most frequently in the caudal lobes of the lungs [51], [52], [62], [63], in countries with efficient eradication programs, lesions are found on abattoir inspection with much greater frequency in the lymph nodes associated with the respiratory system than in the lung parenchyma [63]. In Brazil, a study with 140 cattle naturally infected and detected in slaughterhouse inspection found 55% of the animals with some macroscopic lesions, 49% with lesions only in the mediastinal lymph nodes, 28% in the liver, and 14% in the lung only; 6% carcasses showed lesions in the liver, lung and mediastinal lymph node, and 4% had lesions in the lung and lymph node [64]. Another study, with 38,172 cattle, found 0.16% of caseous TB suggestive lesions and 0.11% of calcified TB suggestive lesions, within the inspected organs (lungs, liver, tongue, and lymph nodes of the head and mediastinal)

and lungs were the most affected [65]. Moreover, in a literature review involving 1,879 million animals in slaughterhouses, 1,233 (0.065%) presented TB suggestive lesions, the most affected lymph nodes were bronchial with 628 of the affected animals (50.93%), followed by mediastinal and parotid (both with 11.19%), retropharyngeal (8.11%), pre-scapular (8.60%), and the sum of hepatic, inguinal and sciatic patients totaled 7.53% of the cases [66].

M. BOVIS SURVEILLANCE

A satisfactory system of control and epidemiological surveillance of bTB relies on slaughterhouse inspection. This requires a comprehensive infrastructure, highly trained staff and a reliable register system for tracing back to the herd of origin [13], [67]. In addition, the prevention and control of bTB requires a strong laboratory capacity and access to appropriate diagnostic tools [68]. The diagnosis of *M. bovis* infection can be made either by the direct detection of the etiological agent in biological material or through the indirect detection of a host immune response to the *M. bovis* [63], [67]. The diagnostic conditions of microscopy and the extended time needed for traditional culture methods have attracted attention to develop rapid methods for *M. bovis* detection in clinical specimens and the early identification of mycobacterial isolates. Therefore, this review will focus on selected rapid diagnostic methods.

INDIRECT DETECTION OF *M. BOVIS*

For over 100 years, the TST has been the primary diagnostic test used in TB diagnosis for both humans and cattle. The TST involves the intradermal injection of purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later [69]. In cattle, the wide use of the

TST is justified by its low costs, high availability, long history of use and, for a long time, the lack of alternative screening methods to detect bTB [67], [70], [71]. However, the TST has several well-known limitations including difficulties in administration and interpretation of results, need for a second-step visit, low degree of standardization, and imperfect test accuracy [67], [70]. Additionally, the TST sensitivity is also influenced by the purity [72], potency [73], and dosage of the PPD [74], the age of the animal [75], production type [76], the sensitization of the animal by environmental mycobacteria [56, 57], and by the genetic background of the animal [78]. Worldwide numerous studies have been carried to evaluate the sensitivity (Se) and specificity (Sp) of the TST in cattle under different epidemiological situations using different antigens [71]. Retrospective analysis of the results from the bTB eradication program in the United Kingdom (UK) demonstrated Sp at animal level is 99.983 (standard interpretation) and 99.871 (ultra-severe interpretation) and that at herd level the Sp is 99.2–99.5% (average test size) and 96.5% for larger (250 animals) herd tests [79]. In Ireland, Se of CCT ranged from 89.6 to 91.2% (standard and severe interpretation, respectively) [80]. A meta-analysis of field studies in US cattle herds produced estimates for CFT Se ranging from 80.4% to 93.0% and CFT Sp from 89.2% to 95.2%: estimates for serial interpretation CFT-CCT Se ranged from 74.4% to 88.4% and CFT-CCT Sp ranged from 97.3% to 98.6% [81]. A retrospective Bayesian latent class analysis conducted on 71,185 cattle from 806 herds chronically infected with bTB distributed across Northern Ireland found CCT Se ranging from 40.5% - 57.7% (standard) to 49.0% - 60.6% (severe) and the Sp ranged from 96.3% - 99.7% (standard) to 49.0% - 60.6% [76]. In Brazil, a study in three dairy herds submitted for depopulation where CCT was

compared with culture and Polymerase Chain Reaction (PCR) confirmation, CCT demonstrated sensitivity of 28.2% and specificity of 57.1% [82]. Overall, it's recommended to exercise caution in extrapolating Se and Sp from one environment and/or the tuberculin from one manufacturer and/or one potency, and/or one type of tuberculin test to another test. Additionally, assessing Se and Sp the conditions and the species in which the test is performed should be considered [83]. Another debate regarding the TST is that it precludes implementation of Bacille Calmette-Guérin (BCG) vaccine–based control programs, and BCG vaccination of cattle would be particularly interesting in developing countries where bTB remains endemic and where the testing and slaughter strategy is not feasible [84], [85]. To address this problem, experiments have been done to test alternative antigens to the traditional PPDs used in TST. Recently a novel peptide-based defined antigen skin test to diagnose bTB and to differentiate infected animals from vaccinated animals presented promising results [86]–[88]. Despite all the limitations, it is highly probable that the TST will remain the screening test of choice for farmed livestock for the considerable future [84].

Supplemental tests are used in combination with the TST screening usually to maximize the detection (increasing sensitivity) of infected animals (parallel testing), or to confirm or negate the TST results (serial testing). The gamma-interferon (IFN-γ) assay, the lymphocyte proliferation assay, and the enzyme-linked immunosorbent assay (ELISA) are blood-based laboratory tests recognized by OIE [69]. The IFN-γ assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity [69]. The TST and the IFN-γ test are both based on the detection of the early cell-mediated immune (CMI) response in TB infection [67].

However, in settings where no or poor disease control measures are applied and where the percentage of late-stage diseased animals is believed to be high, CMI-based tests can fail leaving behind false negative animals since in the late stages of the disease, the CMI response may decrease as opposed to a generally increasing humoral immune response [67]. Therefore, in the advanced and generalized stages of TB infections, a certain proportion of anergic animals may be present, which are potentially highly infectious and do not produce a reaction to CMI-based tests. Consequently, serological tests based on the detection of the humoral immune response such as the ELISA, may be particularly important in developing countries [89].

The first serological assays were generally lacking in sensitivity (18%–73%) [90]. Moreover, the presence of environmental mycobacterial infections usually affected their specificity [91]. Further studies identified more specific diagnostic antigens (Table 1) and introduced the multiplex assay strategies allowing for the development of a variety of ELISAs using single or multiple antigen combinations [92]–[97].

The ELISA is a serological technique that measures the binding of specific antibodies to an antigen. The test has several advantages over the methods traditionally used. ELISA permits testing of many samples in a short time, it is simple, rapid, inexpensive, and allows standardization of the technique in different laboratories [93].

MPB70 and MPB83 are among the most studied MTC antigens [91], [93], [94], [98]. They are major antigens highly expressed by *M. bovis* and considerably less expressed by *M. tuberculosis* [98]. MPB70 is a soluble secreted protein expressed by *M. bovis* [96], and MPB83 is a glycosylated lipoprotein which has been detected as being serodominant [94]. Looking for tests able to differentiate *M. bovis* infection from

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environmental mycobacteria and BCG, the Early Secretory Antigenic Target protein 6 (ESAT6) and the Culture Filtrate Protein 10 (CFP10) are antigens expressed in *M. bovis* but absent from environmental mycobacteria and *M. bovis* BCG [96], [99] and have been proposed for tests to differentiate between infected and vaccinated animals [86], [100] as well as alternative antigens to the PPDs for blood stimulation in the IFN-γ assay [71]. Recently, recombinant MPB70 and SahH (rM70S) and a native 20-kDa protein (20K) were evaluated as ELISA antigens, the 20K ELISA showed 94.4% sensitivity and 98.2% specificity and had an optimal sample-to-positive (S/P) ratio cut-off of 0.531 [97]. The study showed rM70S ELISA 94.4% sensitivity and 97.3% specificity, both ELISA had acceptable diagnostic efficiency and were recommended to be used for bTB diagnosis as ancillary test [97].

Currently the ELISA IDEXX™ seems to be the most prominent serological test used, as it detects the antigens MPB70 and MPB83, however the assay's Se presents great variation by geographic region ranging from 9% in Mexico to 85.4% in the Kuwait [101], [102]. It was hypothesized that the variation in Se was due to genetic factors in relation to *M. bovis* protein coding variation, however this hypothesis was rejected [101]. Therefore, more studies are needed to understand other major factors that appear to impact the Se, such as the stage of the disease and the TST testing a few days before the ELISA is performed [93]–[95], [103].

DIRECT DETECTION OF *M. BOVIS* BY MOLECULAR TECHNIQUES

Molecular epidemiology complements (rather than substitutes) a traditional epidemiological approach [42]. It has been used to confirm epidemiologically suspected transmission, to detect epidemiologically unsuspected transmission, to identify risk

factors and environments where transmission is occurring, to detect laboratory errors and to monitor the efficacy of TB control programs [45]. Additionally, molecular techniques can provide insights into the geographical origin, source, and spread of *M. bovis* among humans and animals [42], [104], [105]. Currently many molecular techniques are used to understand the epidemiology of *M. bovis*, selecting the most appropriate technique in accordance with the existing laboratory conditions and the specific features of the geographic region [42], [106].

PCR-based methods, such as Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) and Spacer Oligonucleotide typing (Spoligotyping), have been extensively used for typing *M. bovis* [106], [107]. More recently, the genome-based Whole Genome Sequencing (WGS) has become the preferential technique to inform outbreak response through contact tracing and source identification for many infectious diseases [108].

Spoligotyping identifies polymorphism based in the presence or absence of spacer units in the Direct Repeat (DR) region [109]. The DR locus is part of the bacterial immune system which provides resistance to foreign DNA (plasmids and viruses) [110]. It is composed of a series of almost identical 36-bp repeats interspersed with unique DNA spacer sequences of similar size (direct variant repeats or DVR units) [111]. The positions of each unique spacer sequence within the DR region are highly conserved [111]. Polymorphisms in various isolates consist in the presence or absence of spacers of a known sequence [112]. This characteristic is used to determine genetic similarities among strains [42]. Spoligotyping is a technique that is fast, robust, low cost and can differentiate strains of *M. bovis* and *M. tuberculosis*, and is useful for identifying sources

of infection, transmission of TB between species in a defined geographical area and is very useful to search for a relationship between strains [11], [113]. Additionally, the results are produced in a simple, digital format easily comparable and exchanged between laboratories. However, spoligotyping has a low discriminatory power, which is the ability of the method to differentiate unrelated isolates, therefore spoligotyping cannot clarify deep questions regarding evolution of the microorganism [63], [106], [114].

The development of high-throughput sequencing technologies has resulted in a dramatic reduction in DNA sequencing costs, making the technology more accessible to the average laboratory [115]. Although PCR-based techniques have been useful for bTB control programs, *M. bovis* WGS will likely replace some of these laborious assays while simultaneously allowing the investigation of outbreaks for which higher resolution is warranted [108]. WGS provides a greater resolution than other molecular markers and can close the gaps between molecular and epidemiological data [116]–[118]. WGS requires bacterial culture, which is a major time limitation, therefore it is not ready to be applied at the point-of-care [118].

The use of WGS to understand outbreaks of *M. tuberculosis* is widespread and provided the basis for phylogenetic analysis resulting in the classification human-adapted MTC in 7 lineages, with *M. tuberculosis* accounting for L1 to L4 and L7, and *M. africanum* comprising L5 and L6 [38], [39]. Currently, *M. bovis* is most frequently classified in Clonal Complexes (CCs). The CCs are groups of bacterial strains that descended from a single cell that was the most recent common ancestor of the resulting complex [119]. To date, 4 different CCs of *M. bovis* have been based on specific

deletions ranging from 806 to 14,094 base pairs (bp), SNPs and spoligotypes [120]–[122]. These were named based on their geographical distribution as: African 1 and 2 restricted to Africa, European 2 commonly found in the Iberian Peninsula, and European 1 distributed globally [120], [121], [123], [124]. However, recent studies have shown that the CCs do not represent the whole diversity of *M. bovis* genomes since some isolates do not carry any CC genetic marker [38], [125], [126]; therefore, studies based on whole genome information are needed to provide better insights into *M. bovis* populational structure and evolution [38]. After the large increase in the availability of WGS few comprehensive studies were performed aiming to analyze *M. bovis* genomes at a global scale [43], [116], [122], [127], [128], but still widespread phylogenetic analysis is needed to better understand *M. bovis* progress worldwide.

Molecular epidemiological investigation has been proved to be a useful tool for TB control and surveillance, which allows us to better understand the dynamic of disease transmission and precise identification of the infectious agent [129]. In addition, the knowledge regarding strain diversity within host species has special contemplation in areas under risk of zTB occurrence, thereby providing new insights for establishing strategic measures for TB control and prevention [42], [130].

In an ecosystem where a pathogen can infect multiple species, it is necessary to understand the role of each host species in the infection dynamics to control the disease [131]. Recent studies using WGS have demonstrated a close genetic relationship among *M. bovis* isolates taken from sympatric cattle and wildlife populations [128], [132]. However, the low genomic variability of *M. bovis* and imbalanced sampling across host species has limited the ability to identify the direction of transmission [132].

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CFT	Caudal Fold Tuberculin
CFT10	Culture Filtrate Protein 10
CMI	Cell-Mediated Immune response
DR	Directed Repeated
ELISA IDEXX™	Enzyme Linked Immunosorbent IDEXX Laboratories
ELISA	Enzyme Linked Immunosorbent Assay
EPTB	Extra Pulmonary Tuberculosis
ESAT6	Early Secretory Antigenic Target Protein 6
GLMM	Generalized Linear Mixed Models
GTA	Guide for Animal Transportation
HDI	Human Development Index
LST	Lesions Suggestive of Tuberculosis
MAPA	Ministry of Agriculture, Livestock, and Supply, Brazil
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat
MS	Ministry of Health, Brazil

MTC	*Mycobacterium Tuberculosis Complex*
NTM	Non-Tuberculosis Mycobacteria
OH	One Health
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PNCEBT	Program for Control and Eradication of Bovine Brucellosis and Tuberculosis, Brazil
PNCTB	National Program for the End of Human Tuberculosis
PPD	Purified Protein Derivative
PPDa	Purified Protein Derivative avian
PPDb	Purified Protein Derivative bovine
SCT	Single Cervical Tuberculin
SDGs	United Nations Sustainable Development Goals
Se	Sensitivity
SMS	Shotgun Metagenomic Sequencing
SNPs	Single Nucleotide Polymorphisms
Sp	Specificity
TB	Tuberculosis
The Union	International Union against Tuberculosis and Lung Disease
TST	Tuberculin Skin Test
UK	United Kingdom
US	United States
WGS	Whole Genome Sequencing
WHO	World Health Organization

zTB   Zoonotic tuberculosis

CHAPTER I – INTRODUCTION

Tuberculosis (TB) due to *Mycobacterium bovis* (*M. bovis*) remains a major endemic infectious disease in livestock worldwide and a serious zoonosis [1]. In 2015, it was estimated that more than 50 million cattle were infected by *M. bovis* worldwide [2]. From July to December 2018, of the 188 countries and territories reporting their bovine tuberculosis (bTB) situation to the World Organization for Animal Health (OIE), 96 countries (51%) reported the presence of the disease [3]. In 2019, there were an estimated 140,000 new cases of zoonotic tuberculosis (zTB) and some 12,500 people died of the disease. Although this represents a small proportion of the total human TB disease burden, it's an important hamper to the goal of the World Health Organization (WHO) to end the TB epidemic by 2030 [4].

The economic burden caused by *M. bovis* is multifaced and multisectoral with consequences on livestock production, animal health, wildlife, and human health [5]. In livestock, it impacts negatively on profitability and can decimate years of genetic improvement towards desirable production traits causing global economic losses estimated around $3 billion annually, including those resulting from trade barriers [6]. It also impacts negatively on the welfare of affected farming families [7]. However, most of the time, cost assessments focus primarily on livestock production losses [8]. In the United States (US), bTB was completely eradicated in many herds at a cost of $450 million over 50 years using a "test and slaughter" strategy combined with meat inspection [9]. In England, the fight against bTB has cost almost £500m annually since 2004 and it is estimated that it will top £1 billion over the next decade [10]. In Argentina,

the annual economic losses due to bTB has been estimated to be $63 million [11]. To date, no study has been performed in Brazil to estimate productivity losses in terms of meat and milk and the economic costs due to *M. bovis* infection.

Since 2001, Brazil has the National Program for Control and Elimination of Bovine Tuberculosis (PNCEBT), which is based on "test and slaughter" and risk-zoning [12]. The diagnostic method adopted is the Tuberculin Skin Test (TST), with the intradermal injection of bovine tuberculin Purified Protein Derivative (PPD) [12]. The Caudal Fold Tuberculin test (CFT), the Single Cervical Tuberculin test (SCT), and the Comparative Cervical Tuberculin test (CCT) are the official tests of detection [12]. In contrast, with similar programs in other countries, PNCEBT has no mandatory confirmatory post-mortem tests, such as culture or molecular diagnosis [13]. Grounded in voluntary actions and no compensatory (indemnity) measures for the farmers, PNCEBT has made slow progress toward bTB eradication [13]. From 2005 to 2014, adopting the CCT, 14 epidemiological studies were conducted in 13 states [14]–[27]. The herd prevalence ranged from 0.5% to 11%, while individual animal prevalence was from 0.035% to 1.3%. In Amazonas state, prevalence of bTB is unknown, there are no initiatives toward bTB eradication, and control measures in cattle and buffalo are limited or absent in most of the municipalities. The state bTB economic burden is unknown, however considering the occurrences of carcass condemnations, due to lesions suggestive of bTB, in regional slaughterhouses it is estimated to be considerable.

Brazil is within the 30 highest human TB High Burden countries, accounting for 0.9% of cases worldwide [28]. In 2018, 4,490 deaths were due TB in Brazil and the mortality rate was 2.2 per 100,000 persons with an average annual reduction from 2.2

to 2.3 deaths per 100,000 persons since 2010 [29]. In 2019, 73,864 new cases of TB were identified in Brazil resulting in an incidence rate of 35.0 cases per 100,000 persons. Although there was a constant downward trend in the last decade, the TB incidence rate in the country increased in the years 2017 and 2018 compared to the previous period [29]. Moreover, incidence rates are not homogeneous between states, which requires the development of specific actions, considering the particularities of each location. In Amazonas state, the TB incidence rates have been rising in the last decade, reaching 74.1 cases per 100,000 persons in 2019 – the highest incidence in the country and mortality rate of 3.8 per 100,000 persons [29]. In addition, Manaus, the capital of Amazonas state, presents the highest incidence rate within the capitals with 104.7 per 100,000 persons and mortality rate of 4.7 per 100,000 persons [29]. In Latin America, it is expected a minimum 2% of the total pulmonary TB cases and 8% of extrapulmonary TB cases are caused by *M. bovis* [11]. Although zTB cases have never been reported in Amazonas [30], the major risk factors associated with zTB, such as occurrence of bTB, low rates of milk pasteurization, and high consumption of products from raw milk are daily routine in the state's municipalities. Therefore, a conservative figure expected would be an occurrence of 55 new cases of pulmonary TB and 34 new cases of extrapulmonary TB due to *M. bovis* in Amazonas in 2019.

The pursuit of understanding TB due to *M. bovis* separately in human and animals and without considering the environmental influence leads to an incomplete understanding of disease risks and, consequently, missed opportunities for the control and elimination of the disease. Besides in cattle and humans, *M. bovis* has a wide variety of hosts, including water buffalo (*Bubalus bubalis*), swine, sheep, camelids, and

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free-ranging and captive wildlife. Its ability to infect a such variety of species can be attributed to the different routes of transmission [31]. Knowledge of the TB determinant factor's and their interactions is important for the establishment of public policies and the planning of effective preventive and control measures for the disease. The One Health (OH) approach seems to be most adequate strategy to understand the dynamic of *M. bovis* for the control of the TB epidemic. The OH is a collaborative, multisectoral, and transdisciplinary approach, that recognizes the interconnection between people, animals, plants, and their shared environment, and the closer cooperation between human and animal health results in benefits that are not achieved through the two professions working independently [32], [33]. Cases of OH approaches include actions like the 2000-2003 study to understand the prevalence of Crimean-Congo Hemorrhagic Fever in Kazakhstan; the 2007 investigation that determined toxin from the blue-green algae was killing sea otters in Monterey Bay, California; the 2015 China's Stepwise Approach to Rabies Elimination (SARE) Workshop; and the 2017 Uzbekistan's initiative through multisectoral approach to prevent zoonotic diseases [34]. An OH approach is clearly warranted for TB [35].

CHAPTER II – LITERATURE REVIEW

THE NATURE AND EVOLUTION OF THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

Mycobacterium Tuberculosis Complex (MTC) is a highly successful clonal group of pathogens that cause TB disease in humans and animals [35, 40]. MTC members have evolved from a common ancestor and alignable regions of MTC genomes are over 99.95% identical, with horizontal gene transfer and large recombination events considered absent [38]. These pathogens have exclusively evolved through single nucleotide polymorphisms (SNPs), indels, deletions of up to 26 Kb, duplication of few paralogous genes families, and insertion sequences (IS), leading to a large variation of virulence and host tropism [38]. MTC includes *M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, *M. mungi*, *M. orygis*, *M. pinnipedii*, *M. suricattae*, and *M. tuberculosis* [37], [39].

Genetic evidence indicates that the most common ancestor of MTC emerged some 40,000 years ago from its progenitor in East Africa, the region from where modern human populations disseminated around the same period [44, 46]. Molecular sequencing of the *M. bovis* genome challenged the natural epidemiological hypothesis that *M. tuberculosis* is a human-adapted variety of *M. bovis* that was acquired from cattle [41]. In fact, *M. tuberculosis* and *M. bovis* genomes sequencing, demonstrated that the old story should be reversed, as *M. tuberculosis* is more ancestral than *M. bovis* [41, 46]. Compared with *M. tuberculosis*, *M. bovis* has a reduced genome due to deletion of genetic information. This irreversible genetic event seems to be responsible for differences in pathogenicity and host range between the species [41, 42, 44, 45]. *M.*

tuberculosis comprises seven human-adapted lineages, on the other hand *M. bovis* has a broader host range and can infect and cause TB in multiple host species, including humans [39].

Originally, human TB was thought to be caused solely by *M. tuberculosis*, later a zoonotic form of the disease caused by *M. bovis* and acquired through contact with unpasteurized dairy products was uncovered [37], [44]. Correspondingly, bTB was believed to be caused only by *M. bovis* [42]. Currently the occurrence of interspecies disease by most members of the MTC is well known. Examples of these include: *M. africanum* (humans and cattle), *M. bovis* (cattle, water buffalo, swine, sheep, camelids, deer, antelopes, African buffalo, badgers, brushtail possums, wild boar, ferrets, hedgehogs, rodents, lagomorphs, rhinoceroses, primates, and humans), *M. caprae* (goats, cattle, wild boar, pigs, and humans), *M. microti* (rodents and humans), and *M. tuberculosis* (humans, cattle, primates, elephants, and buffalo) [30, 40]. Recent discoveries in molecular analysis and genomic sequence mapping have led to a new understanding of the pathogenesis, host range, evolution, and phenotypic differences within the MTC [40], [45].

PATHOGENY OF TB

In humans, TB is a pulmonary and systemic disease caused predominantly by *M. tuberculosis.* TB infection occurs when a susceptible person inhales droplet nucleus containing *M. tuberculosis* organisms [46]. The bacilli that reach the alveoli of the lungs are phagocytosed by alveolar macrophages and most of these bacilli are destroyed or inhibited [47]. A small number of bacilli can resist the bactericidal mechanisms induced by the macrophage and multiply within the phagosome, thus avoiding apoptosis [48].

Pathogenic mycobacteria induce macrophage necrosis allowing the release of bacilli to the extracellular environment, where they are phagocytosed by other macrophages [47], [48]. This cycle ends with the formation of granuloma, the hallmark structure of TB [46]–[48]. The granuloma is a compact aggregate of immune cells that segregate the infecting mycobacteria and ends the progressive infection [46]–[48]. Historically, the granuloma is regarded as a host-protective structure. However, recent studies have shown that *Mycobacterium* have a more active role in this process. It is now known that in fact *Mycobacteria* exploit granuloma formation for their proliferation and dissemination within the host [47], [48]. After this point, the bacilli enter in the state of latency, in which the bacillary forms can survive under the conditions of stress generated by the host [49].

M. tuberculosis mostly causes latent infection in mammals, whereas *M. bovis* mostly produces an acute infection [50]. In fact, the most usual manifestation of TB in adults is the reactivation of a pre-existing, chronic infection. On the other hand, there is controversy as to whether this process of passage from active form to latency occurs in cattle with bTB [50]. To date, some researchers propose the existence of latency in bTB [51], [52], although the evidence in the literature is not convincing [52]. In animal models, *M. bovis* has been more hostile than *M. tuberculosis* on experimental infection of cattle, goats, rabbit, and mice [53]–[55]. Moreover, transcriptome and proteome analysis comparing the *M. bovis* and *M. tuberculosis* have demonstrated the host cellular responses to both pathogens differ significantly [56]–[58].

The pathogenesis of the infection is a key component to understanding *M. bovis* epidemiology [59]. Most of the primary understanding of the pathogenesis of the

disease in cattle came from extrapolation of data from humans and laboratory animals [52], [59]. The severity of the disease and the nature of the symptoms depend on the host species susceptibility, invading genotype and on the prevalence of bTB [60]. The distribution of lesions in affected cattle, humans and laboratory animals demonstrate the preeminence of the respiratory system as doorway and primary site of infection [52], [53]. Pathogenesis studies strongly suggest that the route of transmission of bTB is largely via the respiratory system, requiring transmission via infectious aerosols. Therefore, bTB is mainly a respiratory infection and is believed to occur via "direct" aerosol transmission between animals in close contact [7]. Moreover, epidemiological modelling studies considering no latency period in bTB indicates that the disease basic reproductive ratio would range from 1.5 in a herd with 30 cattle up to 4.9 in a herd of 400 animals [61].

In naturally infected cattle, TB lesions are found most frequently in the caudal lobes of the lungs [51], [52], [62], [63], in countries with efficient eradication programs, lesions are found on abattoir inspection with much greater frequency in the lymph nodes associated with the respiratory system than in the lung parenchyma [63]. In Brazil, a study with 140 cattle naturally infected and detected in slaughterhouse inspection found 55% of the animals with some macroscopic lesions, 49% with lesions only in the mediastinal lymph nodes, 28% in the liver, and 14% in the lung only; 6% carcasses showed lesions in the liver, lung and mediastinal lymph node, and 4% had lesions in the lung and lymph node [64]. Another study, with 38,172 cattle, found 0.16% of caseous TB suggestive lesions and 0.11% of calcified TB suggestive lesions, within the inspected organs (lungs, liver, tongue, and lymph nodes of the head and mediastinal)

and lungs were the most affected [65]. Moreover, in a literature review involving 1,879 million animals in slaughterhouses, 1,233 (0.065%) presented TB suggestive lesions, the most affected lymph nodes were bronchial with 628 of the affected animals (50.93%), followed by mediastinal and parotid (both with 11.19%), retropharyngeal (8.11%), pre-scapular (8.60%), and the sum of hepatic, inguinal and sciatic patients totaled 7.53% of the cases [66].

M. BOVIS SURVEILLANCE

A satisfactory system of control and epidemiological surveillance of bTB relies on slaughterhouse inspection. This requires a comprehensive infrastructure, highly trained staff and a reliable register system for tracing back to the herd of origin [13], [67]. In addition, the prevention and control of bTB requires a strong laboratory capacity and access to appropriate diagnostic tools [68]. The diagnosis of *M. bovis* infection can be made either by the direct detection of the etiological agent in biological material or through the indirect detection of a host immune response to the *M. bovis* [63], [67]. The diagnostic conditions of microscopy and the extended time needed for traditional culture methods have attracted attention to develop rapid methods for *M. bovis* detection in clinical specimens and the early identification of mycobacterial isolates. Therefore, this review will focus on selected rapid diagnostic methods.

INDIRECT DETECTION OF *M. BOVIS*

For over 100 years, the TST has been the primary diagnostic test used in TB diagnosis for both humans and cattle. The TST involves the intradermal injection of purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later [69]. In cattle, the wide use of the

TST is justified by its low costs, high availability, long history of use and, for a long time, the lack of alternative screening methods to detect bTB [67], [70], [71]. However, the TST has several well-known limitations including difficulties in administration and interpretation of results, need for a second-step visit, low degree of standardization, and imperfect test accuracy [67], [70]. Additionally, the TST sensitivity is also influenced by the purity [72], potency [73], and dosage of the PPD [74], the age of the animal [75], production type [76], the sensitization of the animal by environmental mycobacteria [56, 57], and by the genetic background of the animal [78]. Worldwide numerous studies have been carried to evaluate the sensitivity (Se) and specificity (Sp) of the TST in cattle under different epidemiological situations using different antigens [71]. Retrospective analysis of the results from the bTB eradication program in the United Kingdom (UK) demonstrated Sp at animal level is 99.983 (standard interpretation) and 99.871 (ultra-severe interpretation) and that at herd level the Sp is 99.2–99.5% (average test size) and 96.5% for larger (250 animals) herd tests [79]. In Ireland, Se of CCT ranged from 89.6 to 91.2% (standard and severe interpretation, respectively) [80]. A meta-analysis of field studies in US cattle herds produced estimates for CFT Se ranging from 80.4% to 93.0% and CFT Sp from 89.2% to 95.2%: estimates for serial interpretation CFT-CCT Se ranged from 74.4% to 88.4% and CFT-CCT Sp ranged from 97.3% to 98.6% [81]. A retrospective Bayesian latent class analysis conducted on 71,185 cattle from 806 herds chronically infected with bTB distributed across Northern Ireland found CCT Se ranging from 40.5% - 57.7% (standard) to 49.0% - 60.6% (severe) and the Sp ranged from 96.3% - 99.7% (standard) to 49.0% - 60.6% [76]. In Brazil, a study in three dairy herds submitted for depopulation where CCT was

compared with culture and Polymerase Chain Reaction (PCR) confirmation, CCT demonstrated sensitivity of 28.2% and specificity of 57.1% [82]. Overall, it's recommended to exercise caution in extrapolating Se and Sp from one environment and/or the tuberculin from one manufacturer and/or one potency, and/or one type of tuberculin test to another test. Additionally, assessing Se and Sp the conditions and the species in which the test is performed should be considered [83]. Another debate regarding the TST is that it precludes implementation of Bacille Calmette-Guérin (BCG) vaccine–based control programs, and BCG vaccination of cattle would be particularly interesting in developing countries where bTB remains endemic and where the testing and slaughter strategy is not feasible [84], [85]. To address this problem, experiments have been done to test alternative antigens to the traditional PPDs used in TST. Recently a novel peptide-based defined antigen skin test to diagnose bTB and to differentiate infected animals from vaccinated animals presented promising results [86]–[88]. Despite all the limitations, it is highly probable that the TST will remain the screening test of choice for farmed livestock for the considerable future [84].

Supplemental tests are used in combination with the TST screening usually to maximize the detection (increasing sensitivity) of infected animals (parallel testing), or to confirm or negate the TST results (serial testing). The gamma-interferon (IFN-γ) assay, the lymphocyte proliferation assay, and the enzyme-linked immunosorbent assay (ELISA) are blood-based laboratory tests recognized by OIE [69]. The IFN-γ assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity [69]. The TST and the IFN-γ test are both based on the detection of the early cell-mediated immune (CMI) response in TB infection [67].

However, in settings where no or poor disease control measures are applied and where the percentage of late-stage diseased animals is believed to be high, CMI-based tests can fail leaving behind false negative animals since in the late stages of the disease, the CMI response may decrease as opposed to a generally increasing humoral immune response [67]. Therefore, in the advanced and generalized stages of TB infections, a certain proportion of anergic animals may be present, which are potentially highly infectious and do not produce a reaction to CMI-based tests. Consequently, serological tests based on the detection of the humoral immune response such as the ELISA, may be particularly important in developing countries [89].

The first serological assays were generally lacking in sensitivity (18%–73%) [90]. Moreover, the presence of environmental mycobacterial infections usually affected their specificity [91]. Further studies identified more specific diagnostic antigens (Table 1) and introduced the multiplex assay strategies allowing for the development of a variety of ELISAs using single or multiple antigen combinations [92]–[97].

The ELISA is a serological technique that measures the binding of specific antibodies to an antigen. The test has several advantages over the methods traditionally used. ELISA permits testing of many samples in a short time, it is simple, rapid, inexpensive, and allows standardization of the technique in different laboratories [93].

MPB70 and MPB83 are among the most studied MTC antigens [91], [93], [94], [98]. They are major antigens highly expressed by *M. bovis* and considerably less expressed by *M. tuberculosis* [98]. MPB70 is a soluble secreted protein expressed by *M. bovis* [96], and MPB83 is a glycosylated lipoprotein which has been detected as being serodominant [94]. Looking for tests able to differentiate *M. bovis* infection from

environmental mycobacteria and BCG, the Early Secretory Antigenic Target protein 6 (ESAT6) and the Culture Filtrate Protein 10 (CFP10) are antigens expressed in *M. bovis* but absent from environmental mycobacteria and *M. bovis* BCG [96], [99] and have been proposed for tests to differentiate between infected and vaccinated animals [86], [100] as well as alternative antigens to the PPDs for blood stimulation in the IFN-γ assay [71]. Recently, recombinant MPB70 and SahH (rM70S) and a native 20-kDa protein (20K) were evaluated as ELISA antigens, the 20K ELISA showed 94.4% sensitivity and 98.2% specificity and had an optimal sample-to-positive (S/P) ratio cut-off of 0.531 [97]. The study showed rM70S ELISA 94.4% sensitivity and 97.3% specificity, both ELISA had acceptable diagnostic efficiency and were recommended to be used for bTB diagnosis as ancillary test [97].

Currently the ELISA IDEXX[TM] seems to be the most prominent serological test used, as it detects the antigens MPB70 and MPB83, however the assay's Se presents great variation by geographic region ranging from 9% in Mexico to 85.4% in the Kuwait [101], [102]. It was hypothesized that the variation in Se was due to genetic factors in relation to *M. bovis* protein coding variation, however this hypothesis was rejected [101]. Therefore, more studies are needed to understand other major factors that appear to impact the Se, such as the stage of the disease and the TST testing a few days before the ELISA is performed [93]–[95], [103].

DIRECT DETECTION OF *M. BOVIS* BY MOLECULAR TECHNIQUES

Molecular epidemiology complements (rather than substitutes) a traditional epidemiological approach [42]. It has been used to confirm epidemiologically suspected transmission, to detect epidemiologically unsuspected transmission, to identify risk

factors and environments where transmission is occurring, to detect laboratory errors and to monitor the efficacy of TB control programs [45]. Additionally, molecular techniques can provide insights into the geographical origin, source, and spread of *M. bovis* among humans and animals [42], [104], [105]. Currently many molecular techniques are used to understand the epidemiology of *M. bovis*, selecting the most appropriate technique in accordance with the existing laboratory conditions and the specific features of the geographic region [42], [106].

PCR-based methods, such as Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) and Spacer Oligonucleotide typing (Spoligotyping), have been extensively used for typing *M. bovis* [106], [107]. More recently, the genome-based Whole Genome Sequencing (WGS) has become the preferential technique to inform outbreak response through contact tracing and source identification for many infectious diseases [108].

Spoligotyping identifies polymorphism based in the presence or absence of spacer units in the Direct Repeat (DR) region [109]. The DR locus is part of the bacterial immune system which provides resistance to foreign DNA (plasmids and viruses) [110]. It is composed of a series of almost identical 36-bp repeats interspersed with unique DNA spacer sequences of similar size (direct variant repeats or DVR units) [111]. The positions of each unique spacer sequence within the DR region are highly conserved [111]. Polymorphisms in various isolates consist in the presence or absence of spacers of a known sequence [112]. This characteristic is used to determine genetic similarities among strains [42]. Spoligotyping is a technique that is fast, robust, low cost and can differentiate strains of *M. bovis* and *M. tuberculosis*, and is useful for identifying sources

of infection, transmission of TB between species in a defined geographical area and is very useful to search for a relationship between strains [11], [113]. Additionally, the results are produced in a simple, digital format easily comparable and exchanged between laboratories. However, spoligotyping has a low discriminatory power, which is the ability of the method to differentiate unrelated isolates, therefore spoligotyping cannot clarify deep questions regarding evolution of the microorganism [63], [106], [114].

The development of high-throughput sequencing technologies has resulted in a dramatic reduction in DNA sequencing costs, making the technology more accessible to the average laboratory [115]. Although PCR-based techniques have been useful for bTB control programs, *M. bovis* WGS will likely replace some of these laborious assays while simultaneously allowing the investigation of outbreaks for which higher resolution is warranted [108]. WGS provides a greater resolution than other molecular markers and can close the gaps between molecular and epidemiological data [116]–[118]. WGS requires bacterial culture, which is a major time limitation, therefore it is not ready to be applied at the point-of-care [118].

The use of WGS to understand outbreaks of *M. tuberculosis* is widespread and provided the basis for phylogenetic analysis resulting in the classification human-adapted MTC in 7 lineages, with *M. tuberculosis* accounting for L1 to L4 and L7, and *M. africanum* comprising L5 and L6 [38], [39]. Currently, *M. bovis* is most frequently classified in Clonal Complexes (CCs). The CCs are groups of bacterial strains that descended from a single cell that was the most recent common ancestor of the resulting complex [119]. To date, 4 different CCs of *M. bovis* have been based on specific

deletions ranging from 806 to 14,094 base pairs (bp), SNPs and spoligotypes [120]–[122]. These were named based on their geographical distribution as: African 1 and 2 restricted to Africa, European 2 commonly found in the Iberian Peninsula, and European 1 distributed globally [120], [121], [123], [124]. However, recent studies have shown that the CCs do not represent the whole diversity of *M. bovis* genomes since some isolates do not carry any CC genetic marker [38], [125], [126]; therefore, studies based on whole genome information are needed to provide better insights into *M. bovis* populational structure and evolution [38]. After the large increase in the availability of WGS few comprehensive studies were performed aiming to analyze *M. bovis* genomes at a global scale [43], [116], [122], [127], [128], but still widespread phylogenetic analysis is needed to better understand *M. bovis* progress worldwide.

Molecular epidemiological investigation has been proved to be a useful tool for TB control and surveillance, which allows us to better understand the dynamic of disease transmission and precise identification of the infectious agent [129]. In addition, the knowledge regarding strain diversity within host species has special contemplation in areas under risk of zTB occurrence, thereby providing new insights for establishing strategic measures for TB control and prevention [42], [130].

In an ecosystem where a pathogen can infect multiple species, it is necessary to understand the role of each host species in the infection dynamics to control the disease [131]. Recent studies using WGS have demonstrated a close genetic relationship among *M. bovis* isolates taken from sympatric cattle and wildlife populations [128], [132]. However, the low genomic variability of *M. bovis* and imbalanced sampling across host species has limited the ability to identify the direction of transmission [132].

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Previous investigations of outbreaks by *M. tuberculosis* in humans [133] and *M. bovis* in cattle [134] demonstrated that even with access to whole sequence data, obtaining directional estimates of transmission might only be possible at the population level and will require dense targeted sampling and thorough epidemiological metadata [131]. Additionally, due to small sample sizes, recent studies were limited in their ability to identify other important epidemiological factors such as host preference. Previous research suggests that host preference amongst MTC members has a basis in host innate immune responses [135]. Moreover, a recent study revealed a distinct response from bovine's macrophage to infection by *M. bovis* and *M. tuberculosis* [58]. Much more data is needed to draw conclusions about the *M. bovis* virulence, species adaptability and directional estimates.

The arrival of high-throughput sequencing platforms has led to studies investigating microbial communities in a broad range of different biological ecosystems, including bovine milk [136]. The microbiome of milk from cattle has been intensely studied, aiming to assess and improve animal health and ensure product quality and safety for human consumption [137], [138]. The Shotgun Metagenomic Sequencing (SMS), which is a method to evaluate bacterial diversity and detect the abundance of microbes in various environments, allows comprehensive sampling of all genes in all organisms present in microbiomes such as milk samples without some of the limitations imposed by culture methods [139]. A few studies on milk microbiota using SMS approach are now available [137], [138]. These studies not only allowed exploration of bacterial communities, but also for archaeal, fungal, and viral communities. They also

produced a functional profile of these microbial communities, including data on microbial metabolism, virulence, and antibiotic resistance [138].

BOVINE TB SITUATION IN SOUTH AMERICA

In South America, bTB is not of uniform importance throughout the region [140]. Scattered information from the past and results from ongoing studies in specific regions show that the prevalence of bTB varies widely between countries and within each country (besides Brazil, already reported earlier in this work). In 2002, a study in Paraguay found 0.7% of 11,000 animals from 10 districts were bTB positive [140]; according to the OIE report bTB is limited to some geographical areas in that country [141]. In 2004, Ramos using SCT tested 1284 cattle and found no reactors in Bolivia in Santa Cruz State [142]. In Venezuela, the national prevalence and incidence were 4.51% and 0.10%, respectively in 2006 [143]. In Argentina bTB is most prevalent in dairy industry areas. Between 1969 and 2004, an average of 10 million carcasses were submitted to official veterinary inspection annually. The percentage of cattle condemned due to TB decreased from 6.7% to 1.2% during this period [140]. In Uruguay, a retrospective study detected a total of 58 bTB outbreaks from 2011 to 2013 [143]. In Ecuador, where there is no bTB national control program, available data from studies aiming to determine the apparent prevalence of bTB showed the rate ranging from 0.33% in 1977 to 8.63% in 2007 [144]. In 2011, the bTB prevalence in Colombia was less than 1% and limited to restricted areas in states and in 2017 the country presented 9,199 accredited TB-free herds [145].

HUMAN TB

TB is an ancient scourge, which has challenged humanity since prehistory and remains a major public health problem [36]. Worldwide, the disease is one of the top 10 causes of death and the leading cause from a single infectious agent [146]. According to the WHO, nearly a quarter of the world's population is infected with *M. tuberculosis* and thus at risk of developing TB disease. In 2019, approximately 10 million people fell ill and 1.2 million died from TB, so it remains the top killer of people with HIV and a major cause of deaths related to antimicrobial resistance [4]. Moreover, the cumulative reduction of TB incidence rate between 2015 and 2018 was only 6.3%, which is considerably below of the End TB Strategy milestone of a 20% reduction between 2015 and 2020 [146]. The challenges to end the TB endemic by 2030 include global migration, an increase of multi-drug resistant tuberculosis (MDR-TB), a large number of missing cases, funding gaps for diagnosis, treatment, care and research, and the zTB [3, 36].

Besides the health problem, TB is also a major economic problem. TB cost the world economy $616 billion from 2000 – 2015, reaching 1% of the gross domestic product (GDP) in several countries, and it is predicted to cost almost $1 trillion US to the global economy between 2015 and 2030 [147]. In Brazil, the average cost of treating one new case of TB is approximately $103 US and the treatment of a multi-resistant patient the cost is 27-times higher [148]. Costs for public service accounted for 65% of hospitalizations, 32% on treatment and only 3% on prevention. The average cost for a family is $1,113.92 US, which means the commitment of about 33% of their income on expenses related to TB [149].

The true global load of human TB caused by *M. bovis* is unknown, because TB caused by *M. tuberculosis* and TB caused by *M. bovis* are indistinguishable clinically, radiologically, and histopathologically [150]. Therefore, to determine the role of each pathogen in TB burden is necessary to identify isolates to species level. However, isolation and confirmatory culture of the pathogen is not routinely performed in the regions where human infections by *M. bovis* are more prevalent [11], [129], [151], [152].

In Latin America, the estimated proportion of zTB due to *M. bovis* accounts for 3% and 8% of pulmonary and extrapulmonary TB cases, respectively [11], [140]. In 2008, a study based on a questionnaire filled out by laboratories in Argentina, Brazil, Chile, Colombia, Costa Rica, Dominican Republic, Ecuador, Peru, Uruguay and Venezuela, and a search of published literature (1970–2007), revealed that *M. bovis* was most likely never isolated from humans in Chile, Colombia, Costa Rica, the Dominican Republic, Peru and Uruguay [153]. Only Ecuador (two cases), Brazil (three cases), Venezuela (one case), and Argentina (10,240 cases) reported bacteriologically confirmed cases of *M. bovis* infection in humans [153]. With the exception of Argentina, the low coverage of tuberculin surveys in cattle, and technical limitations to isolate *M. bovis*, likely contributes to the underestimation of this infection in the majority of countries in Latin America [153].

In Mexico, zTB has a dramatic impact in children [154]. A retrospective study examining 124 TB isolates collected from 1995-2009 revealed 35 of these patients infected with *M. bovis*, based on PCR analyses. Of the 35 *M. bovis* cases, 74% were extra-pulmonary TB, 51% were children, 69% had malnutrition, 51% had consumed unpasteurized milk, 6% had contact with animals, 11% were relapses, and 31% died

[155]. In 2017, a study with 2,736 clinical samples isolated by culture and identified by spoligotyping two samples as *M. bovis.* The isolates presented SNP patterns similar to those found in cattle in different parts of Mexico [156].

In Brazil, zTB is underestimated as well. Conducting an electronic literature search, we found less than a dozen published studies addressing the issue within the last 46 years. In 1974, a seminal study found 3.5% of zTB out of 200 TB cases analyzed [157], although more recent studies have shown that zTB prevalence may be lower. A retrospective study of *M. bovis* diagnoses made at several Brazilian reference laboratories, identified only one case of TB due to *M. bovis* during a 20-year period (1987-2006) [153]. In a 5- year study (2001-2006), a total of 355,383 cultures were performed and no *M. bovis* was isolated [153]. In a Southern state, from 1997 to 2005 approximately 5,000 mycobacterial isolates were analyzed by RFLP and no *M. bovis* was confirmed [153]. In a cross-sectional study, mycobacteria specimens from 189 TB patients living in an urban area in Brazil from 2008-2010, found a low prevalence (1.6%) of *M. bovis* [158]. In 2013, a study in an urban area of Brazil, found three (1.6%) of 189 TB patients with coinfection of *M. bovis* and *M. tuberculosis* [159]. Moreover, a survey with 3,046 suspected TB patients failed to identify any contribution of *M. bovis* to patient TB cases in the Brazilian cities of Rio de Janeiro and Juiz de Fora [160]. As a limitation, all national studies have been restricted largely to urban areas in the southeast region of Brazil, the most developed area of the country. Therefore, rural and undeveloped areas should be included [151] in both national and international studies on zTB to clarify the magnitude of the problem and, when occurring, to identify the main transmission drivers or risk factors in these areas [159].

In 2018, 72,788 new TB cases were diagnosed in Brazil, which corresponds to an incidence coefficient of 34.8 cases/ 100 thousand persons. The two states with the highest TB incidence coefficient were Amazonas (72.9 cases / 100 thousand persons) and Rio de Janeiro (66.3 cases / 100 thousand persons), whose capitals also presented the highest coefficients, being 102.6 cases / 100 thousand persons in Manaus and 89.9 cases / 100 thousand persons in Rio de Janeiro [161]. Even with universal health care and free treatment for TB, *Mycobacterium* culture, identification and susceptibility tests are performed only in TB reference centers, and usually only for selected cases. Therefore, the proportion of new cases of pulmonary TB with laboratory confirmation in Brazil in 2018 was 72.7%, with 74.3% in the northern region of the country. Particularly in Amazonas state, from the 3,077 new cases only 72.4% had laboratory confirmation. In a conservative approach 1.4% of human TB cases accounts for zTB [162], therefore, from those cases with no laboratory confirmation at the very least, 12 human TB cases might be due to *M. bovis*, in Amazonas state.

Moreover, although TB arising from any *M. tuberculosis* complex must be compulsively reported in Brazil [3], the National Surveillance System records do not show what species are responsible for any one particular case. Consequently, a retrospective analysis of the available data will not differentiate TB caused by *M. bovis* from that caused by *M. tuberculosis*. Determining which human TB cases are being induced by *M. bovis* is a major step necessary to elucidate *M. bovis*-induced human TB epidemiology, which will allow more efficient treatment and prevention strategies.

Historically, zTB due to *M. bovis* has been associated with the consumption of raw milk or products made from the raw milk from *M. bovis* infected animals. Few

studies in Brazil have evaluated the presence of *M. bovis* in cheese. In 2103, a study to assess the occurrence of *M. bovis* in raw cheese samples found a positivity rate of 10% as idented by qRT-PCR [163]. In another study 10% of artisanal cheese samples were positive [163], and 2.8% were positive by culture [164]. Both cheese studies occurred in the northwest region of the country. In southern Brazil, a study found 25% of raw and 4% of pasteurized milk samples positive for nine different species of non-tuberculous mycobacteria including: *M. nonchromogenicum, M. peregrinum, M. smegmatis, M. neoaurum, M. fortuitum, M. chelonae, M. flavescens, M. kansasii and M. scrofulaceum* [165]. On the other hand, a study on 130 samples of raw cheese from a farmers' market in Sao Paulo did not detect *M.bovis* contamination [166].

In Brazilian Amazon, especially in small rural towns, raw milk is consumed daily. Household preparations of fermented products with raw milk, like curd or sweet dishes, is a common practice. Therefore, isolation of clinically relevant *Mycobacterium sp.* from raw milk represents potential risk of zTB. Thus far, no studies have been conducted to evaluate the contamination of milk in Amazonas state.

A worldwide systematic review conducted in 32 databases confirmed the strong association between zTB and the extrapulmonary site of disease [167]. In 2018, 12% of all confirmed new cases of TB in Brazil were extrapulmonary, and Amazonas had the same incidence rate [168]. In Amazonas, several studies have found Mycobacteria other than *M. tuberculosis*, causing TB in humans, however *M. bovis* was never reported [169]–[171].

All of this information reinforces the importance of studies to enable understanding of the epidemiology of TB caused by *M. bovis*. Thus, the results of the

research to be conducted will generate important knowledge about the complex

epidemiology of TB in Brazilian Amazon, allowing us to establish regional policies that

effectively reduce the prevalence of the disease in regional livestock, and consequently

reduce the possibility of co-infections with *M. bovis* in the human population.

APPENDIX

Table 2.1: Performance of several antigens used on ELISA for diagnosis in bovine tuberculosis in cattle

Antigen	Animals tested (infected/ non-infected)	Sensitivity (%)	Specificity (%)	Reference
ESAT-6 and MPB70	320/155	98.6	98.5	Koo *et al*., 2005 [92]
CMP70	62/3	84	100	Cho *et al*., 2007 [93]
ESAT-6, MPB70, and MPB83	107/362	67.3-83.2	86.5-95	Souza *et al*., 2012 [95]
MPB83 and MPB70	7/8	63	98	Waters *et al*., 2011 [94]
MPB70, MPB83, ESAT6, CFP10, and tuberculin PPDb	162/1278	74.2	94.9	Fontana *et al*., 2018 [96]
rM70S and 20-kDa	18/975	94.4	97.3	Cho 2020 [97]
		94.4	98.2	

CHAPTER III – HYPOTHESES

The direct correlation between infection by *M. bovis* in cattle and the disease in human population has been well documented in industrialized countries [11], [172], [173]. In developed countries, control and eradication of bovine tuberculosis programs, along with pasteurization of milk, dramatically reduced the incidence of disease caused by *M. bovis* in cattle and humans [9], [11]. Yet, in developing countries, *M. bovis* transmission risk factors among bovines and between bovines and humans are numerous, especially in the tropics [31], [152], [172], [173]. Zoonotic Tuberculosis is widespread where the disease's control measures on the herds are yet to be adopted or just sporadically utilized and pasteurization rarely practiced [44].

The economic and heath impact of TB due to *M. bovis* in animal species including wildlife is significative [5], [9], [44]. The real magnitude of the *M. bovis* impact in human health is unknown although in 2016, WHO estimated that there were 147,000 new cases of zTB in people and 12,500 deaths due to the disease [68]. Good epidemiological studies are needed in order to understand *M. bovis* dynamic within hosts and environment, identify risk factors, and design intervention strategies to end animal and human TB.

The Brazilian nationwide tuberculosis control measures date back to 1952 with the publishing of the Regulation for Industrial Inspection of Products of Animal Origin (RIISPOA), which forbade marketing milk and its derivate products without being previously pasteurized and established the guidelines for the slaughterhouse inspection [174]. The National Brucellosis and Tuberculosis Control and Eradication Program

(PNCEBT), which is based on the sacrificing of all animals displaying positive reaction to tuberculosis tests, was establish in 2004. However, despite these efforts, milk pasteurization and veterinary inspection on slaughterhouses are still not routine in a large part of the country. The disease persists in affecting the herds with serious implications for public health and especially for the country's economy as pertaining to the international market viewpoint [175]. In recent report about Performance of Veterinary Services, the OIE cited an alert for the necessity to re-assess the PNCEBT as to their effectiveness [176].

The Brazilian Program for the Control of Human Tuberculosis (PNCTB), is based on the improvement of the surveillance to increase the detection of new cases, enhance healing, and reduce the noncompliance with treatment. Currently, the diagnosis of active TB in Brazil is based on sputum Acid-Fast Bacilli (AFB) microscopy or rapid assays such as Xpert MTB/RIF®. *Mycobacterium* culture, identification of and susceptibility tests are performed in Tb reference centers, usually for HIV patients and other vulnerable populations. However, in 2019 only 72.2% of the new TB cases had laboratorial confirmation, even with universal health coverage.

Additionally, the Program is designed for detection, treatment, and prevention of tuberculosis caused only by *Mycobacterium tuberculosis*, the agent of tuberculosis in humans. AFB and rapid assays are unable to differentiate the *M. tuberculosis* complex into the distinct species within the *M. tuberculosis complex* meaning that most cases of TB have no defined pathogenic agent or are misclassified. Moreover, culture remains the golden standard method for *Mycobacterium* species identification and the standard medium routinely used in culture, the solid egg-based, glycerol-containing (without

pyruvate supplementation) Lowenstein-Jensen (L-J) or Ogawa media, do not favor isolation of other species of the complex such as the *M. bovis*, the major causative agent of zTB. Therefore, disease caused by *M. bovis* is systemically underdiagnosed. Additionally, there is no systematical survey or data collection about zTB, not even the Standard Operational Proceedings (SOP) have a field determined to report that occurrence. Furthermore, physicians may even be unaware of the risk of zoonotic infection once there is no interaction between human health and animal health sectors to allow the identification of geographical areas or patient groups with a high risk of exposure. All those factors likely contribute to underestimation of zTB cases in Brazil.

Exposure to the *Mycobacterium* not necessarily result in the occurrence of TB. A set of factors such as absence of BCG, poor nutrition, poor ventilation, crowding, poor nutrition, and low immunity need to be combined with exposure to the agent. Moreover, the set of determinants that produce TB infection in one individual may not be the same set of conditions that were responsible for the occurrence of TB in others [177]. Additionally, the Human Development Index (HDI), has been associated with the incidence of TB; in several countries there is a significant inverse relationship between TB incidence and HDI [178]–[180]. Intriguingly, the common-sense epidemiological frames of TB component causes do not sufficiently explain why Amazonas has the highest incidence rate in Brazil. The State has the lowest demographic density in the country and a HDI of above 0.700 which is considered high [181]. Poverty might not completely explain the status of TB in this area. Amazonas State ranks the 18th HDI, and neighborhood States with similar or lower HDI such as, Roraima and Pará respectively present TB incidence rates considerably lower than Amazonas State.

Regarding HIV infection, the North region of Brazil responded for only 6.7% of identified cases from 1980 to 2020 [182]. Moreover, a state-wide populational study in 2016 found negative association between the TB incidence rates and proportion of poor and the unemployment rate [183]. Therefore, there is no set of factors that appropriately explain the magnitude of TB in Amazonas state. Complementary factors such as environmental conditions and human behavior should be studied to clarify this question.

The Amazonas state presents a set of risk factors associated with zTB such as high prevalence of bovine TB within livestock, little or no control of bovine TB program, low rates of milk pasteurization, and a population with the habit of consuming perishable food, especially cheese (curd type) produced from raw milk obtained from regional dairy herds, which suggest the likely influence of *M. bovis* in the profile of tuberculosis of the human population of Amazonas capital. The totality of these factors lead us to the hypothesis that the *M. bovis* represents a risk to human health in Amazonas State.

THE OVERALL GOAL

The overall goal of this project is to describe the status of the *M. bovis* and investigate its potential risk to human health in Amazonas State, Brazil. To address this goal the following hypotheses were tested.

HYPOTHESES

1. The prevalence of *M. bovis* in Amazonas state is higher than the national average.

2. The prevalence of *M. bovis* is significantly higher in buffalo than in cattle populations.

3. Significant risk factors are associated with the prevalence of *M. bovis* in cattle and buffalo in Amazonas.

4. The genetic profile of *M. bovis* in the Brazilian Amazon is different from the rest of the country.

5. The adoption of ELISA diagnosis test as supplemental to the standard Tuberculin Skin Test, can improve the detection of *M. bovis* in herds and animals.

6. Unpasteurized milk as consumed in Amazonas state can pose a potential risk of transmission of *M. bovis* to humans.

To test the six hypotheses, 5 studies were conducted. Study one was designed to address hypotheses 1, 2, and 3; studies two and three were conducted to address hypothesis 4; study four was conducted to address hypothesis 5, and study five was conducted to address hypothesis 6.

CHAPTER IV – EPIDEMIOLOGICAL STUDY OF *MYCOBACTERIUM BOVIS*

INFECTION IN BUFFALO AND CATTLE IN AMAZONAS, BRAZIL

This chapter was published as an original article (*Carneiro et al. Epidemiological study of Mycobacterium bovis infection in buffalo and cattle in Amazonas, Brazil. Front. Vet. Sci. doi: 10.3389/fvets.2019.00434, 2019*)

ABSTRACT

Objective - The aim of this study was to determine bTB prevalence and the main risk factors for the *Mycobacterium bovis* prevalence in cattle and buffalos in Amazonas State, Brazil.

Methods - Tissue samples from 151 animals (45 buffalo and 106 cattle from 5 herds with buffalo only, 22 herds with cattle only, and 12 herds with buffalo and cattle) were obtained from slaughterhouses under State Veterinary Inspection. *M. bovis* were isolated on Stonebrink medium. The positive cultures were confirmed by polymerase chain reaction testing.

Results - The apparent herd and animal prevalence rates were 56.4% and 5.40%, respectively. Regarding animal species, the apparent prevalence rates were 3% in cattle and 11.8% in buffalo. Generalized Linear Mixed Models (GLMM) with random effect were used to assess the association with risk factors on the prevalence. Species (buffalo), herds size (>100 animals) and the presence of both species (buffalo and cattle) in the herd were the major risk factors for the infection by *Mycobacterium bovis* in the region.

Conclusions – 1) The three stated hypotheses were supported by our findings. 2) The findings reveal an urgent need for evidence-based effective intervention to

reduce bTB prevalence in cattle and buffalo and prevent its spread to the human population. 3) Studies are needed to understand why buffalo are more likely to be infected by *M. bovis* than cattle in Amazon. 4) Adoption of zoning, use of data from the inspection services to generate information regarding bTB focus, adoption of epidemiological tools, and discouragement of practices that promote the mixing of cattle and buffalo, were recommended.

INTRODUCTION

Bovine TB remains one of the world's major health problems in livestock. The disease affects the national economy of countries where disease is endemic by causing a decrease in productivity, condemnation of meat in slaughterhouses, and decreasing the ability for international trade [5]. During 2015 to 2016, 179 countries reported the presence of the disease in livestock and/or wildlife, demonstrating its wide geographical distribution [69].

M. bovis is the causative agent of bTB and is also responsible for the zTB which is a major impediment for the success of the global efforts to end TB by the year 2030 [184]. Although estimates of the global burden of zTB are imprecise, in 2016 WHO estimated that there were 147,000 new cases of zTB in humans and 12,500 deaths due to the disease [68]. The human burden of disease cannot be reduced without controlling bTB in the animal reservoirs [68].

In many industrialized countries, the implementation of national bTB programs, based on regular tuberculin testing and removal of infected animals, had led to the successful eradication or a major reduction in the incidence of bTB in cattle herds [13]. However, these control measures have been only partially effective in countries or regions with a wildlife reservoir of infected animals, such as the UK, New Zealand and the United States of America (USA) [185]–[187]. Furthermore, these measures are not affordable in most countries of the world, particularly in countries which have a high prevalence of bTB in their domesticated livestock population [172].

In Brazil, the PNCEBT was establish in 2004 and is based on the sacrificing of all animals displaying positive reaction to tuberculosis tests [175]. In recent years

epidemiological studies were conducted to determine the bTB status in several Brazilian states [15]–[20], [24]–[27], however, no studies were conducted in Amazonas State. Moreover, a detailed understanding of the risk factors involved in the *M. bovis* transmission is an identified gap in bTB studies. Understanding the epidemiology of the disease is fundamental for the development of efficient disease control strategies [85], [172].

Statistical modeling studies are important to elucidate the transmission dynamics of bTB within and between herds [85], [188]–[190]. Additionally, mathematical modeling studies have been carried out to analyze disease transmission and provide insight into useful control measures [191]–[194]. Therefore, we hypothesize that: 1) The prevalence of *M. bovis* in Amazonas state is higher than the national average. 2) The prevalence of *M. bovis* is significantly higher in buffalo than in cattle populations, and 3) Significant risk factors are associated with the prevalence of *M. bovis* in cattle and buffalo in Amazonas.

To test the above three hypotheses, the objective of this study was to ascertain the prevalence of bTB and, through statistical modelling, unveil the main risk factors of the disease in cattle and water buffalos in Amazonas State, Brazil. Ultimately, our goal is to propose evidence-based measures to improve the regional programs for the eradication of TB caused by *M. bovis* in livestock and humans.

MATERIALS AND METHODS

Study population

In Amazonas State, cattle and buffalo, are predominantly managed in extensive and semi-confined systems, there are no herds raised in a total confined system. In an

extensive system, animals remain in the pasture most of the time and the feeding system is based strictly on grazing with mineral salt being offered in feeders on the pasture. Herd health is based on palliative care of animals that present wounds or signs of illness, and the preventive care is restricted to semi-annual vaccination of Foot and Mouth Disease. Cattle are predominantly mixed *Bos indicus* or mixed *Bos taurus indicus*; buffalo are predominantly mixed breeds Murrah, Carabao, and Mediterranean. In semi-confined models, animals are gathered daily in pens where food supplementation and mineral salt are provided in separate feeders. Within the Semi-confined systems, herd health is more appropriate, animals are observed daily for injuries or signs of illness, the preventive care usually is composed of control of parasites, vaccination against Foot and Mouth Disease and Brucellosis. Cattle are predominantly of the Nelore and Girolando breeds, for beef and dairy, respectively. Buffalo are predominantly Murrah (dairy and beef) and Mediterranean (dairy).

In common, the husbandry systems of the two species are influenced by flooding during the raining season. During the rainy season (November to June) herds remain at the mainland areas. During the dry season (July to mid-November) weaned calves, steers, heifers, and dry cows are transported to shared floodplain grassland for beef or recovery purpose. Apui is the only municipality in this study not influenced by flooding. Buffalo and cattle are raised adopting the same management farming system, but due to having more resistance to flooding in regard hoof problems, buffalos are moved from the mainland to the floodplains earlier, and moved back later, than cattle. On average, buffalos spend an additional three months in floodplains compared to cattle.

Herds (n=39) from three intermediary regions and thirteen municipalities were included on this study. Twenty-two herds (56.4%) were composed only by cattle, twelve herds were composed of buffalo and cattle (30.8%), and five (12.8%) herds were composed only by buffalo. The total number of animals inspected during the sampling were 832 (229 buffalo and 603 cattle), and from those 151 samples tissues (45 buffalo and 106 cattle) were obtained (Table 5.1). The median age group of inspected animals in both species were from 25 to 36 months old, and the mean herd size was 142 for cattle and 84 for buffalos.

Of all the animals in the study, 48.3% were from small size herds, 28.4% from medium herds, and 23.1% from large herds. Additionally, 82.7% of the animals were from farms with herds of only one specie (cattle or buffalo). With regard to the purpose, 49.6% of the animals were from herds with mixed purpose, beef and dairy animals represented 31.1% and 19.2% of the sampling, respectively (Table 5.2).

Criteria for inclusion

The study was based on a convenience sampling of adult animals sent for commercial slaughter at three major slaughterhouses in Amazonas State.

From herds with a report of the official tuberculin skin test (TST) performed and reactive buffalo or cattle, samples of all animals sent to the slaughterhouses, with or without Lesion Suggestive of Tuberculosis (LST), were collected. The Caudal Fold Test (CFT), the Simple Cervical Test (SCT), and the Comparative Cervical Test (CCT) are the official tests of detection. The CFT and SCT were adopted as screening tests for beef and dairy cattle, respectively, while the CCT was adopted as a confirmatory test for animals positive at the screening test [13]. From herds with unknown TST status,

samples were collected from all animals with visible tubercles and from animals with suspicious granulomatous lesions.

The inspection of the animals was performed by SIE trained officials, LST were defined as granulomas a mass or nodule of chronically inflamed tissue, yellow or tan, and either caseous, caseo-calcareous or calcified. The same criteria for detection of lesions were used for cattle and buffalo.

Study design

This study is a cross-sectional study performed from March of 2017 to February of 2018. Two samples per animal were collected, one from the suspicious tissue and other from the respiratory system lymph nodes found with increase of volume or LST or from the medial retro-pharyngeal lymph nodes in case of no alterations found in lymph nodes. The option for the medial retro pharyngeal lymph nodes is based on our experience in Michigan [195]. The unit of analysis was the animal. The individual animal was considered bTB positive if the culture growing was confirmed by the polymerase chain reaction (PCR) as *M. bovis*, in either tissue samples. For the herd-level analysis, the herd was considered infected when it presented at least one animal confirmed positive by the PCR analysis. The animals were slaughtered for commercial purposes, there were no animals sacrificed due to this study.

Preparation and culture of samples

Lesions from suspected animals (10 to 25 mg) were processed and inoculated in duplicate into Stonebrink medium [35]. The Stonebrink medium has the same composition as Lowenstein–Jensen, except that glycerol is replaced by 0.5% sodium pyruvate, further incubated at 37° C and evaluated weekly for 90 days to verify bacterial

growth. One medium per sample were used and the colonies with characteristics suggestive of M. bovis were submitted fur DNA extraction.

DNA extraction

The bacterial colonies were washed with 500 μL of Tris-EDTA (TE) buffer in micro-tubes and inactivated in a dry bath for 1 hour at 87° C, with subsequent centrifugation at 14,000 rpm for 2 minutes. The pellet that formed was discarded and the supernatant containing the mycobacterial DNA was transferred to new micro-tubes and stored at -20° C for subsequent analysis.

Microorganism identification by PCR

The mycobacterial DNA samples were submitted to standard PCR according to Sales et al. 2014 [196], using primers Mb.400.F (5'AACGCGACGACCTCATATTC3') and Mb.400.R (5'AAGGCGAACAGATTCAGCAT3'), which amplify a 400 base pair (bp) DNA fragment flanking the region of differentiation 4 (RD4), specific to *M. bovis* [112]. The PCR products were stained with Gel Red and submitted to 1% agarose gel electrophoresis in 1X TAE buffer and visualized in a PhotoDocumentor under ultraviolet light.

Sample size

The sample size should be determined based on expected prevalence in samples from slaughterhouses, however, we are unable to find a previous study with this sample source in the region. In Amazonas state, only one study about prevalence of bTB in buffalos (*Bubalus bubalis*) was found, based on comparative cervical test (CCT) showing a prevalence of 20.4% [197]. Recent studies about bTB prevalence in the region, also based on CCT, showed results ranging from 0.12 (cattle) to 7.2%

(buffalos) in Rondônia and Para State, respectively [19], [198]. Thus, as this study is based on a convenience sampling of cattle and buffalo, an expected prevalence of 10% was used for sample size calculations. With a test sensitivity of 97%, Type I error of 0.5%, and power of 80%, the minimum sample size needed was 139 animals.

Risk factors

The risk analysis was based on data obtained directly from the Guide for Animal Transportation (GTA) and secondary data provided by the Amazonas State Animal Health Agency (ADAF). From each carcass sampled, epidemiological information, such as: origin, specie (cattle or buffalo), herd size, herd age, presence in the farm of both species, farming system, purpose, habitat, herd history of TB, and presence or absence of regular herd health practices, was collected.

Origin was defined by the municipality described on the GTA mandatory for the movement of animals from the farm to slaughterhouses. The species involved were cattle and buffalo (*Bubalus bubalis*), the last raised in the region as livestock for the same purposes as cattle. Herd size was divided into three categories: 1) Small, herds ≤ 99 animals, 2) Medium, herds from 100 to 199 animals, and 3) Large herds with more than 200 animals. The same criteria were used for cattle and buffalo. Herd age was divided in 4 categories: 1) Animals ≤ than 12 months, 2) Animals, 13-24 months age, 3) Animals, 24-36 months age, and 4) Animals older than 36 months. The median age rank of the herd was used for the analysis. The study looked at the species composition of the herd, classifying if the herd is composed only of cattle, only of buffalo or a mix of cattle and buffalo.

Farming systems were divided in three categories: 1) Extensive, characterized by farms with mixed breed herds, low technological level and productivity, 2) Semi-confinement, characterized by farms with a predominant breed, adequate technological level and productivity, and 3) Confinement, characterized by farms with well-defined breeds, specialized for beef or dairy, excellent technical level and productivity.

The purpose of the farm was classified as adopted by ADAF as, Dairy – farms with the main activity to produce milk; Beef – farms of beef cattle; and Mix – farms without mainly objective defined, either can be dedicated to beef, in full or partial cycle (breading, rearing, and fattening) and to produce milk. In mixed farms, beef and dairy animals share environments and facilities.

Regarding the habitat, farms were classified according to the grazing area of the animal. In the Amazon region, herds can be moved between two ecosystems according the river flooding: The floodplains areas flooded during a six-month period characterized by natural pastures of high nutritional value and the mainland areas not under influence of the rivers and characterized by artificial pasture planted after the removal of the native vegetation. Cattle and buffalo herds were classified according to the exposure to Floodplain grazing.

Based on secondary data from ADAF, animals were classified according to the historic presence or absence of bTB in their herds of origin. As the Brucellosis State Program requires vaccination of heifers which can only be done under veterinary supervision, herds with a register of vaccination were classified as having regular veterinary assistance, otherwise they were classified as not having regular herd health.

Statistical analysis

The prevalence was calculated by counting the data (animals *M. bovis* positive) per the reference population during the period of the outcome, according to method described by Dohoo and co-authors [199].

Given the nature of the outcome and number of risk factors, a multi-variable logistic regression model and a Generalized Linear Mixed Model (GLMM) with random effect was used to assess the influence of the risk factors on the prevalence, using 95% confidence intervals ($P \leq 0.05$).

A summary of statistics was computed for each of the risk factors of interest (SAS® 9.4, SAS Institute Inc., Cary, NC, USA). Univariable logistic regression for distinguishable data was conducted for each of the risk factors to assess their degree of association with the outcome variable [200].

The risk of *M. bovis* infection was evaluated using logistic regression for distinguishable data. The dependent variable (*M. bovis* status) was defined as positive if the animal had at least one sample culture positive confirmed by PCR and negative if hadn't reach the inclusion criteria. Due to sampling conducted at the farms, a random-effects term was included during modeling to account for extra-binominal variation attributable to lack of independence between individual animals within farms [200].

The likelihood ratio statistic was used for model development. Therefore, inclusion or exclusion of risk factors were done to test the model. Only those animals, having a complete data set were used for multivariable analysis. Rather than using a fully-saturated model containing all risk factors assessed, a starting model containing a selected subset of risk factors was utilized [200]. The starting model included farm and

individual-animal-level risk factors having risk ratios (RR) with a p-value ≤ 0.5 on univariable logistic-binomial regression. A forward method of variable evaluation using the likelihood ratio statistic was conducted to assess risk factor inclusion or exclusion from the final model. After a variable was added only the ones with a p-value ≤ 0.35 were kept on the model. The goodness-of-fit of the final model was evaluated by calculating the likelihood ratio statistic between the starting and final models and comparing it to the chi-square distribution. Ultimately, the most parsimonious model, was chosen to represent the data collected.

Model development (Table 5.2) provides summaries of herd and individual-animal-level risk factor data compiled for the 151 animals (106 cattle and 45 buffalo) involved in the study. The Generalized Linear Mixed Models (GLMM) with random effect at the individual-animal level were presented at Table 5.3.

The project obtained all necessary approvals from MSU-IRB and IACUC and from IFAM's CEPSH and CEUA.

RESULTS

The overall animal rate prevalence was 5.4%. At individual-animal level, a total of 151 animals (45 buffalo and 106 cattle) were considered suspect of bTB and had tissues collected for laboratory analysis, and from those a total of 45 animals (27 buffalo and 18 cattle) were confirmed by culture and PCR as positive for *M. bovis* infection. Prevalence within species was 3.0% in cattle and 11.8% in buffalo (Table 5.4).

The overall herd prevalence was 56.4%, twenty two out of 39 herds had at least one animal confirmed as infected by *M. bovis*. The apparent prevalence in herds composed only by cattle, by buffalo and cattle, and only by buffalo was respectively,

45.4%, 66.7%, and 80% (Table 5.5). As reported before there were no significant differences between LST samples and no LST [201].

Results from the univariate logistical analysis revealed animals from dairy herds (p = 0.004), frequent veterinary assistance (p = 0.0004), and history of bTB (p = 0.004) were more likely to be infected with *M. bovis*. Additionally, animals that attend the floodplains (p = 0.001), from extensive farming systems (p = 0.006), and from herds with more than 100 animals (p = 0.05) were also more likely to be infected. Moreover, animals equal or older than 25 months were 2.7 times more likely to be infected, and buffalo and cattle living together are 2.63 times more likely to have *M. bovis* infection (Table 5.6).

DISCUSSION

The observed herd prevalence 56.4% and animal rate prevalence 5.40% were the highest reported in Brazil to date [15]–[20], [24]–[27]. Considering only cattle, the 3.0% animal prevalence this study is the highest found in the country, where before the range was 0.04%-1.3% [21], [23]. It should be noted that the number of animals and herds were less than to previous studies, which may represent a limitation in this study. On the other hand, our results were based on microbiological and molecular diagnosis, while the other Brazilian studies were based only on TST screening, meaning that our results represent specificity superior to the previous studies in Brazil. In view of that if the true prevalence is the same than the observed on TST screenings, we would expect a lower prevalence than in the previous studies. Considering the sensitivity of 28.2% and specificity of 57.1% found in a controlled field study [82], the practice of TST as a

screening test for bTB in Amazonas can result in a worrisome number of false-negative animals remaining in herds.

The absence of compensatory measures on the PNCEBT, is a factor to be considered as a hamper for the producers' adherence to the program, successful countries on bTB eradication adopted the screening and elimination police as well as compensatory measures to incentivize animal owners within the programs [13]. Moreover, the only study found in Brazil assessing the use of TST as screening for buffaloes, found 10.81% of false positive and 33.33% of false negative on caudal fold test (CFT) and 0% of false-positive and 66.66% of false-negative on the CCT [202]. These testing limitations for buffalo can represent a major challenge to elimination of bTB in Amazonas. Regardless, the observed herd and animal prevalence rates show the need for effective intervention to reduce the rates of disease in livestock populations. Additionally, it should be pointed out that there a number of slaughterhouses without inspection services in inner cities and there is a local preference for regional cheese made from raw milk. Both these practices substantially increase human exposure to *M. bovis* in Amazonas State.

This is the first bTB epidemiological study in Brazil which includes both cattle and buffalo, to our knowledge. The significantly higher prevalence in buffalo (p>.0001) agrees with previous studies [197], [198], [203]. Factors that might contribute to these results can be inherent to the species, such as behavior. Buffalo are very social and commonly under pasture have a tendency to aggregation. Buffalo are also better adapted to protect themselves from the heat than cattle, in order to reduce the thermic stress, they spend lot of time wallowing in the mud, which can be a potential source of

spreading *M. bovis* within the herd. This is consistent with other studies stating

respiratory transmission via the inhalation of contaminated aerosols or fomites is the

most efficient form of transmission, requiring few numbers of organisms as an effective

dose [31], [172].

Another factor might be the differences in herd management between cattle and

buffalo. Local farmers understand buffalo are more resistant to harsh environmental

conditions than cattle, consequently buffalo farmers may provide less feed and routine

herd health management to buffalo compared to cattle.

A third major factor to consider is genetic differences between Buffalo and cattle

or related to the *M. bovis*. Are buffalo more susceptible to *M. bovis* infection than cattle?

In cattle, *Bos indicus* seems to be more resistant than *Bos taurus* [15], [25], [51], [189],

[204], does the same occur in buffalo? Or it may not be a host factor. Does *M. bovis*

more able to infect buffalo than cattle? Studies to clarify these questions are needed.

Regarding control polices, actions adequate to the reality must be in place, such as:

inspection services must be more alert during inspection of buffalo carcasses in abattoir

and milk in milk plants, as well as information from SIE should be used to identify

infected herds.

Cattle and buffalo from large size herds were more likely to have bTB than

animals from small size herds consistent with other studies conducted in Brazil [14],

[15], [20], [24], [25] and around the world [7], [204]–[209]. Herd size is a major risk

factor, since the number of animals in the herd increase the possibility of the

transmission of the *M. bovis* increases. Moreover, in Amazonas, large herds are more

commercial than small size herds, meaning that they have frequent introduction of

animals from other herds and movement of animals increases bTB transmission risk within the herd. Similar results were found in the neighboring State of Rondônia [19]. Modern modeling studies in England reveal that movement of infected animals was responsible for 84% of newly infected farms [192]. Due to the large territory a good measure to control and eradicate the bTB should the use of the current Foot and Mouth disease zoning for implementation of a bTB zoning and implementation of control measures specifics by the zone, such as: classification of the zones according bTB prevalence, tuberculosis test requirements by zone, and movement control between the zones.

The presence of cattle and buffalo herds on the same farm increases the risk of *M. bovis* infection regardless of the specie. The presence of different livestock species increases the potential for interactions and inter-dependency among cattle and buffalo management; greater exposure leads to greater incidence. Modeling studies suggest that the environment is seriously contaminated when the practices that promote the mixing of cattle and buffalo occur, which also suggests that the cross-infection route promotes the persistence of bTB infection in cattle and buffalo populations [193]. Experience in Australia showed that the complete eradication of bTB from cattle herds was possible only after the elimination of buffalo (Bubalus bubalis) population [13]. This measure is not feasible for Brazilian circumstances, but the practices that promote the mixing of cattle and buffalo must be discouraged.

In this study, animals managed in semi-intensive and extensive systems were 52.32% and 47.68% of the sampling, respectively. Cattle and buffalo from extensive systems were 2.76 more likely to have been infected by *M. bovis* than animals raised in

semi-intensive systems. This can be explained by the fact that animals in extensive systems are more likely to frequent the floodplains where multiple herds share the same pasture thereby increasing their exposure. In addition, in extensive systems, the TST and slaughter of reactors are less frequent than in semi-intensive systems. In order to determine if farming systems are influenced by other risk factors, the multivariable logistic regression demonstrated that once other factors are controlled, extensive systems are in fact protective. Although the risk factor didn't meet the eligibility criteria (p-value = 0.50) to remain in the final model, the result is coherent since semi-intensive herds are more commercial with frequent introduction of new animals from different herds and these findings agree with other studies in Brazil [16], [18], [20], [21], [25].

Based on previous studies of bTB risk factors, the purpose (milk, beef, and mix) is an important risk factor for *M. bovis* prevalence [15], [16], [18], [20], [21], [25], [26], however, in this study when other risk factors are controlled the purpose of the farm wasn't significant (p> 0.81). The regional characterization of the farms in three categories might be an explanation for our results. The "Mix" category adopted to farms with no defined objective (milk or beef) represented almost half of the sampling and can be responsible for confounding within the model. The appropriate characterization of the farming system should be evaluated, considering other factors like breeds predominant in the herd, infrastructure, and the characteristic of neighboring herds. This may provide more accurate representation of the data for models aiming to figure better strategies to break the chain of infection of *M. bovis*.

CONCLUSIONS

1. The three stated hypotheses were supported by our findings.

2. The findings reveal an urgent need for evidence-based effective intervention aiming to reduce bTB prevalence in cattle and buffalo herds and to prevent the spread of *M. bovis* to the human population.

3. Species, herd size, and production system need to be considered when developing disease surveillance and control program in Amazon.

4. State zoning according the bTB prevalence and adoption of measures specific for zones is highly recommended.

5. Information from Inspection Services should be used to identify infected herds.

6. Practices that promote the mixing of cattle and buffalo must be discouraged.

7. Studies are needed to understand why buffalo are more likely to be infected by *M. bovis* than cattle in Amazon.

8. Epidemiological tools, such as modeling should be adopted for bTB control and eradication in Amazon.

This study can stimulate a discussion about the many factors potentially impacting bTB eradication schemes in Brazil and possibly stimulate new research in the areas identified.

APPENDIX

Table 4.1: Distribution of the sampling by origin, number of animals inspected, sample by species, and percent of the sampling, Amazonas State, Brazil.

Region	Municipality	Animals inspected	Buffalo	Cattle	%
Labrea	Apui	122	0	26	17.22
	Manicore	19	0	1	0.66
	Novo Aripuana	83	0	14	9.27
Manaus	Autazes	108	24	2	17.22
	Careiro	24	1	1	1.32
	Careiro da Varzea	121	0	14	9.27
	Iranduba	7	0	4	2.65
	Manacapuru	98	0	4	2.65
	Manaquiri	6	0	6	3.97
	Pres. Figueiredo	80	0	26	17.22
Parintins	Itacoatiara	56	2	8	6.62
	Parintins	50	9	0	5.96
	Urucara	58	9	0	5.96
TOTAL		832	45	106	100.00

Table 4.2: Description and descriptive statistics for animal-level risk factors evaluated for 151 animals (106 cattle and 45 buffalo) in 39 herds in Amazonas State.

Risk Factor [a]	Description	N	%
Specie	Cattle	106	70.20
	Buffalo	45	29.8
Herd size	Small	73	48.34
	Medium	43	28.48
	Large	35	23.18
Herd age	≤ than 12 months,	0	0
	13-24 months age,	42	27.81
	24-36 months age,	54	35.76
	≥ 36 months	55	36.42
Cattle & Buffalo	No	125	82.78
	Yes	26	17.22
Farming System	Confined	0	0
	Semi-confined	79	52.32
	Extensive	72	47.68
Purpose	Beef	47	31.13
	Dairy	29	19.21
	Mix	75	49.67
Habitat	Floodplains	71	47.02
	Mainland	80	52.98
History	No	129	85.43
	Yes	22	14.57
Herd health	No	46	30.46
	Yes	105	69.54

[a] Admitted to the starting multivariable model because it passed screening (p<0.50)

Table 4.3: Generalized Linear Mixed Model with random effects of farm and individual-animal-level risk factors associated with the infection by M. bovis in 832 animals (106 cattle and 45 buffalo) in 41 farms in Amazonas State.

Risk Factor	Description	B	SE(b)	*P-value*	OR	95% CI
Specie	Buffalo	2.5768	1.5379	0.0968	13.15	0.623-277.28
	Cattle	0	.	.		.
Herd size	Large	1.6474	1.3817	0.2358	5.19	0.336 – 80.399
	Medium	1.7940	1.6770	0.2872	6.01	0.216 – 167.20
	Small	0	.	.		.
Cattle & Buffalo herds	No	-1.8588	1.4870	0.2140	0.15	0.008 – 2.973
	Yes	0	.	.		.
Random Effects		4.071	2.0659	.		.

Table 4.4: *M. bovis* prevalence by species in Amazonas State, Brazil.

Species	Animals Inspected	Animals from which the samples were collected	Animals with LST	Positive (culture + PCR)	Study Prevalence
Cattle	603	106	13	18	3.0%
Buffalo	229	45	33	27	11.8%
TOTAL	832	151	46	45	5.40%

LST = Lesions Suggestive of Tuberculosis

Table 4.5: *M. bovis* prevalence by herd in Amazonas State, Brazil.

Herd	Prevalence (%) *
Buffalo	4/5 (80%)
Cattle	10/22 (45.4%)
Buffalo & Cattle	8/12 (66.7%)
TOTAL	22/39 (56.4%)

*The herd was considered infected when it presented at least one animal confirmed positive by the PCR analysis.

Table 4.6: Univariate Logistic Regression of farm and individual-animal-level risk factors associated with the infection by *M. bovis* in 832 animals (106 cattle and 45 buffalo) in 39 herds in Amazonas State.

Risk Factor	Description	B	SE(b)	*P-value*	OR	95% CI
Buffalo & Cattle	Yes	0.96	0.45	0.03	2.63	1.07 – 6.45
	No	0				
Farming	Extensive	1.01	0.37	0.006	2.76	1.33 – 5.71
	Semi-confined	0				
Habitat	Floodplains	1.20	0.37	0.001	3.35	1.59 – 7.03
	Mainland	0				
Herd age	≥ 25 months	0.99	0.50	0.04	2.71	1.007 – 7.31
	< 25 months	0				
Herd health	Yes	1.98	0.56	0.0004	7.24	2.40 – 21.80
	No	0				
Herd size	Large	0.84	0.44	0.05	2.33	0.98 – 5.54
	Medium	0.44	0.43	0.96	1.55	0.65 – 3.68
	Small	0				
History	Yes	1.41	0.49	0.004	4.13	1.554 – 11.013
	No					
Purpose	Dairy	1.51	0.53	0.004	4.54	1.59 – 12.96
	Mix	0.66	0.45	0.14	1.93	0.79 – 4.68
	Beef	0				
Specie	Buffalo	1.06	0.20	<.001	8.40	3.73 – 18.89
	Cattle	0				

CHAPTER V – MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM BOVIS* INFECTION IN CATTLE AND BUFFALO IN AMAZON REGION, BRAZIL

ABSTRACT

Objective - The aim of this study was to characterize *Mycobacterium bovis* from cattle and buffalo tissue samples, from two Brazilian states, and to analyze the genetic diversity of them by spoligotyping.

Method - Tissue samples from tuberculosis suspect animals, 57 in Amazonas State (12 cattle and 45 buffaloes) and six from Pará State (5 cattle and one buffalo) from slaughterhouses under State Veterinary Inspection, which were isolated in culture medium Stonebrink. The positive cultures were confirmed by PCR and analyzed by the spoligotyping technique and the patterns (spoligotypes) were identified and compared at the *Mycobacterium bovis* Spoligotype Data base (http://www.mbovis.org/). There was bacterial growth in 44 (69.8%) of the tissues of the 63 animals, of which PCR for RD4 identified 35/44 (79.5%) as *Mycobacterium bovis*.

Results - Six different spoligotypes were identified among the 35 *Mycobacterium bovis* isolates, of which SB0295, SB1869, SB0121, and SB1800 had already been described in Brazil, SB0822, and SB1608 had not been described. The most frequent spoligotype in this study (SB0822) had already been described in buffaloes in Colombia, a neighboring country of Amazonas state. The other identified spoligotypes were also described in other South American countries, such as Argentina and Venezuela, and

described in the Brazilian states of Rio Grande do Sul, Santa Catarina, São Paulo, Minas Gerais, Mato Grosso do Sul, Mato Grosso, and Goiás, indicating an active movement of *Mycobacterium bovis* strains within Brazil.

Conclusions – 1) Our hypothesis was supported by our findings. 2) A high genetic diversity of *M. bovis* isolates were found in the Brazilian Amazon. 3) The data corroborates with the previous information that buffaloes are more infected than cattle in the region, and 4) Genotypes isolates in this study were reported in neighboring countries suggesting the need for more studies to clarify the rotes of transmission between regions.

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic, infectious disease caused by *Mycobacterium bovis (M. bovis)*, a member of a group called the *Mycobacterium tuberculosis* Complex (MTC), which includes tuberculosis-causing mycobacteria such as *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae*, *M. bovis*, *M. suricattae*, *M. mung*i and *M. orygis* [38], [40], [210].

BTB predominantly affects cattle and buffaloes, but may occasionally infect other mammalian species, including humans [11], [31], [211]. It may spread through direct contact with infected animals, causing the spread of disease among herds or herds to wild animals and vice-versa [31], [63], [210], [212], [213], or being transmitted through indirect contact with contaminated equipment, water and food [36], [63], [213]–[215].

Globally recognized, bTB persists in both developed and developing countries [44], [152], [173], [216]. In Brazil, the Ministry of Livestock and Food Supply (MAPA) was established in 2001 and modified in 2017 as the National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis (PNCEBT), in order to reduce the prevalence and incidence of bTB [13]. The regulation determines the slaughter of all bovines and buffaloes that present a positive reaction to the tuberculin test (ante mortem diagnosis) and as gold standard, isolation in culture medium for identification and confirmation of *M. bovis* infection (post diagnosis -mortem) [12].

Molecular techniques are increasingly used to support conventional methods, both for the identification and confirmation of *M. bovis* strains, and for molecular epidemiology. Molecular genotyping by spoligotyping is a technique developed by Kamerbeek *et al* [109] which discriminates genotypes of *M. bovis* by amplification of the

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polymorphic DR (Direct Repeats) chromosomal locus in the MTC which contains DR sequences interspersed with variable spacer sequences, followed by a reversed line blot hybridization (RLBH). The presence or absence of the spacers is identified. Spoligotyping, by discriminating *M. bovis* genotypes, through RLBH patterns, may aid in bTB control programs, providing epidemiological data among isolates.

Due to the geographic isolation and unique pattern of occupation through rivers, we hypothesize that the genetic profile of *M. bovis* in the Brazilian Amazon is different from the rest of the country. To test this hypothesis, this study was designed to determine the spoligotypes of *M. bovis* isolates from cattle and buffalos in the Amazon region of Brazil.

METHODS

Sample collection

From July 2016 to February 2017, a total of 922 animals were inspected (635 cattle and 287 buffalo), from those 63 samples of cattle (n = 17) and buffalo (n = 46) tissues were obtained. From the herds with report of TST reactive animal samples of all animals sent to the slaughterhouses, with or without lesions suggestive of tuberculosis (LST), were collected. From herds with unknown TST status samples were collected only from animals with LST (Figure 4.1). Three abattoirs were selected based on logistics and willing to participate. The inspection of the animals was performed by trained officials of Amazonas State Veterinary Inspection Service (SIE), LST were defined as granulomas small, spherical, tan, and firm nodules usually with a mineralized core. The same criteria for detection of lesions were used for cattle and buffalo.

Two samples per animal were collected (one from the suspected tissue and one from the retropharyngeal lymph node) the unit of analysis was the animal. For the analysis, herd was considered infected when it presented at least one animal confirmed positive by the PCR analysis. The animals were slaughtered for commercial purposes. Thus, there was no animal sacrifice due to this study.

Herds from ten municipalities of Amazonas and four municipalities in Para were involved. The median age group of inspected animals in both species were from 25 to 36 months old, the mean herd size was 142 for cattle and 84 for buffalos. Unfortunately, we have only data available from Amazonas State, the municipalities on the study have 3,818 cattle herds and 1,315 buffalo herds.

The samples were shipped in a sterile plastic package containing the Guide for Animal Transportation (GTA) number, which has info about animal species, sex, and age but no information about tissue and race. The samples were transported under refrigeration in a thermic container with artificial ice to the Animal Immunology Laboratory of Embrapa Beef-Cattle, located in Campo Grande - MS, for further analysis.

Preparation and culture of samples

Lesions suggestive of tuberculosis (10 to 25 mg) were macerated in 2 mL tubes containing ceramic beads (MagNA Lyser green beads) and 1 ml of sterile water in a MagNA Lyser Instrument (Roche) for three cycles of 30 seconds at 6.000 rpm. Later, 1 ml of 1N NaOH was added, and the tube was incubated at 37°C for 15 minutes. The tube was centrifuged at 3,000 rpm for 15 minutes, and the supernatant was discarded. The decontamination by Petroff method was performed. Briefly, the pellet was suspended in 1 mL of sterile distilled water and 100 ul of 0.2% phenol red solution were

added. After that, 50-100 ul of 1% HCl were added until change of color was visualized − from pink to amber yellow. The pH was adjusted to 7.0 with neutralizing solution and 300 ul of the material was inoculated in duplicate into the Stonebrink medium. [217]. The Stonebrink medium has the same composition as Lowenstein–Jensen, except that glycerol is replaced by 0.5% sodium pyruvate, further incubated at 37° C, and evaluated weekly for 90 days to verify bacterial growth. One medium per sample were used. The colonies with characteristics suggestive of *M. bovis* were submitted to DNA extraction.

DNA extraction

The bacterial colonies were washed with 500 μl of Tris-EDTA (TE) buffer in micro-tubes and inactivated in a dry bath for 1 hour at 87° C, with subsequent centrifugation at 14,000 rpm for 2 minutes. The pellet that formed was discarded and the supernatant containing the mycobacterial DNA was transferred to new micro-tubes and stored at -20° C for subsequent analysis.

This DNA extraction method by has been reported by our laboratory and others [113], [218]–[220].

Microorganism identification by PCR

The mycobacterial DNA samples were submitted to standard PCR, using primers Mb.400.F (5'AACGCGACGACCTCATATTC3') and Mb.400.R (5'AAGGCGAACAGATTCAGCAT3'), which amplify a 400 base pair (BP) DNA fragment flanking the region of differentiation 4 (RD4), specific to *M. bovis* [221], [222]. The PCR products were stained with Gel Red and submitted to 1% agarose gel electrophoresis in 1X TAE buffer and visualized in a PhotoDocumentor under ultraviolet light.

Spoligotyping

The spoligotyping was performed on *M. bovis* isolates, following the instructions of Kamerbeek et al. 1997 [109]. Hybridization of the PCR product was performed on a spoligotyping membrane with oligonucleotides of spacer sequences, using a miniblotter according to the manufacturer's instructions (MapMyGenome; Hyderabad, India). The membrane was incubated with streptavidin-peroxidase and the spacers were detected by ECL chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific), followed by exposure of an x-ray film to the membrane. The patterns visualized in the x-ray film were compared to those contained in the database on the Mbovis.org website (http://www.mbovis.org/) of Complutense University of Madrid, Spain. To analyze and visualize the hypothetical relationship of genetic patterns of the strains, we have applied an eBURST algorithm using the PhyloViz free software (Figure 4.2) [223].

RESULTS

Sixty-three samples from 17 cattle and 46 buffalo, from 25 herds, were isolated in Stonebrink culture medium. Fourteen showed no growth, 13 contaminated cultures were discarded due to presenting growing compatible with environmental contamination and 36 show growth of colony compatible with *Mycobacterium*. In addition, in Amazonas State 7 from 10 municipalities presented positive results and in Para 2 from 4 municipalities presented positive results, showing that the disease is widespread in the region.

PCR results confirmed a total of 35 animals (3.8%) positive for *M. bovis* in 4 isolates of cattle and 27 buffaloes in the state of Amazonas and 4 cattle from Para State

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(Table 4.1). That means a prevalence within species of 1.26% in cattle and 9.41% in buffalo, and a prevalence higher than predicted (2%) (Table 4.1). The result seems to confirm the regional belief that *M. bovis* has higher occurrence in buffalo over cattle in the area. The municipalities with highest prevalence were Autazes (17.86%), Urucara (12.06%), Itacoatiara (5.88%), and Apui (4.2%) in Amazonas State and Prainha (3%) in Para.

The spoligotyping was performed on 35 *M. bovis* isolates and 6 types of spoligotypes were identified: SB0822, SB0295, SB1869, SB0121, SB1800 and SB1608 (Table 4.2). The geographical distribution of the strains is disposed in Figure 4.3. The municipality of Autazes presented the largest variety of strains with 4 spoligotypes, followed by Parintins and Urucará with 2 spoligotypes each. The other municipalities presented only 1 spoligotype each, Manacapuru (SB0822), Itacoatiara (SB1869), Careiro da Várzea (SB0822), and Apuí (SB1800) in Amazonas state, and the municipalities of Alenquer and Praínha in Pará state presented the same spoligotype SB0822. The district of Novo Ceu in the municipality of Autazes presented the largest variety of isolates, with four different spoligotypes in the studied animals, SB0295 (n = 5), SB0822 (n = 4), SB1869 (n = 2), and SB0121 (n=1), highlighting a condition to facilitate the dissemination of distinct genotypes in the region.

The spoligotype SB0822, although considered unusual, was the most commonly observed in this study. This spoligotype was present in the Amazonas municipalities of Careiro da Varzea in one cattle, Manacapuru in two cattle, Urucara in six buffaloes, and Autazes/ Novo Ceu district in four buffaloes. In the state of Pará, one cattle was

observed in the municipality of Alenquer and three in the municipality of Prainha, all with LST.

DISCUSSION

The study was based on a convenience sampling of adult animals sent to three major slaughterhouses in Amazonas State, those slaughterhouses represent more than 70% of all regional cattle and buffalo slaughtered on the region. All herds for the two species, but one, on this study came from the Geographical region known as Low Amazon River which comprehends the East region of Amazonas State and West Region of Para State. The sampling process was performed throughout the rain and dry season which would reduce any potential bias due to the seasonality during the sampling. Moreover, during the dry season, cattle and buffalo herds are concentrated on the floodplains grassland where they have access to high quality pasture and naturally gain weight and improve the immune defenses what could represent a counterbalance to the overexposure to animals from diverse herds with unknown bTB status. On the other hand, during the Rainy season, although less exposed to bTB transmission between herds, the probability of transmission within the herd can increase due to less favorable offer of pasture and proximity of animals during dairy offer of food supplementation.

The regional herds, cattle and buffalo, are mainly managed in extensive system characterized by farms with low technological level and productivity and few managed in Semi-intensive systems characterized by farms with good technological level and productivity. In common, the two systems have the influence of the river floods. During the water floods period (November to June) herds remains at the mainland areas,

during the dry season (July to mid-November) weaned calves, steers, heifers, and dry cows are transported to floodplain's grassland for beef or recovery purpose which represents an additional challenge regarding the infection by *M. bovis* because the floodplain's grassland are shared between herds from several owners with no guarantee of sanitary control. Apuí is the only municipality on this study out of the influence of the Amazon River, with herds in a Semi-intensive system managed only in grasslands not subject to flooding season. Although, not including animals bellow two years old, the sample can be considered representative to determine the status of bTB on regional herds, since the median age of the sample for both species in the study are similar the median age of the respective reference population (25-36 months) and though bTB can occurs in young cattle and buffalo its commonly affect adult animals.

All the herds in the sampling areas are from open herds with frequent introduction of animals from other herds and regions. All, but one, share pasture during the low season of Amazon River and/ or. Those factors can represent a major factor for the bTB dissemination. Modern modeling studies in England reveal that movement of infected animals was responsible for 84% of newly infected farms [85]. Disease control measures basically are reduced to the vaccination against Foot and Mouth disease twice a year, control measures against tuberculosis, such as diagnostic and elimination of positive animals, are not adopted regularly. Some herds of the study presented control of brucellosis by vaccination of the heifers with B19 vaccine.

The study was based on a convenience sampling performed during the routine inspection service at the slaughterhouses. Samples were collected from all animal that came from herds with TST reactive status. However, due to logistic and financial

reasons, animals from unknown Tb status herds, samples were collected only from animas that had lesions. No information about the bTB prevalence on the reactive herds were provided. The authors recognize the situation might be a limitation of the study since the prevalence can be artificially increased if the samples without LST from TST reactive herd presented a significant difference of positive samples however in our analysis this situation was not observed.

The prevalence in buffalo seems agree with previous studies in the region [197], [198] however, at the first study, from 266 skin test reagent animals only 14 were sacrificed for microbiological analysis and the second study, only performed the TST test in our study all TST reagent animals were tested for microbiological and molecular diagnosis of *M. bovis*. In our study the higher prevalence in buffalos might be explained by three factors: an environmental, on this study buffalo herds had less herd health than cattle herds; a behavior factor, buffalo under pasture have a high tendency to stay closer to each other than cattle which favors the transmission of the *M. bovis*, or a genetic factor, buffalo can be more susceptive to specific *M. bovis* strain.

The geographical location of Novo Ceu at the border of two municipalities and with great availability of pasture in flood lands which attracts in transit herds that mingle in the area can be the reason for the prevalence rate and certainly should be particularly observed by the regional Bovine TB control program. The municipalities of Alenquer, Praínha, Careiro da Várzea, Urucará, and Manacapuru, and Itacoatiara, located at the border of the Amazon River, presented shared spoligotypes reflecting the intense transit of animals through the river. Moreover, the municipality of Apuí, located at the Southwest region of Amazonas state and not linked to the Amazon basin, presented a

unique profile suggesting that other factors might drive the *M. bovis* distribution in that area.

The spoligotype SB0822, although considered unusual, was the most commonly observed in this study. This spoligotype was present in the Amazonas municipalities of Careiro da Varzea in one cattle, Manacapuru in two cattle, Urucara in six buffaloes, and Autazes/ Novo Ceu district in four buffaloes. In the state of Pará, one cattle was observed in the municipality of Alenquer and three in the municipality of Prainha, all with LST. The spoligotype SB0822 had not been described in Brazil, but has been previously described in France [224], cattle in Spain [104] and Portugal [225], and buffalo in Colombia [226], suggesting an active transmission of the strain between the animals of the Amazon region and the neighboring country; however, as there are no roads nor known transit of cattle or buffaloes from Colombia to the region of the lower Amazon river. This link between Colombia and the area of study needs to be further explored.

Other spoligotypes considered unusual were observed in this study, as SB1869, which had already been described in São Paulo by Rocha [227], was identified in five buffaloes (14.3%) in the state of Amazonas, one in Itacoatiara, two in Autazes, and two in Autazes/ Novo Ceu district (Table 4.2). Also considered unusual, SB1608 and SB1800 spoligotype were isolated in this study, the spoligotype SB1608 isolated in buffalo in the municipality of Parintins had not previously been described in Brazil and was described in wild animals in Portugal [228], SB1800 was described in Brazil [224] and identified in cattle in Apuí-AM (Table 2).

SB0295 was the second most frequent spoligotype observed, with nine buffaloes in Amazonas, was most frequently found in animals without LST (7/9). This spoligotype

is considered the second most frequent in Brazil [113], described in the state of Paraiba [229], Bahia [230], Mato Grosso [25], Goias [16], Mato Grosso do Sul [231], Santa Catarina [18] and the state of São Paulo [227], but not described or identified in the states of Amazonas and Para. Outside of Brazil it was described in buffaloes in Argentina [113] and alpacas (*Lama pacos*) [232], and wild boars (*Sus scrofa*) in Spain [233].

The spoligotype SB0121 is considered the most prevalent in several studies, and the most frequent in Brazil [113] was observed in two (5.7%) buffaloes in the municipality of Autazes, as well as described in other studies in the states of Bahia [230], Paraíba [234], Mato Grosso [231], Mato Grosso do Sul [231], Goias [228], Minas Gerais [234], and Rio Grande do Sul [112]. Outside of Brazil, SB0121 has been described in Colombia [226], Argentina [113], Venezuela [113], Mexico [235], Portugal [236], and France [237]. In addition, the spoligotype SB0121 was identified as an agent of human tuberculosis in USA [238] and England [239].

The SB0822 and SB0295 spoligotypes were together responsible for 74.3% of strains isolated in the Amazon region. SB1869, considered not very frequent, was the third most common spoligotype observed in this study, with 14.3% of the isolates in opposite to other studies describing SB0121 as the most prevalent, which comprised only 5.7% of the isolates in this study.

Considering that the evolution of the mycobacteria has occurred by successive loss of DNA, the founder spoligotypes would have more spacers than their descendants. The e-BURST can be used with multilocus data to define groups or CC of related isolates derived from a common ancestor, the patterns of descent linking them

together, and the ancestral genotype. The lack of the spacer N°37 from SB0121 would have evolved in the SB0295. By the other hand, the SB0295 could be considered a subgroup founder of spoligotypes SB1608 (lack of spacer N°15) and SB1869 (lack of spacers N°1 and 2). The spoligotype SB0121 could be the founder of SB0822 and SB1800 however, to explain their relationship would have to consider the presence of other spoligotypes not detected in this study (Figure 2). The spoligotyping was the option of choice for genotyping in this study, because after the growth of the isolates in culture, this technique was produced in a short period of time, demonstrating speed and ease. Offering data of diverse strains of *M. bovis* in broad scale, confirmed the existing polymorphism between strains of the Amazon region.

In this study, it was possible to observe a high genetic diversity of *M. bovis* isolates in the Amazon region, including the detection of unusual spoligotypes in Brazil. It also detected a spoligotype found in Colombia, border country with the Amazon region, as well as identical genotypes in the two states of Pará and Amazonas. These facts suggest a possible dissemination of *M. bovis* genotypes by trade / transport of cattle between regions or perhaps a wildlife reservoir might be playing a role in the spatial distribution of *M. bovis* genotypes in the region.

CONCLUSIONS

1. The findings support the hypothesis that the *M. bovis'* genetic profile in the Brazilian Amazon is different from the rest of the country.
2. A high genetic diversity of *M. bovis* isolates were found in the Brazilian Amazon.
3. The data corroborates with the previous information that buffaloes are more infected than cattle in the region.

4. Genotypes isolates in this study were reported in neighboring countries suggesting the need for more studies to clarify the rotes of transmission between regions.

APPENDIX

Table 5.1: *M. bovis* culture, PCR, and prevalence by species.

Species	Animals Inspected	Animals from which the samples were collected*	Animals with LST**	Culture +	PCR +	Study Prevalence
Cattle	635	17	13	9	8	1.26%
Buffalo	287	46	33	27	27	9.41%
TOTAL	922	63	46	36	35	3.8%

*Two samples per animal were collected. One cattle and 9 buffaloes without LST were PCR +. **LST = Lesions Suggestive of Tuberculosis

Table 5.2: Distribution of the 35 *M. bovis* isolates from the Amazon region, according to place of origin, number of animals inspected, species, presence of lesion, and spoligotype found.

Municipality/ State	Animals inspected	Species	Lesion	Spoligotype (n)
Alenquer/ PA	150	Cattle	+	SB0822 (1)
Praínha/ PA	100	Cattle	+	SB0822 (3)
Apuí/ AM	162	Cattle	+	SB1800 (1)
Autazes/ AM	184	Buffalo	+	SB1869 (1)
		Buffalo	-	SB1869 (1)
		Buffalo	+	SB0121 (1)
Autazes (NCD)/ AM	213	Buffalo	+	SB1869 (1)
		Buffalo	-	SB1869 (1)
		Buffalo	+	SB0822 (2)
		Buffalo	-	SB0822 (2)
		Buffalo	+	SB0295 (2)
		Buffalo	-	SB0295 (3)
		Buffalo	+	SB0121(1)
Careiro da Várzea/ AM	98	Cattle	-	SB0822 (1)
Itacoatiara/ AM	17	Buffalo	+	SB1869 (1)
Manacapuru/ AM	298	Cattle	+	SB0822 (2)
Parintins/ AM	50	Buffalo	+	SB1608 (1)
		Buffalo	-	SB0295 (3)
Urucará/ AM	58	Buffalo	-	SB0295 (1)
		Buffalo	+	SB0822 (5)
		Buffalo	-	SB0822 (1)

Autazes (NCD)/ AM: Autazes (Novo Céu District) / Amazonas.
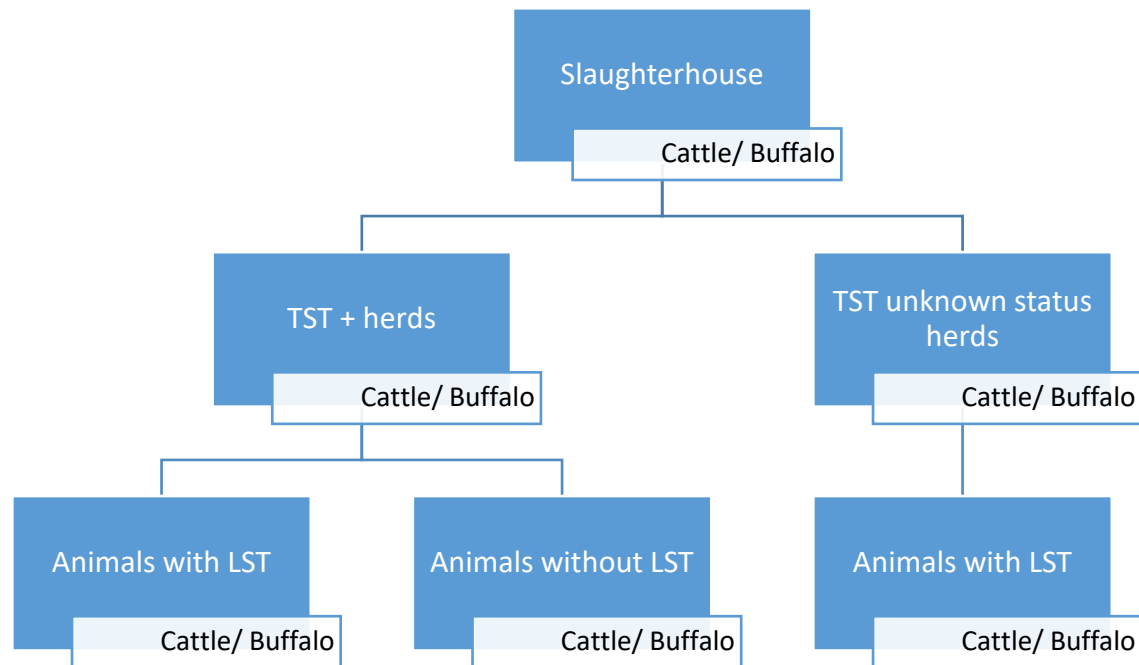
Figure 5.1: Diagram of the sampling process

ST	SB
1	SB0121
2	SB0295
5	SB0822
6	SB1800
7	SB1608
8	SB1869

Amazonas | Para
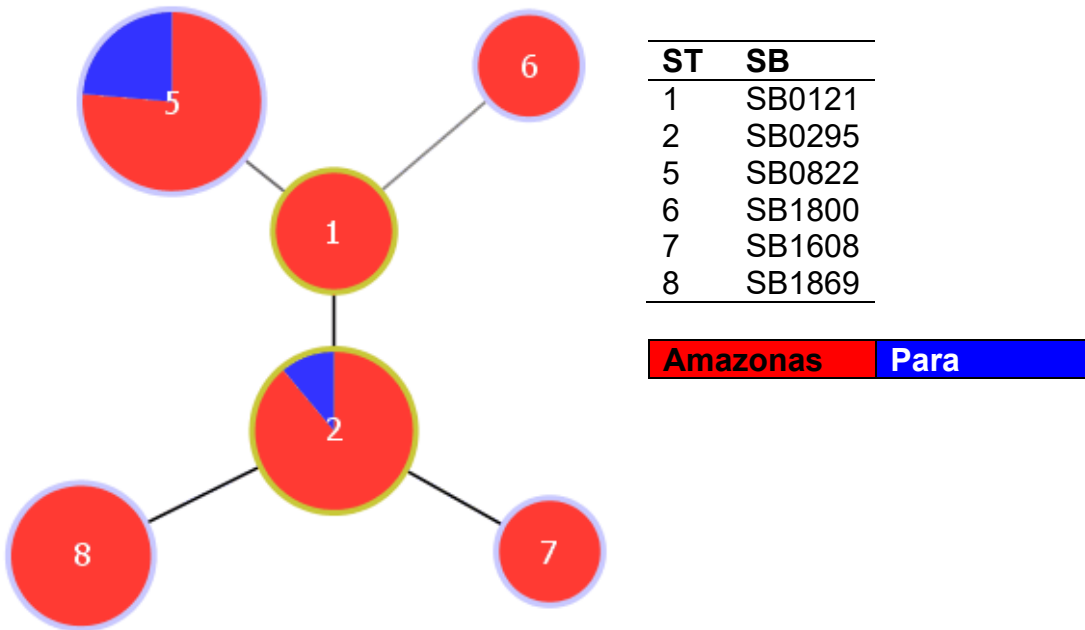
Figure 5.2: e-Burst of the 35 *Mycobacterium bovis* isolates from the Amazon region of Brazil.
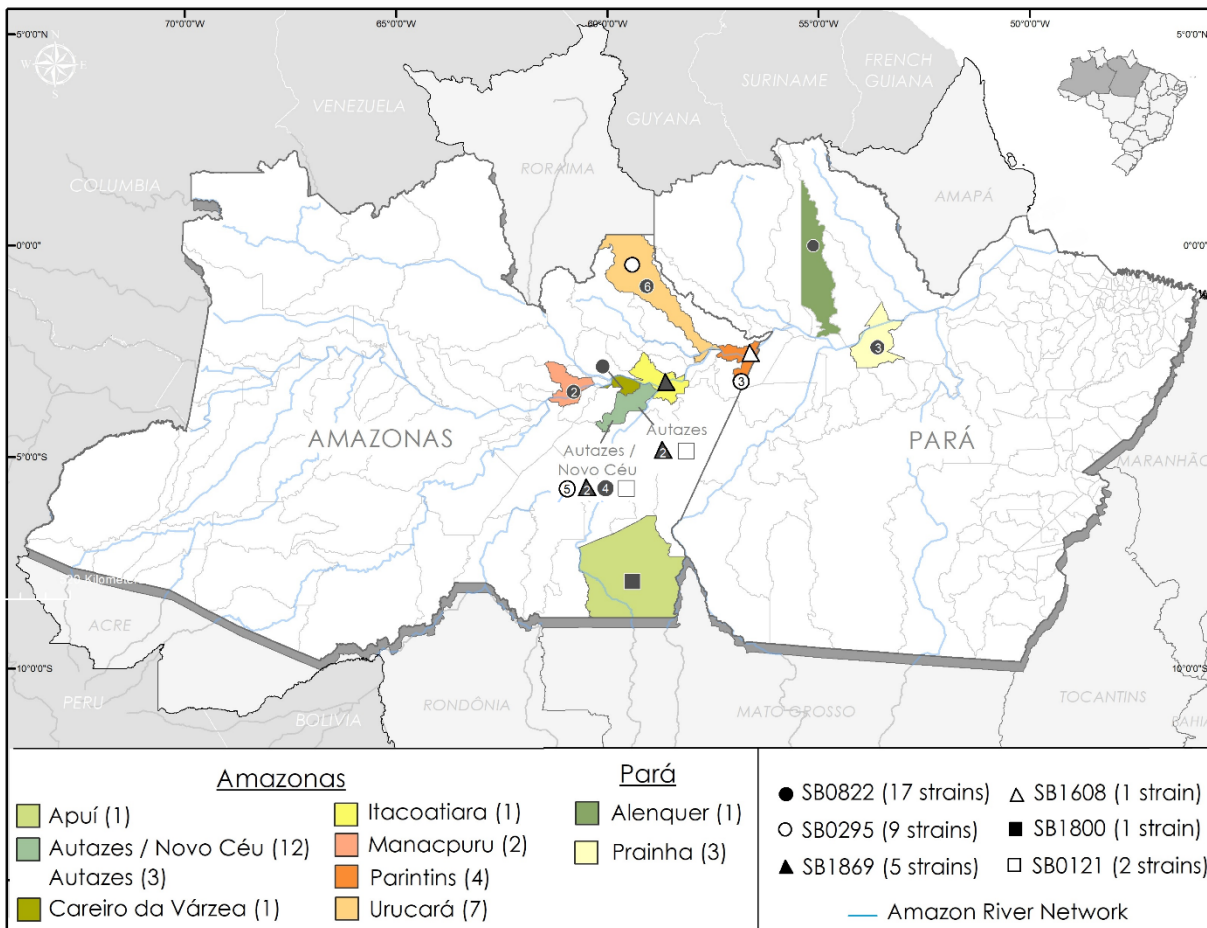
Figure 5.3: Geographic origin and spoligotypes of the *Mycobacterium bovis* isolates from municipalities of Amazon region, Brazil.

CHAPTER VI – GENETIC DIVERSITY AND POTENTIAL PATHS OF TRANSMISSION OF *MYCOBACTERIUM BOVIS* IN AMAZON: THE DISCOVERY OF *M. BOVIS* LINEAGE LB1 CIRCULATING IN SOUTH AMERICA

This chapter was published as an original article (*Carneiro et al. Genetic diversity and potential paths of transmission of Mycobacterium bovis in Amazon: the discovery of a M. bovis Lb1 lineage circulating in South America. Front. Vet. Sci. doi: 10.3389/fvets.2021.630989, 2021*).

ABSTRACT

Objectives – The study aimed to provide a better understanding of *Mycobacterium bovis* (*M. bovis*) populational structure and transmission dynamics affecting cattle and buffalos to contribute in efforts to improve their sanitary status.

Methods – We sequenced the whole genome of 22 *M. bovis* isolates (15 from buffalo and 7 from cattle) from 10 municipalities in the region of the Lower Amazon River Basin in Brazil and performed phylogenomic analysis and Single Nucleotide Polymorphism (SNP)-based transmission inference to evaluate population structure and transmission networks. Additionally, we compared these genomes to others obtained in unrelated studies in the Marajó Island (*n*=15) and worldwide (*n*=128) to understand strain diversity in the Amazon and to infer *M. bovis* lineages.

Results – Our results show a higher genomic diversity of *M. bovis* genomes obtained in the Lower Amazon River region when compared to the Marajó Island, while no significant difference was observed between *M. bovis* genomes obtained from cattle and buffalo (p ≥ 0.05). Two putative transmission cluster were identified. Inter-species transmission between cattle and buffalo was observed. Two *M. bovis* lineages were

detected in our dataset, namely Lb1 and Lb3. Most of the strains of this study (13/22) and all 15 strains of the Marajó Island carried no clonal complex marker

Conclusions – 1) The results of our study supported the hypothesis that the genetic profile of M. bovis, is different in the Brazilian Amazon than the rest of the country. 2) The *M. bovis* CCs classification cannot cover the whole diversity of *M. bovis* strains present in the Amazon region. 3) The first description of inter-species transmission between cattle and buffalo in the Amazon brings implications to the bTB control program. 4) The detection of *M. bovis* Lb1 reveals, for the first time, the circulation of this lineage in South America and requires further investigation. 5) The recent lineage classification better describes the diversity of *M. bovis* in the Amazon.

INTRODUCTION

Mycobacterium bovis (*M. bovis)* is a member of the *Mycobacterium tuberculosis* complex (MTC) and is the leading causative agent of bovine tuberculosis (bTB), an OIE (World Organisation for Animal Health) notifiable disease that affects mainly cattle, buffalo, and other domesticated and wild animals, but can also be transmitted to humans (zTB) [1, 146]. bTB is distributed worldwide but has very low prevalence in most industrialized countries and has even been eradicated in few nations. However, the disease remains a major problem in developed countries with wildlife reservoirs that end up transmitting the pathogen to domestic livestock and vice-versa, and in developing countries where inefficient bTB control programs result in high disease endemicity and spread [1], [151]. In the Brazilian Amazon, few studies aiming to better understand bTB's epidemiology were performed [19], [240]–[242]. The prevalence within animals in the area ranged from 0.1% [19] to 5.4% [242] and the major risk factors associated with bTB were the introduction of new animals into the herds [19], the buffalo species, herds with more than 100 animals, and the presence of cattle and buffalo in the same farm [242].

MTC members evolved from a most recent common ancestor with *M. canettii*, and are characterized as clonal species demonstrating high genomic similarity [243]. Currently, the use of whole-genome sequencing (WGS) to understand tuberculous mycobacteria populational structure is widespread and provided the basis for outbreak tracing and phylogenetic analysis resulting in the classification of human-adapted MTC into 8 lineages, with *Mycobacterium tuberculosis* (*M.* tuberculosis) accounting for L1 to L4 and L7-L8, and *Mycobacterium africanum* comprising of L5 and L6 [38], [244]. On

the other hand, *M. bovis* has been historically classified by Clonal Complexes (CCs), which are identified by genomic deletions, few Single Nucleotide Polymorphism's (SNPs), and/or spoligotypes patterns [108]. Accordingly, four different *M. bovis* CCs have been described presenting distinct geographical distribution patterns: African 1 and 2 restricted to Africa, European 2 commonly found in the Iberian Peninsula, and European 1 distributed globally [120], [121], [123], [124]. With the advent of WGS, recent studies at a global scale provided insights into the population structure and evolution of *M. bovis* lineages [38], showing that CCs do not represent the whole genomic diversity of the isolates [38], [245], [246] and suggesting the existence of at least four *M. bovis* lineages, named Lb1 through Lb4, and three "unknown groups" [38]. With the populational structure of *M. bovis* based on WGS starting to be unveiled, additional studies covering different geographic locations are needed to better comprehend worldwide disease spread and to provide new insights regarding the use of genomes to understand disease transmission at the herd and farm levels.

Molecular epidemiological investigation has been proved to be a useful tool for TB control and surveillance, which allows us to better understand the dynamics of disease transmission and precise identification of the infectious agent [129]. In addition, the knowledge regarding strain diversity within host species has special contribution in areas under risk of zTB occurrence, thereby providing new insights in strain distribution that may help establishing strategic measures for TB control and prevention [42], [130]. In Brazil, few and dispersed molecular epidemiologic studies of *M. bovis* have been reported [201], [247]–[250], and the WGS characterization of the pathogen is just starting to be unraveled [128], [251], [252]. While few studies focused on areas of high

dairy herd productivity [251], [252], a recent study evaluating *M. bovis* genomes obtained from buffalo and cattle of the Marajó Island, North Brazil, was performed and showed the existence of a monophyletic group without CC classification [128].

We hypothesize that the genetic profile of *M. bovis,* characterized by WGS is different in the Brazilian Amazon from the rest of the country. To test this hypothesis, this study was designed to apply whole-genome and SNP-based phylogenomic analyses to obtain novel information regarding the genetic diversity of *M. bovis* strains circulating in buffalo and cattle from the region of the Lower Amazon River Basin. We believe that such information will guide policy development and strategies to contain the disease in livestock, and thus reduce the risk associated with transmission to humans.

MATERIALS AND METHODS

Mycobacterium bovis isolate selection

A total of 24 *M. bovis* isolates were selected, representing one herd of each municipality involved in two previous studies [201], [242] that obtained a total of 63 *M. bovis* isolates from Amazonas State (12 from cattle and 45 from buffalo) and from Pará State (5 from cattle and 1 from buffalo). The selection of *M. bovis* isolates maintained the proportionality according to species (cattle and buffalo) and the local prevalence from the previous studies [201], [242]. Accordingly, these isolates were from tissue samples of 8 cattle and 16 buffalos collected at the slaughterhouse from herds with or without known tuberculin skin test (TST) status originating from 12 different municipalities: Alenquer (n=1), Apui (*n=1*), Autazes (*n=1*), Careiro da Varzea (*n=1*), Itacoatiara (*n=2*), Manacapuru (*n=1*), Novo Ceu (*n=8*), Parintins (*n=3*), Prainha (*n=2*),

Presidente Figueiredo (*n*=1), and Urucara (*n*=3). Samples were collected from June

2016 to October 2017.

DNA extraction

M. bovis isolates were reactivated in Stonebrink media and incubated until

positive growth at 37ºC. DNA extractions from colonies suggestive of *M. bovis* for

genomic sequencing were performed according to the protocol of van Embden et al.

[253], with modifications. Initially, for inactivation, 2-3 colonies were resuspended in 400

µl TE buffer (10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid - EDTA, pH

8.0) and heated at 80°C for 30 minutes. Subsequently, 50 µl of lysozyme (10 mg/ml)

were added and incubated at 37°C for one hour. Then, 75 µl of 10% SDS (sodium

dodecyl sulfate) and 10 µl of proteinase K (10 mg/ml) were added and incubated at

65°C for 10 minutes. Next, 100 µl of 5M NaCl (sodium chloride) and 100 µl of CTAB

(cetyltrimethylammonium bromide) were added, followed by stirring and incubation at

65°C for 10 minutes. After that, 750 µl chloroform/isoamyl alcohol (24:1) were added,

stirred and centrifuged at 12,000 g for 5 minutes. The aqueous phase (surface) was

transferred to another tube, 450 µl of isopropanol were added and incubated at -20°C

for 30 minutes and then centrifuged again at 12,000 g for 15 minutes at room

temperature. The supernatant was discarded, and the pellet washed once with 1 ml of

ice-cold ethanol (70%) with centrifugation at 12,000 g for 5 minutes. After drying the

tube by evaporation at room temperature, the DNA was resuspended in 20 µl of TE

buffer and stored in a freezer at -20°C. The quality and concentration of the extracted

DNAs were evaluated using Nanodrop (Thermo Fisher Scientific). Procedures were

performed in a Biosafety Level 3 Laboratory located at the Embrapa Gado de Corte, Campo Grande, Brazil.

Genome sequencing

WGS was performed at the Laboratory of Molecular Biology Applied to Mycobacteria, of Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. Briefly, DNA quantification was performed using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA) and the Agilent High Sensitivity DNA Kit (Agilent, California, USA). WGS of *M. bovis* isolates was carried out on a NextSeq instrument (Illumina, San Diego, CA) using 2 × 150 paired-end chemistry and the Nextera XT library preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Sequencing reads were deposited in Sequence Read Archive (BioProject number PRJNA675550), NCBI and accession numbers are described in table S1.

Genome quality assessment and RDs (regions of difference) identification

Obtained reads were trimmed using a Trimmomatic version 0.38 [254] for adapters and low-quality base removal (sliding window 5:20). Trimmed reads were then evaluated for reads size, per base and read sequence quality, presence of adapters, and GC content using FastQC [255]. The GC content had to be around 65% (which is common to mycobacterial genomes) and without multiple peaks (i.e. possible contamination with sequences of different GC content) per quality criteria.

As to confirm that genomes were from *M. bovis*, reads were mapped against *M. tuberculosis* H37Rv using Burrows-Wheeler Aligner (bwa-mem) [256] and the positions according to reference genome of RD1 (4,354,000 - 4,358,331 nt), RD4 (1,696,017–1,708,748 nt), and RD9 (2,330,880 – 2,332,100 nt) were evaluated for coverage as

previously described [257]. The genome was considered as *M. bovis* species when RD1 was absent (i.e region was intact) and RD4 and RD9 were present (i.e., regions were deleted) [258]. Obtained coverage against *M. tuberculosis* H37Rv was also used as quality criteria, considering 95% as a minimum mapping percentage for a genome to be included in the analysis.

Spoligotyping and Clonal Complexes (CCs)

Spoligotypes were also investigated *in silico* using SpoTyping [257]. Identified genetic spacers were processed in the *M. bovis* Spoligotype Database (www.mbovis.org) to retrieve a spoligotype pattern and SB number. The CCs African 1 (Af1) and 2 (Af2) and European 1 (Eu1) and 2 (Eu2) were evaluated as previously described [38]. Briefly, the SNP in the *guaA* gene was investigated in the bam files generated from read mapping against *M. tuberculosis* H37Rv, by checking the position 3,813,236. For the RDs, the same bam files were used to investigate read depth using samtools depth [259] and GNU parallel 2018 [260] for the following regions: RDEu1 (1,768,074 – 1,768,878 nt), RDAf1 (665,042 – 668,394 nt), and RDAf2 (680,337 – 694,429 nt) also as described previously [38].

Variant calling

Trimmed *M. bovis* reads were mapped against *M. bovis* AF2122/97 (NC_002945.4) using bwa-mem [256]. Duplicated reads were removed using Picard v2.18.23 (https://github.com/broadinstitute/picard). SNPs were called using Samtools v1.9 mpileup [259] and VarScan v2.4.3 mpileup2cns [261], selecting read depth of 7, mapping quality and minimum base quality of 20, and strand bias filter on, followed by annotation using snpEFF [262]. INDELs (insertions and deletions), as well as SNPs,

from repetitive regions (PE/PPE, transposases, integrases, maturase, phage and repetitive family 13E12 genes) were removed from the analysis using a previously described awk command [38]. Genomes were also evaluated based on the number of heterogenous SNPs, considering 15% as a maximum amount for a genome to be included in the analysis.

Phylogenetic reconstruction

As to evaluate genetic diversity of *M. bovis* in the geographic region, 15 quality-approved *M. bovis* genomes previously sequenced and obtained from cattle and buffalo of the Marajó Island [128] (ENA accession number ERP116404) were used to evaluate the phylogenetic relatedness with the *M. bovis* genomes sequenced in this study. The Marajó Island is geographically close to the targeted region analyzed herein (Figure 1), together with the Lower Amazon River Basin are the region of major concentration of bufallo in the country, and these *M. bovis* genomes [128] were the only strains of the North of Brazil sequenced up until this study. These genomes were quality-assessed and SNPs were obtained as described above. A matrix of concatenated SNPs of the *M. bovis* genomes were constructed as described [38] and used to estimate a maximum likelihood (ML) phylogeny. RAxML version 8.2.12 [263] was used to construct this phylogenetic tree, selecting the GTRCAT model and autoMRE for best-scoring ML tree and a maximum of 1,000 bootstrap inferences. Genomes of *Mycobacterium caprae* (*M. caprae)* (ERR1462591, ERR1462625, ERR1462617, ERR1462581) were also included in the SNP matrix to serve as outgroup.

Minimum spanning tree

A pairwise SNP-distance matrix and a minimum spanning tree were constructed using PhyloViZ [223] with default parameters and using a concatenated SNP matrix of the *M. bovis* genomes as input.

Phylogenomic analysis with lineage representatives of *M. bovis* genomes

M. bovis genomes obtained from this study and those from Marajó Island [128] were compared against a collection of 106 genomes representing the four *M. bovis* lineages (Lb1 – Lb4) and unknown groups 1, 2 and 3 from a recent study that evaluated worldwide distribution and evolution of *M. bovis* genomes [38]. In addition, 21 quality-approved *M. bovis* genomes from France [264], which include representatives of the recently suggested CC called European 3 [265], were also included in the analysis. Genomes of *M. caprae* and *M. tuberculosis* H37Rv (outgroup) were included to construct the phylogenetic tree using the ML approach as described above.

Pairwise SNP-comparisons

From a SNP-distance matrix, pairwise distances distributions between *M. bovis* genomes obtained from cattle versus buffalo (Amazon dataset) and between *M. bovis* genomes originating from the Lower Amazon River Basin and Marajó Island were compared using the non-parametric Mann Whitney test in R software. A result was considered statistically significant when p-value ≤ 0.05.

RESULTS

Out of the 24 *M. bovis* isolates sequenced, two were excluded because of low coverage against the reference genome of *M. tuberculosis* H37Rv or >15% heterogeneous SNPs. These occurred because one isolate resulted in only 38 Kb of

sequencing (i.e. failed to be properly sequenced) and the other showed the presence of mixed-strain infection, respectively. The 22 remaining quality-approved *M. bovis* genomes originated from 10 municipalities: Apui (*n*=1), Autazes (*n*=1), Careiro da Varzea (*n*=1), Itacoatiara (*n*=2), Manacapuru (*n*=1), Novo Ceu (*n*=8), Parintins (*n*=3), Prainha (*n*=2), Presidente Figueiredo (*n*=1), and Urucara (*n*=2) (Figure 6.1).

The genotypic characterization by spoligotyping revealed 5 distinct profiles (Table 6.1). The predominant spoligotype was SB0822, detected in 10 isolates, followed by SB0295, representing 6 isolates; the patterns SB1190 and SB1800 were identified in 2 isolates each; the pattern SB0121 had one representative; and one isolate without described spoligotype pattern. Surprisingly, out of 19 *M. bovis* isolates previously typed using an experimental technique [201], 12 were discordant though disagreement between *in silico* and experimental spoligotyping has been previously described [266], [267]. Finally, comparing hosts, our results show a higher number of spoligotypes patterns in buffalo when compared to cattle (Table 6.1). Given the low sample size, further studies should be conducted to confirm if buffalo have consistently higher diversity of spoligotype patterns when compared to cattle in this region.

Among the CCs, only the CC Eu2 was found among 41% (9/22) of the isolates. The remaining 13 samples (59%) were not identified as belonging to any known CC (i.e. Eu1, Eu2, Af1 or Af2). Within buffalo, 40% (6/15) of the samples were Eu2, 60% (9/15) had no CC marker. Within cattle, 42.8% (3/7) of the samples demonstrated the marker of CC Eu2, and the remaining of the isolates (4/7) were not classified within any described CC. In the generated phylogenetic tree, with *M. bovis* strains from the Lower Amazon River Basin and Marajó Island, the host classes (buffalo and cattle) are found

dispersed among different clades (Figure 6.2), while *M. bovis* genomes from Marajó Island clustered together, appearing more closely related, and genomes from different Amazonas municipalities did not follow a clear clustering pattern according to geographic region.

Pairwise-SNP comparisons (Figures 6.3 and 6.4) and minimum spanning tree (Figure 6.5) of the *M. bovis* genomes obtained herein and from the Marajó Island [128] showed a highly diverse genomic dataset, with pairwise SNP-distances varying from 8 to 711 (from 8 to 100 among *M. bovis* genomes from Marajó Island and from 12 to 711 among *M. bovis* genomes of the Amazon) (Figure 6.3). Although the overall distribution was not significantly different (Figure 6.4 A), *M. bovis* genomes from the Lower Amazon River region tend to show higher pairwise SNP-distances than *M. bovis* genomes from the Marajó Island (Figure 6.3). There was also no significant difference of *M. bovis* genetic diversity distribution using the dataset from the Lower Amazon River region as a function of host (Figure 6.4 B; host information from Marajó Island was not available in ENA).

Reflecting this high diversity, based on current *M. tuberculosis*-based SNP threshold to infer transmission links (~12 SNPs) [268]–[270], only three possible active transmission clusters can be observed, one in Marajó Island (host unknown), one connecting the municipalities of Urucará and Parintins (between two buffalo, TB051 and TB050), and one connecting the municipalities of Careiro da Várzea, Novo Céu and Itacoatiara (involving cattle and buffalo, TB54, TB46 and TB41), demonstrating recent transmission between different herds and hosts (Figure 6.5). Isolates comprising each

transmission clusters also had the same spoligotype pattern (SB0822, SB0822 and SB0295, respectively).

Based on the proposed four major global lineages of *M. bovis* (Lb1, Lb2, Lb3, and Lb4) [7] our setting would be composed by two lineages, Lb1 with two buffalo isolates (TB052 and TB042) and Lb3 with six buffalo and three cattle isolates with the CC marker Eu2 and seven buffalo and four cattle isolates without CC markers (Figure 6.6). All isolates from Marajó Island were also identified as being from Lb3 without a CC marker. The presence of Lb1, infecting two buffalo, is the first description of this *M. bovis* lineage in Brazil [38] and requires further investigation into the actual origin of these isolates.

In order to evaluate if the high genetic diversity observed in *M. bovis* from the Lower Amazon River region was only due to the presence of the two highly divergent Lb1 genomes, we compared the distributions of pairwise-SNP distances between the Lower Amazon River region and Marajó Island for the Lb3 only (Figure 6.4 C). No significant difference in *M. bovis* genetic diversity was observed (Figure 6.4 C). However, the maximum SNP-distance between two genomes observed in the Marajó Island was 100, while in the *M. bovis* genomes of the Amazon (Lb3 only) continued to be high. There was also no significant difference in the genetic diversity of *M. bovis* Lb3 when comparing genomes obtained from cattle and buffalos (Figure 6.4 D).

DISCUSSION

To investigate the clonality and population structure of *M. bovis* in the study area at first, we relied on the use of spoligotyping in the characterization of 22 *M. bovis* isolates from 15 buffalo and 7 cattle from June 2016 to October 2017. The spoligotype

SB0822 in Brazil was first described in our previous study [201] and we agree with the recent study in Marajó Island in Pará state [128] which seems to demonstrate that SB0822 is the predominant spoligotype in the Amazon region, opposite to a national study with 143 samples from 10 states that found only one occurrence of SB0822 [247]. Moreover, SB0822 has been previously described in France [224], cattle in Spain [104] and Portugal [225], and buffalo in Colombia [226], and overall agree with the history of the livestock in the region where the first animals introduced came from Cape Verde (a former Portugal's colony) initially to Marajó Island and from there expanded to the floodplains of the Lower Amazon, on the banks of the Amazon River [271].

Our results also show a higher number of spoligotype patterns of *M. bovis* in the Lower Amazon River Basin compared to the Marajó Island, which can be explained due to the frequent movement of animals in our area of study and the isolation of the herds in the Marajó Island. It is important to highlight that the diversity of *M. bovis* found within buffalo in our sampling is unlikely a result of recent introduction of animals from other Brazilian states since there has been no buffalo imported from other states to the Amazon region and this is probably maintained by constant reinfection from reservoir animals. Accordingly, SB0121 (the most prevalent spoligotyping in Brazil) was found in only one sample, which may reflect the low transit of animals from other states to the area of this study.

Our results show overall higher genetic diversity of *M. bovis* genomes obtained from different municipalities of Lower Amazon River region when compared to the Marajó Island. This is most likely due to the geographic isolation of the island with lower chances of animal importation over time. We also show possible transmission links

between buffalo and cattle from different herds but from close geographic proximity. As wildlife reservoirs have not been identified in Brazil thus far, this transmission may have occurred due to infected animal transit and/or introduction into different herds, showing the presence of a two-host system allowing inter-species transmission in the region.

The detection of an inter-species transmission link, the intertwined phylogenetic dispersal of *M. bovis* obtained from cattle and buffalo, and the absence of significant difference in *M. bovis* genetic diversity in cattle versus buffalo suggest that contact rate between different hosts and consequent geographic proximity likely played a more important role in determining the host range of *M. bovis* in this region than host species, agreeing with recent studies [108], [128]. Currently the most accepted hypothesis is that *M. bovis* is not a specialized pathogen, i.e., can affect several host species irrespective of its genetic makeup. However, we cannot neglect the possibility of differential host susceptibility to different strains or lineages of *M. bovis*, which may allow one particular strain or lineage to thrive in a specific host. Herein and in the study in Marajó Island [128], for instance, there is only 40% power to determine a possible significant difference in distribution of *M. bovis* strains between buffalo and cattle. Therefore, studies with a higher sample size comparing the dynamics of *M. bovis* infection within buffalo and cattle are needed to definitively clarify this research question.

The results from our study support the fact that current CCs cannot represent the whole diversity of *M. bovis* strains. Interestingly, this is the first time that is described in Brazil isolates of *M. bovis* originating from the *M. bovis* lineage Lb1 [38]. In a recent global phylogenomic study of *M. bovis*, strains of Lb1 were shown to emerge from older nodes than Lb3 in the phylogenetic tree and were detected in Eritrea, Ethiopia,

Tanzania, Uganda, Tunisia, France, Spain, Italy, Switzerland, and the United States [38]. However, some strains identified in this lineage carry the CC Af2 marker [38] which is found at high frequency in bTB cases from Mali, Cameroon, Nigeria, and Chad [272], demonstrating the important ties of this lineage to the African continent. One hypothesis to be looked at is that due to the proximity of these countries with Portugal and its colonies; these strains might have been first introduced in the Amazon region during Brazil colonization.

Results from this study, along with others [38], [246], [264], [265], can be used to refine the understanding of *M. bovis* lineage Lb1. With current genomic data, this lineage is composed of two main clusters: one made of *M. bovis* genomes carrying the CC Af2 marker, and the other without known CC markers [38], [246]. Herein, the Lb1 cluster without CC Af2 marker includes 19 out of the 21 French *M. bovis* genomes of SB0120 sequenced by Hauer et al [264] and included in this study. Recently, Branger et al. [273] suggested that this phylogenetic cluster be called CC European 3, also based on their previous work [264]. In this study and in others [38], [246], this cluster is composed of *M. bovis* genomes of many spoligotype patterns (e.g. SB0120, SB0134, SB0828, SB0948, SB1517; Table 6.1) and of BCG vaccine strains [246], [264]. Although 19 specific SNPs have been provisionally suggested to be specific of Lb1 [38] and 5 SNPs of its non-Af2 cluster (i.e. CC Eu3; cluster I in Hauer et al) [264], further studies using comprehensive global datasets should be conducted to confirm or identify definitive genetic markers. The remaining two French *M. bovis* genomes, SRR7851366 and SRR7851376, grouped with genomes of Lb3 and "unknown 3" group, respectively;

their disparate position on the phylogenetic tree corroborates the finding of Hauer et al. [264].

The finding of isolates from Marajó Island belonging to Lb3 without CC marker reinforces the previously described [128] existence of a unique *M. bovis* clade in the island that was likely introduced in a single event. In contrast, our results suggest that *M. bovis* was introduced into the Lower Amazon River region as three different events, for which the temporal order remains to be evaluated. One introduction is related to the neighbor cities of Parintins and Urucara, with strains Lb1. Another introduction occurred with Lb3 strains without the CC Eu2 marker, probably with the same origin from the Marajó Island. And finally, an additional introduction is observed with Lb3 strains carrying the CC Eu2 marker, likely from cattle imported from other states and spreading to buffalo. In 2019, a study with 90 samples of cattle lesions suggestive of bTB from the states of Goias, Mato Grosso, Mato Grosso do Sul, Minas Gerais, São Paulo, Tocantins, and Pará found that 14.4% (13/90) belonged to the CC Eu1 and 81.1% (73/90) to the CC Eu2, while 4.65% (4/90) were not identified as any of the four known complexes [119]. As the isolates without CC markers were not classified into lineages, the data collected seems insufficient to reveal the true epidemiological picture of the bTB in Brazil. Knowing the genetic profile and understanding the transmission routes of *M. bovis* in the Amazon and elsewhere is essential in order to focus on public health and veterinary resources to contain bTB.

A limitation of this study is the sample size. The small number of *M. bovis* isolates may not be representative of the whole bacterial and animal populations of the

Lower Amazon River Basin. Results must be interpreted in light of this fact, and efforts should continue to isolate and study additional *M. bovis* strains from the region.

CONCLUSIONS

1. The results of our study supported the hypothesis that the genetic profile of *M. bovis,* is different in the Brazilian Amazon than the rest of the country.

2. The *M. bovis* CCs classification cannot cover the whole diversity of *M. bovis* strains present in the Amazon region.

3. The presence of *M. bovis* strains Lb1 infecting buffalo requires further investigation into the actual origin of these isolates, showing that the true global diversity of *M. bovis* strains remain to be discovered, likely influenced by cattle trade over history, and.

4. The *M. bovis* classification in lineages by SNP-based phylogenetic analyses seems to better cover the diversity of *M. bovis* strains present in the Amazon region compared to CC classification.

APPENDIX

Table 6.1: Distribution of *Mycobacterium bovis* in Amazon by municipality, host, and spoligotype.

Municipality	Species	Spoligotype
Apui	Cattle	SB0822
Autazes	Buffalo	Unknown*
Careiro da Varzea	Cattle	SB0295
Itacoatiara	Buffalo	SB0295
	Cattle	SB0295
Manacapuru	Cattle	SB0822
Novo Ceu	Buffalo	SB0822 (3)
	Buffalo	SB1190 (2)
	Buffalo	SB0295 (2)
	Buffalo	SB0121
Parintins	Buffalo	SB0822 (2)
	Buffalo	SB1800
Prainha	Cattle	SB0822
	Cattle	SB0295
Presidente Figueiredo	Cattle	SB0822
Urucara	Buffalo	SB1800
	Buffalo	SB0822
TOTAL		**22**

* unknown spoligotype pattern: we have submitted the pattern to M.bovis.org database, but up until this publication, a new SB number has not been provided.

Table 6.2: Distribution of *M. bovis* in Amazon by Municipality, year, host, Clonal Complexes, and Lineages.

Municipality	Year	Host species	Lb1	Lb3	
				Eu2	No CC marker
Apui	2017	Cattle	-	-	1
Autazes	2017	Buffalo	-	-	1
Careiro da Várzea	2017	Cattle	-	1	-
Itacoatiara	2017	Buffalo	-	1	-
	2017	Cattle	-	1	-
Manacapuru	2017	Cattle	-	-	1
Novo Ceu	2016	Buffalo	-	5	3
Parintins	2017	Buffalo	1		2
Prainha	2017	Cattle	-	1	1
Presidente Figueiredo	2017	Cattle	-	-	1
Urucara	2017	Buffalo	1	-	1
TOTAL			**2**	**9**	**11**

Eu2 = Clonal Complex European 2.

CC = Clonal Complex.

Lb1 and Lb3 = lineages of *Mycobacterium bovis* 1 and 3.
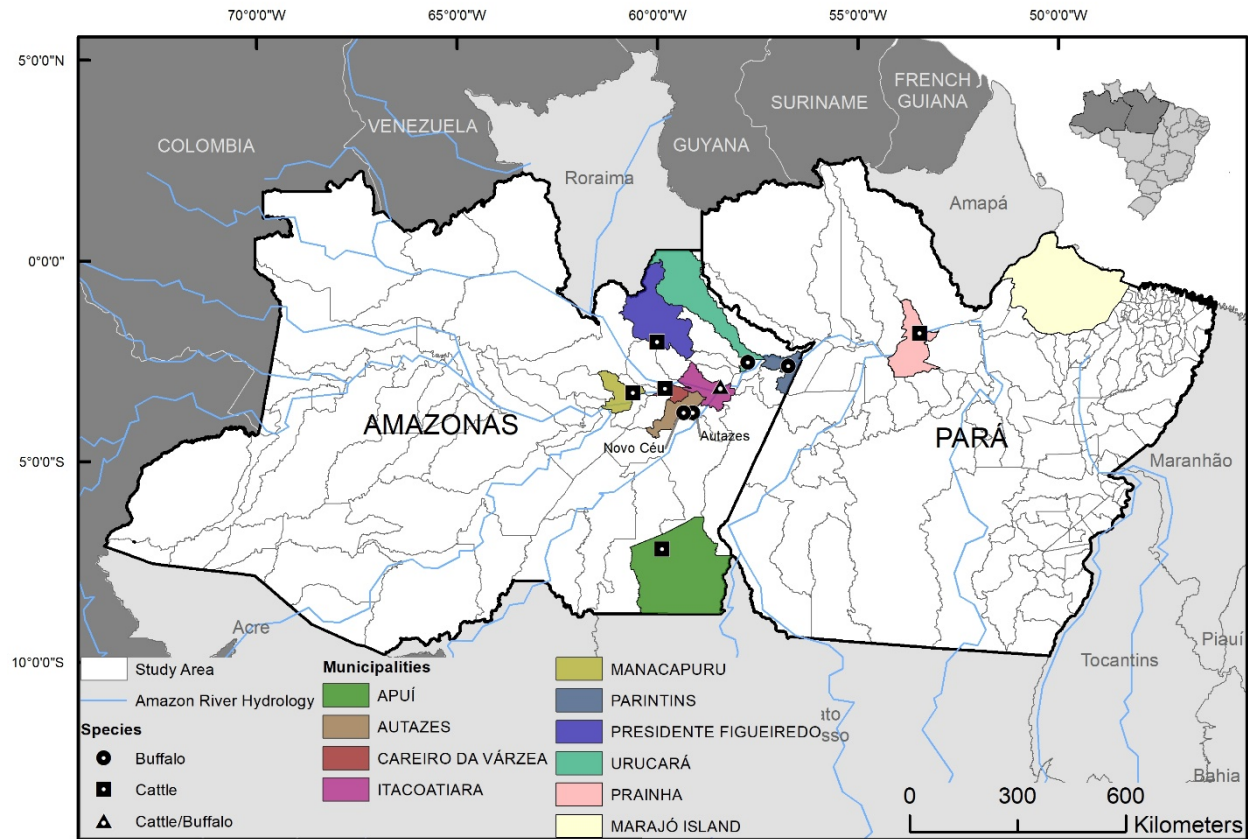
Figure 6.1: Geographic origin and host species of the *Mycobacterium bovis* isolates from municipalities of Lower Amazon River Basin, Brazil. Isolates were selected from 35 M. bovis strains previously isolated from cattle and buffalo [201]**.**
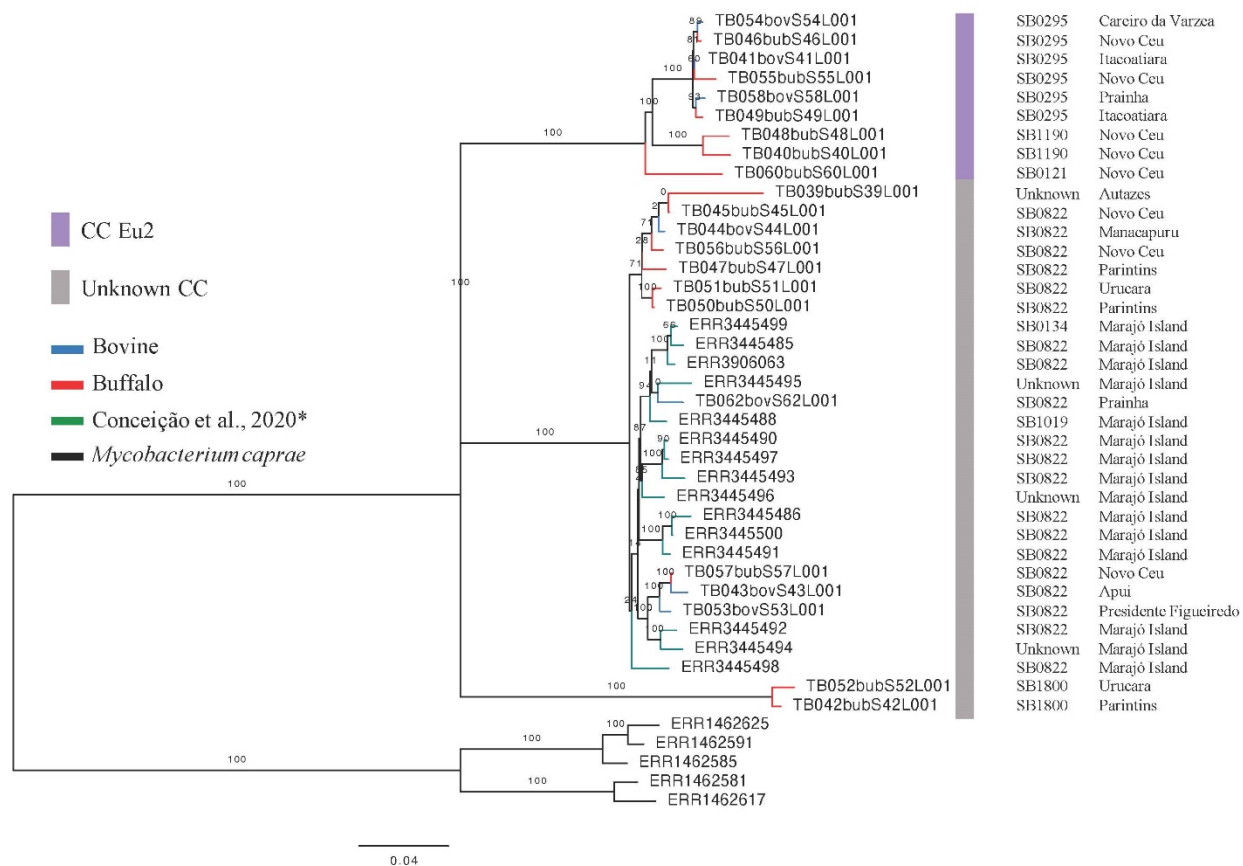
Figure 6.2: Phylogenetic analysis of *Mycobacterium bovis* genomes from North of Brazil. Maximum likelihood (ML) phylogenetic tree from concatenated SNPs (single nucleotide polymorphisms) of *Mycobacterium bovis* genomes sequenced in this study and by Conceição et al. [128] from Marajó Island. Blue tips: *M. bovis* genomes obtained from cattle and sequenced in this study; red tips: *M. bovis* genomes obtained from cattle and sequenced in this study; green tips: *M. bovis* genomes from unknown hosts sequenced by Conceição et al., 2020 and obtained from cattle or buffalo from Marajó Island, Pará; black tips: *Mycobacterium caprae* genomes (outgroup). Purple bar: *M. bovis* genomes carrying markers of European 2 clonal complex; grey bar: *M. bovis* genomes carrying no markers of known clonal complexes. SB: spoligotype patterns. The phylogenetic tree was generated using RAxML and annotated using FigTree v1.4.3 [274]. Horizontal bar shows substitutions per nucleotide. *unknown spoligotype pattern: we have submitted the pattern to M.bovis.org database, but up until this publication, a new SB number has not been provided.
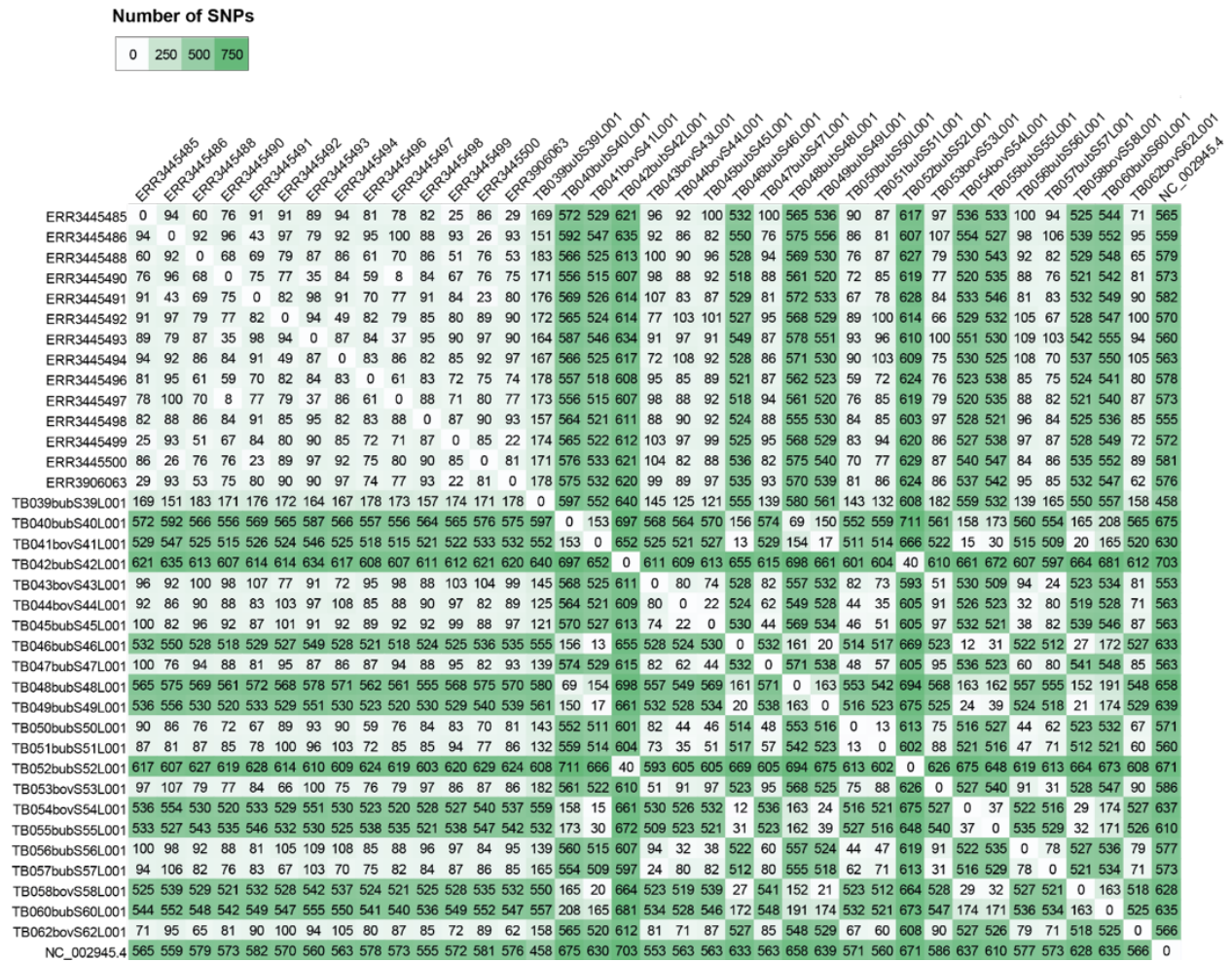
Figure 6.3: Pairwise single nucleotide polymorphism (SNP)-distance between *Mycobacterium bovis* genomes of Northern Brazil. Genomes of *M. bovis* from the Lower Amazon River Basin (sequenced in this study) and from Marajó Island (sequenced by Conceição et al. [128]) are included. Genomes starting with ERR are from Marajó Island, while genomes starting with TB are from the Lower Amazon River Basin. NC_002945.4: reference genome *Mycobacterium bovis* AF2122/97. Pairwise comparisons were generated from concatenated SNP matrix using PHYLOViZ [223].
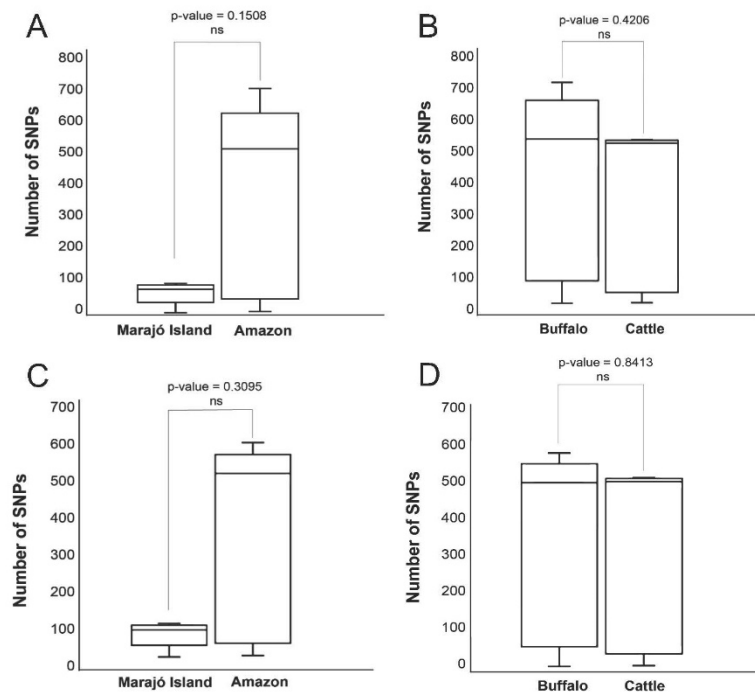
Figure 6.4: Distributions of pairwise single nucleotide polymorphism (SNP) distance of *Mycobacterium bovis* genomes of North Brazil. (A) Comparison of *M. bovis* pairwise-SNP distance between genomes originating from the Lower Amazon River Basin (sequenced in this study) and from Marajó Island (sequenced by Conceição et al., 2020[128]). (B) Comparison of *M. bovis* pairwise-SNP distance between genomes obtained from buffalo and cattle in the Lower Amazon River Basin. (C) Comparison of *M. bovis* lineage 3 (Lb3) pairwise-SNP distance between genomes originating from the Lower Amazon River Basin (sequenced in this study) and from Marajó Island (sequenced by Conceição et al., 2020[128]). (D) Comparison of *M. bovis* Lb3 pairwise-SNP distance between genomes obtained from buffalo and cattle in the Lower Amazon River Basin.
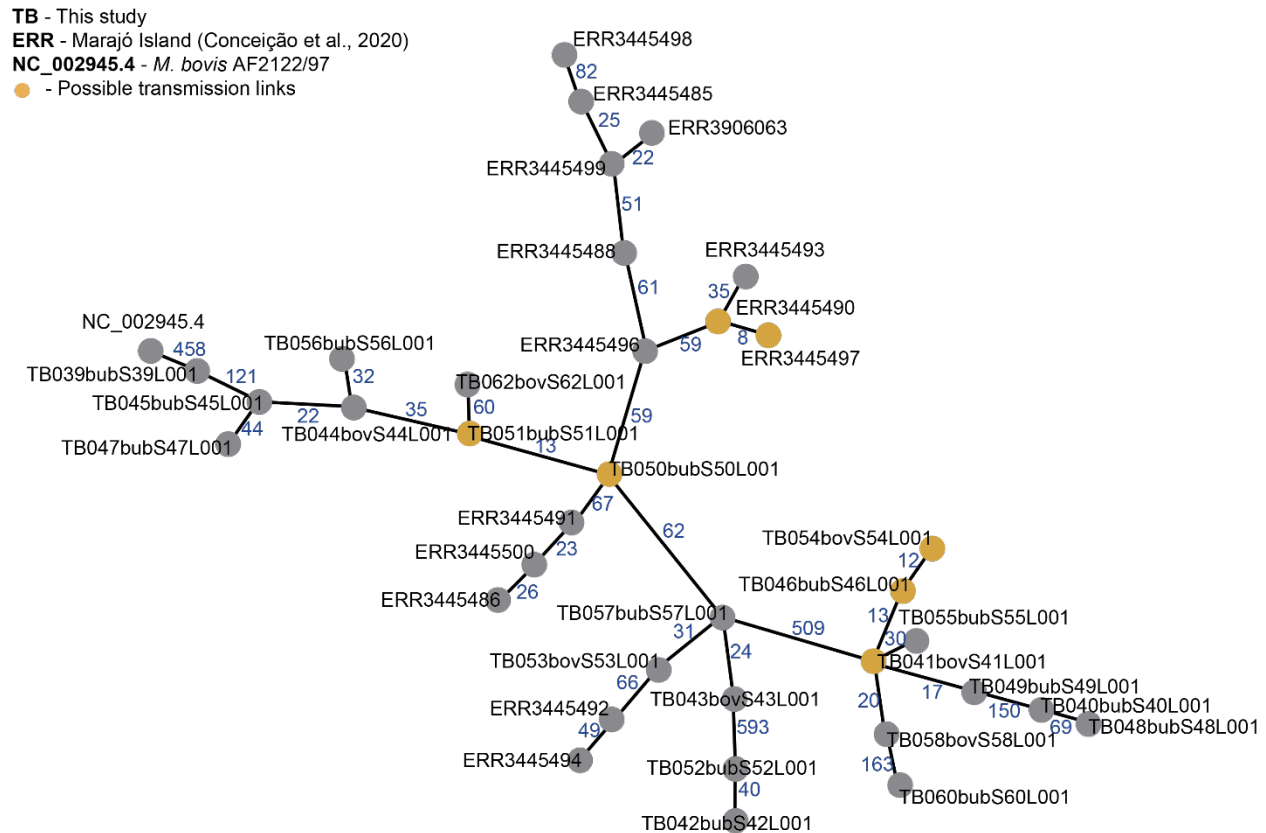
Figure 6.5: Minimum spanning tree (MST) of *Mycobacterium bovis* genomes from North Brazil. Genomes of *M. bovis* from Amazonas state (sequenced in this study, starting with TB) and from Marajó Island (sequenced by Conceição et al. [128], starting with ERR) are included. NC_002945.4: reference genome *Mycobacterium bovis* AF2122/97. Nodes represent *M. bovis* genomes and edges the number of SNPs separating two genomes. Orange nodes represent possible transmission links using an approximate cut-off of 12 single nucleotide polymorphisms (SNP) distance between two *M. bovis* genomes. MST was generated from a concatenated SNP matrix using PHILOViZ [223]. Possible transmission links: TB054, TB046 and TB041 are from the municipalities of C. da Varzea, Novo Ceu and Itacoatiara; TB051 and TB050 are from Urucará and Parintins; ERR3445490 and ERR3445497 are from Marajó Island.
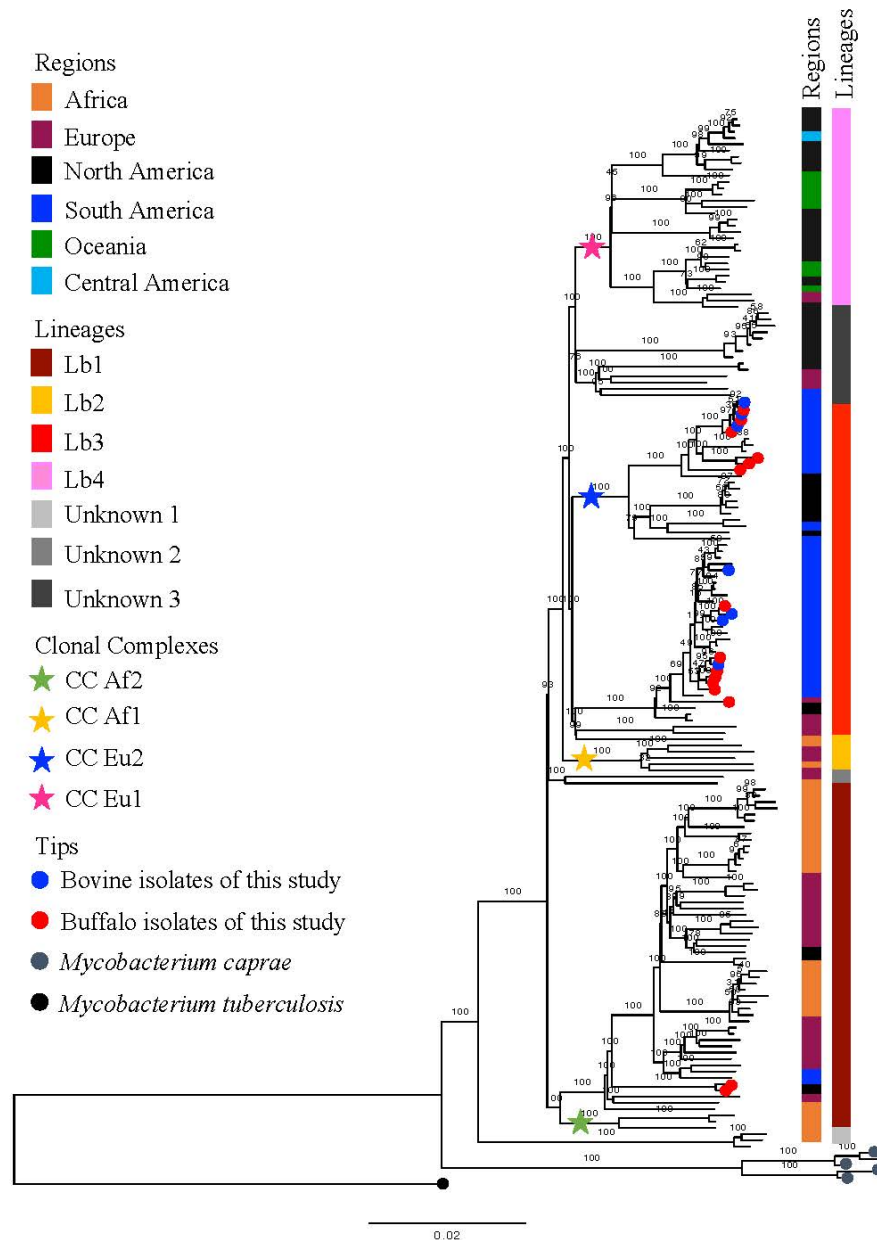
Figure 6.6: Phylogenetic analysis of *Mycobacterium bovis* lineages. Maximum likelihood (ML) phylogenetic tree from concatenated SNPs (single nucleotide polymorphisms) of *Mycobacterium bovis* genomes from North of Brazil (Amazon, this study, and Marajó Island from Conceição et al. [128]) and worldwide isolates. *Mycobacterium caprae* genomes were included in the analysis, and *Mycobacterium tuberculosis* H37Rv was used as outgroup. The phylogenetic tree was generated using RAxML and annotated using FigTree v1.4.3 [274]. Horizontal bar shows substitutions per nucleotide. Lineages are classified according to Zimpel et al., 2020 [38].

CHAPTER VII – STUDY ON A SUPPLEMENTAL TEST TO IMPROVE THE DETECTION OF BOVINE TUBERCULOSIS IN INDIVIDUAL ANIMALS AND IN HERDS

This chapter was published as an original article (*Carneiro et al. Study on supplemental test to improve the detection of bovine tuberculosis in individual animals and herds. BMC Vet Res 17, 137. doi:10.1186/s12917-021-02839-4, 2021)*

ABSTRACT

Objectives – The aim of this study was to evaluate the sensitivity and specificity of a multiplex enzyme-linked immunosorbent assay (ELISA) relative to the tuberculin test used for the diagnosis of tuberculosis in cattle in Brazil.

Methods – The study included a convenience sample of 400 Nelore females raised for beef on five farms, in different municipalities of Para State, in Brazil. The comparative cervical tuberculin test was done and on the day of inoculation of the Purified Protein Derivative, blood samples were obtained and stored for further analysis of the ELISA IDDEX Mycobacterium bovis immunoassay.

Results – Lack of agreement between comparative cervical tuberculin test and ELISA IDEXX TM was observed. The 2 animals positive on the comparative cervical tuberculin test did not react at the ELISA IDEXX TM and 22 negative reactors by comparative cervical tuberculin test were positive by the ELISA IDEXX TM. The ELISA IDEXX TM showed sensitivity that is significantly lower than the official screening test the single cervical tuberculin. ELISA IDEXX TM also detected infected animals and herds undetected by the comparative cervical tuberculin test. The parallel use of

comparative cervical tuberculin test and ELISA IDEXX TM increased sensitivity and the feasibility bTB screening.

Conclusions – 1) Our results support our hypothesis. 2) The results obtained here suggest that the ELISA IDEXX TM may be a supplemental test for the detection of Mycobacterium bovis infection in regions without routine testing and slaughter, where the disease generally progresses to more advanced stages and antibody responses are likely to be more prevalent. 3) Evidence to support the validation of the ELISA IDEXX™ as a supplemental test for bTB eradication programs was provided.

INTRODUCTION

Bovine tuberculosis (bTB), is a worldwide disease caused by *Mycobacterium bovis* (*M. bovis*) and affects mainly cattle but also several other species; including humans causing the zoonotic tuberculosis (TB) [51], [275]–[277]. The disease is difficult to control due to the lack of an effective vaccine, the presence of wildlife reservoirs, and the absence of a diagnostic assay with sufficient sensitivity (Se) and specificity (Sp) to detect sick animals at all stages of infection [277], [278]. The success of bTB eradication and control programs is based on early detection and the removal of reactors from a herd [13] thus routine testing and cull strategy have been applied globally [276]. Therefore, screening-test accuracy is critical to eradication programs. The Single Cervical Tuberculin (SCT) test; the Comparative Cervical Tuberculin (CCT) test in Europe; the Caudal Fold Tuberculin (CFT) test in North America, Australia and New Zealand [279]; and the SCT and CFT tests in Brazil [13], are the prescribed tests for international trade [280]. From a practical point of view, the diagnostic performance, the feasibility of execution and practicality of each test, as well as the costs and associated biological risks, should be considered for better strategic use [279].

Since late 19th century, the Tuberculin Skin Test (TST) has been the primary antemortem test available to support bTB eradication campaigns [281], [282]. Advantages of the TST and reasons for its wide use are low costs, high availability, long history of use and, for a long time, the lack of alternative methods to detect bTB [101], [281]–[283]. On the other hand, TST limitations includes difficulties in performance and result interpretation, need for a second visit, low degree of standardization, and reduced accuracy [284].

Due to the TST limitations in terms of Se and Sp, the credibility of the diagnosis is frequently questioned given the occurrence of false-positive and false-negative reactions, therefore, it is necessary to confirm reactive animals using other methods, ensuring the reliability of the diagnosis [13], [278], [279], [282]. Research continues into the development of new, more accurate, more sensitive tests, less subject to the individual operative performance and subjective interpretation [94], [96], [285]–[288]. As the TST are limited to cell-mediated immune responses (CMI) in the early stages of the infection, a serological test with good Se and Sp aiming at the detection of antibodies to M. bovis in animals in late stages of the disease, would be a viable complementary technique to improve the detection of bTB [281].

Over the last few years, the potential for use of an antibody assays to detect *M. bovis* infection in cattle is being consolidated [94], [96], [287]–[294]. The enzyme-linked immunosorbent assay (ELISA) has proven to be useful as ancilbent serial (to enhance Sp) and parallel (to enhance Se) tests in several species [295]–[299]. Moreover, a booster effect on the antibody response caused after injection of tuberculin has been reported and recommended as a strategic option to increase the Se of serological assays [295]–[297]. The ELISA using MPB83 and MPB70 antigens (IDEXX M. bovis Ab Test, IDEXX Laboratories, Westbrook, Maine, United States (US)) is an immune enzymatic assay promising Se and Sp superior to most tuberculosis diagnoses for both primary and supplementary diagnosis in cases of inconclusive results of the diagnosis by simple or comparative cervical tests [94], [101], [300].

The Brazilian guidelines for control and eradication of animal tuberculosis determines intradermal tuberculin testing as the standard method of diagnosing bTB

[12]. The primary screening is performed using the CFT test (beef only), and the SCT or the CCT test (dairy and beef). The CCT is also adopted as a confirmatory test. In recent years, epidemiological studies adopting TST were made and were aimed at determining the bTB status in several Brazilian states. The herd prevalence ranged from 0.3% to 7.5% and the animal prevalence ranged from 0.03% to 1.3% [12]. Available data from 2017 regarding cattle organs and/or carcass condemnation in Para state demonstrated that 12,183 carcasses and 837,744 cattle organs were condemned in that year. Lungs (46.83%), kidneys (17.06%), heads (10.11%), livers (9.74%), and intestines (5.59%) were the major condemned organs. TB was the cause of 4.88% of the condemnations [301].

Until 2019, no other ancillary indirect methods for bTB diagnoses were approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA). Adoption of complementary field bTB diagnostic tests can improve the detection of infected animals and herds. Due to absence of mandatory test and elimination police the reference standard diagnosis test (culture) is not available, to overcome this obstacle a Bayesian latent class analysis is recommended [300].

To the best of our knowledge, there is no in vivo study comparing the CCT test with ELISA IDEXX™ to evaluate it as a supplemental test for bTB in beef cattle in Brazil. Therefore, our hypothesis is that the adoption of ELISA diagnosis test as supplemental to the standard Tuberculin Skin Test, can improve the detection of *M. bovis* in herds and animals.

To test the above hypothesis, the objective of this study was to determine the Se and Sp of the CCT test and a commercial ELISA multiple test (ELISA IDEXXTM), under

field conditions using a Bayesian approach in order to provide evidence-based data to support adoption of ancillary tests in beef cattle aiming at the improvement of national bTB eradication programs.

MATERIAL AND METHODS

Study Design

A total of 400 female Nellore (*Bos taurus indicus*), aged over 24 months, raised on farms in the northeastern region of Para state and Belem's metropolitan area, Brazil were included in the study. Due to logistical reasons, the 400 cows were selected using a convenient sampling procedure. The animals came from five farms located in the following distribute in the following municipalities: in the Para's northeastern, the municipalities of Capitao Poco (Farm #1, n = 80), Garrafao do Norte (Farm #2, n = 80), and Sao Francisco do Para (Farm #3, n = 80), and in Belem's metropolitan mesoregion the municipalities of Castanhal (Farm #4, n = 80), and Santa Izabel (Farm #5, n = 80), The sampling was carried out from March 13 to May 4, 2013.

Blood collection

Samples were collected from all cows in the experimental group composed of cows from each farm, 10.0 mL of blood was collected by puncture of the external jugular vein, without excessive tourniquet of the vessel, using siliconized vacutainer tubes without anticoagulant and properly identified. The samples were centrifuged for 15 minutes at a

speed of 3,000 G, then separated by aspiration of the serum, aliquoted in 2 ml Eppendorf microtubes, identified and stored at -20 ºC for subsequent serological testing (ELISA).

Enzyme-Linked Immunosorbent Assay – ELISA

The ELISA IDEXX™ *M. bovis* was performed as described previously (16) and according to the manufacturer's instructions.

Shortly, two microtiter plate wells were used for the positive control, two for the negative control and a blank well to reference the microplate reader. The reading was performed on a TP Reader Basic / Thermoplate microplate reader, at a wavelength of 450nm at an accuracy of ± 2nm and an absorbance resolution of 0.001A at an accuracy of ± 0.03A. The results of optical density (OD) provided by the reader were recorded and used to calculate the validation of the test and then the results of the samples according to the specifications of the Kit manufacturer.

Comparative Cervical Skin Test (CCT)

On the same day of blood collection, inoculations of avian (A) and bovine (B) PPD tuberculin were performed intradermally, at a dose of 0.1 mL in the cervical region, in places previously demarcated by hair removal, at 15 to 20 cm between the two inoculations. Avian PPD was inoculated cranially and bovine PPD caudally, on the same side of all animals in the herd to be tested (8). The skin thickness of the inoculation site was measured using calipers before injection. The increase in skinfold thickness were determined by the same researcher at 72 h post-injection. Interpretations of the test results were made according to the Brazilian standard for screening tests for bTB (32).

Statistical analysis

The descriptive statistics of the data, represented by the frequencies (%) of reagents or non-reagents animals, both between the referred exams (ELISA and CCT) and between the different farms (1, 2, 3, 4 and 5), was obtained by Freq procedure of the SAS program (SAS® 9.2, SAS Institute Inc., Cary, NC, USA).

The gold standard in TB is isolation of the organism from the specimens. However, isolation of the organism was not feasible due to logistic reasons. In comparing one or more diagnostic test where there is no data from a golden standard a Bayesian method is used compare the performance of the tests. Therefore, this approach was used to estimate the Se and Sp of the CCT and ELISA. We followed the conservative assumption that results were not independent.

Inferential statistical analyses were performed using analysis of variance (ANOVA), with the Glimmix mixed analysis procedure, from the SAS program. Considering that the two exams were performed on the same animal, the statistical model was constituted by the TEST and ANIMAL classificatory variables. The interaction between the type of test performed within each farm (TEST * FARM), in order to observe any possible different effect between the tests at each of the farms, was also included in the model. The comparison between the frequencies of the groups was performed using the Least Square Means test (LSMeans) of the SAS. The significance level of 5% was used.

RESULTS

The CCT results showed that 2/400 animals were classified as positive (reactors). In the experiment carried out on Farms 3 and 4, the CCT results were similar, out of 80

tested animals per farm, 1 animal in each of them had a positive result, however the CCT reactive animals were not reactive on the ELISA test. On Farms 1, 2 and 5, all animals were CCT non-reactive, therefore only 2/5 herds were considered positive for bTB according the CCT on this study. The results obtained by ELISA IDEXX™, however, were different from those indicated by the CCT. A total of 22 out of the 400 animals (5.5%) were considered positive. At the herd-level all the 5 herds (100%) had at least one positive animal (Table 7.1).

Bayesian analysis showed that the Se of ELISA IDEXX™ was significantly higher (P = 0.003) than CCT's Se. On the other hand, no significant differences in Sp between CCT and ELISA IDEXX (Table 7.2).

The parallel interpretation of the results of the 2 tests, show an increase of Se at herd-level from 40% on the official CCT to 100% and at animal-level from 0.5% to 6.00% (Tables 7.3 and 7.4).

DISCUSSION

This study assessed the performance of bTB screening test habitually used in eradication programs (CCT test) and a potential supplemental test (ELISA IDEXX™) under field conditions in Brazil using a Bayesian approach.

IDEXX ELISA presented higher Se than CCT even in the absence of the booster effect. Previous studies for the detection of antibodies against *M. bovis* had showed that contingent on the epidemiological situation the Se of the antibody detection tests even the absence of the booster effect could be bigger than the one obtained using official TST that detect cellular immune mediated response (CMI) [296], [297]. Recently, under a high bTB prevalence situation serological tests presented higher Se than official techniques in

the absence of booster effect [287], [298]. In our study, adopting a parallel interpretation the apparent prevalence ranged from 1.25% - 11.25% within the herds may explain the higher Se presented by IDEXX ELISA.

No overlap was found between the 22 animals with positive IDEXX ELISA and the 2 animals with positive CCT the results agree with similar findings of other studies [302], [303]. The absence of agreement between the positive results of the two tests might be due to the fact that tests aim to detect different immune response (humoral and cell-mediated immunity) [302], [303]. The detection of CMI response to infection with *M. bovis*, as assessed by the TST usually fail to detect chronic stages of the infection [295], [298], [304]. Particularly in situations such as in the area where the research was carried out, with absence of a test-and-slaughter routine the disease trend to progress to more advance stages and antibody responses are likely to be more prevalent [285]. Moreover, a factor that may have influenced our results might be the age, all tested animals were adult females over than 24 months, unfortunately no further information was obtained allowing to determine the mean age of the animals by farm what is a limitation of this study.

Under the current PNCEBT guidelines adopting the CCT, only 2 herds and 2 animals involved in this study would be considered bTB positive. On the other hand, considering only the ELISA, all herds would be classified as bTB positive and 22 CCT negative animals would be diagnosed as bTB positive. Therefore, the exclusive use of CCT, would leave behind 3 infected herds and 22 false negative animals, the potential of the serological tests to identify non-reactive tuberculin skin test results is being reported [82], [96], [294] and suggests that their application to test non infected herds would help

to increase the performance of the screening strategy in current bTB eradication programs.

If a parallel interpretation of the diagnostic techniques, combining detection of cellular and humoral immune response, is adopted the proportion of TB-infected herds and cattle increased compared to the values reached with techniques separately. Precisely, the CCT was able to detect only 40% (2/5) of infected herds and IDEXX ELISA detected 100% (5/5) of the herds with at least 1 *M. bovis* infected animal (Table 7.3 and 7.4). Furthermore, considering the PNCEBT classification, the herds would be classified from negligible to very low risk (CCT only), to medium risk (CCT + ELISA) [12]. At animal-level, the parallel interpretation of the CCT and the ELISA IDDEX classified as positive 6% (24/400) of the tested animals while the official approach detected only 0.5% (2/400), these results are compatible with previous studies [82], [287], [294] and reinforce the recommendation of serological tests as supplemental test for diagnosing and managing tuberculosis infection [82], [295]–[297], [302], [303].

A limitation of this study was the fact that it was not possible to isolate *M. bovis* from animal tissues which could have provided us data to serve as a gold standard. Although, the study design does not allow the assessment of ELISA as ancillary serial test, the Bayesian analysis show no difference in Sp between CCT and IDEXX ELISA (Table 7.2). Thus, the diagnostic power of animals truly negative for bTB is confirmed by the CCT, standing out as a good confirmatory test for the diagnosis of *M. bovis* infection. Additionally, a trial to test a serial diagnosis scheme would allow to confirm the occurrence of booster effect in beef cattle.

A study to assess the reliability of the combination CFT (the usual screening test for beef cattle) and ELISA in parallel and serial schemes would be of a great value. Adoption of supplemental tests would represent significant logistical improvements on bTB program, reducing farm visits, CCT reading errors, time for removing the infection from the herd, and allowing to store serum sample for confirmatory test for a long period of time. Furthermore, cattle would have to be reunited and handled once, being an additional benefit when working with beef, representing a great benefit for the farmers and the veterinarians.

Although the study needs to be extended, circumstantial evidence was obtained to support the recommendation of adoption of serological tests as supplemental to the traditional TST schemes as an efficient epidemiological tool for outbreak management and disease control that may contribute to a fast-track for bTB eradication.

CONCLUSIONS

1) Our results support our hypothesis that the adoption of ELISA diagnosis test as supplemental to the standard Tuberculin Skin Test, can improve the detection of *M. bovis in* herds and animals.

2) The IDEXX ELISA multiplex presented significant higher sensitivity than the official comparative cervical test CCT and no significant differences on specificity was observed.

3) The ELISA detected reactor animals and herds missed by the CCT. Parallel use of TST and ELISA increased the sensitivity, the feasibility of screenings for *M. bovis* infection diagnosis, and speed up the cleansing of herds.

4) The IDEXX ELISA test can be a supplemental test for *M. bovis* infection detection in regions with no test-and-slaughter routine where the disease usually progress to chronic phase and antibody responses are likely to be more prevalent.

5) Results provided evidences to support the validation of the IDEXX ELISA as supplemental test by the Brazilian eradication program.

APPENDIX

Table 7.1: SCT (PPDb), CCT and ELISA bTB results in 400 Nelore cattle by farm.

Farm	Exam % (n/n)					
	SCT (PPDb)*		CCT**		ELISA***	
	-	+	-	+	-	+
1	96.25 (77/80)	3.75[c] (3/80)	100 (80/80)	0.0[c] (0/80)	98.75 (79/80)	1.25[c] (1/80)
2	92.50 (74/80)	7.50[bc] (6/80)	100 (80/80)	0.0[bc] (0/80)	93.75 (75/80)	6.25[bc] (5/80)
3	86.25 (69/80)	13.75[b] (11/80)	98.75 (79/80)	1.25[b] (1/80)	90.0 (72/80)	10.0[b] (8/80)
4	62.50 (50/80)	37.50[a] (30/80)	98.75 (79/80)	1.25[a] (1/80)	91.25 (73/80)	8.75[a] (7/80)
5	88.75 (71/80)	11.25[bc] (9/80)	100 (80/80)	0.0[bc] (0/80)	98.75 (79/80)	1.25[bc] (1/80)
TOTAL	85.25 (341/400)	14.75[A] (59/400)	99.50 (398/400)	0.50[C] (2/400)	94.50 (373/400)	5.50[B] (22/400)

Different uppercase letters between lines and different lowercase lines between columns differed between them (P < 0,0001). *SCT (PPDb) – Simple Cervical Test, **CCT – Comparative Cervical Test, *** ELISA IDEXX *M. bovis*

Table 7.2: Comparison of the results of the CCT x SCT (PPDb) in 400 Nelore cattle

| | | SCT (PPDb) | | TOTAL |
		Negative	Positive	
CCT	Negative	341 (85.25%)	57 (14.25%)	398 (99.50%)
	Positive	0 (0.0%)	2 (0.50%)	2 (0.50%)
TOTAL		341 (85.25%)	59 (14.75%)	400 (100.0%)

P <.0001; Kappa = 0.0564

Table 7.3: Comparison of the results CCT x ELISA in 400 Nelore cattle

| | | ELISA | | TOTAL |
		Negative	Positive	
CCT	Negative	376 (94.0%)	22 (5.50%)	398 (99.50%)
	Positive	2 (0.50%)	0 (0.0%)	2 (0.50%)
TOTAL		378 (94.50%)	22 (5.50%)	400 (100.0%)

P <.0001; Kappa = -0.0093

Table 7.4: Comparison of the results SCT x ELISA in 400 Nelore cattle

		ELISA		TOTAL
		Negative	**Positive**	
SCT (PPDb)	Negative	325 (81.25%)	16 (4.0%)	341 (85.25%)
	Positive	53 (13.25%)	6 (1.50%)	59 (14.75%)
	TOTAL	378 (94.50%)	22 (5.50%)	400 (100.0%)

P <.0001; Kappa = 0.0739

Table 7.5: Bayesian estimates of Prevalence, Sensitivity, and Specificity of bTB tests with a 95% Confidence Interval

Parameter	Estimative	CI
AP	0.0033	(0.0005; 0.0087)
Se_{CCT}	0.7329	(0.5986; 0.8497)
Se_{ELISA}	0.8882	(0.8063; 0.9513)
$Se_{CCT} - Se_{ELISA}$	-0.1553	(-0.3049; -0.0158)
Sp_{CCT}	0.9557	(0.9372; 0.9718)
Sp_{ELISA}	0.9479	(0.9256; 0.9665)
$Sp_{CCT} - SP_{ELISA}$	0.0078	(-0.0186; 0.0357)

AP – Apparent Prevalence; Se_{CCT} – Comparative Cervical Test Sensitivity; Se_{ELISA} – ELISA IDEXX^{TM} Sensitivity; Sp_{CCT} – Comparative Cervical Test Specificity; Sp_{ELISA} - ELISA IDEXX^{TM} Specificity

Table 7.6: Single and parallel interpretation of CCT + ELISA IDEXX[TM] at Herd-level in 400 Nelore cattle

Results	Tests		
	CCT*	ELISA**	CCT+ ELISA
Positive	40% (2/5)	100% (5/5)	100% (5/5)
Negative	60% (3/5)	0% (0/5)	0% (0/5)

*CCT – Comparative Cervical Test, ** ELISA IDEXX[TM] *M. bovis*

Table 7.7: Single and parallel interpretation of CCT + ELISA IDEXX^{TM} at Animal-level in 400 Nelore cattle

Results	Tests		
	CCT*	ELISA**	CCT+ ELISA
Positive	0,50% (2/400)	5,50% (22/400)	6% (24/400)
Negative	99,50% (398/400)	94,50% (378/400)	94% (376/400)

*CCT – Comparative Cervical Test, ** ELISA IDEXX^{TM} *M. bovis*

CHAPTER VIII – ASSESSMENT OF MILK CONTAMINATION BY *MYCOBACTERIUM BOVIS* IN AMAZONAS STATE, BRAZIL

ABSTRACT

Objective – This study aimed to assess the contamination of milk, from cattle and buffalo, by *Mycobacterium bovis* (*M. bovis*) and other species of the *Mycobacterium tuberculosis Complex* (*MTC*), in Amazonas State, Brazil.

Material and Methods – A cross-sectional study, performed including 250 samples of milk (91 from cattle and 159 from buffalo). The samples were joined in 21 pools according to species and geographic location. After DNA extraction, Shotgun Metagenomic Sequencing (SMS) was performed on the pooled milk samples and the taxonomic classification of microorganisms' high-throughput sequencing reads was performed by Kraken-2 and MegaBLAST. To confirm the presence of *M. bovis* in pools of raw milk, the BLASTN algorithm was used to identify the specific genomic positions at the TbD1 and RD1 regions and the flanking RD4 region of *M. bovis*.

Results – Genetic material from MTC species was identified in all 21 pools of raw milk. *M. bovis*-consistent genetic material was identified in seven pools (33.3%) of raw milk (1 cattle and 6 buffalo). Raw milk from buffalo presented significantly higher amounts *M. bovis* genetic material than that from cattle.

Conclusions – 1) Our results support the hypothesis that the common practice of consumption of raw milk and its derivatives in Amazonas, Brazil, presents an eminent risk for infection with zoonotic tuberculosis; 2) Urgent measures to enforce the regulation for milk pasteurization are needed in the region, 3) Great efforts and resources should be directed towards controlling bTB in cattle and buffalo, and; 4) SMS

as a potential as a screening tool for active surveillance of *Mycobacterium Tuberculosis Complex* species in milk from cattle and buffalo was demonstrated.

INTRODUCTION

Despite regulations prohibiting the sale, raw milk and products made with raw milk are routinely consumed in Brazil, making the clandestine sale and consumption of raw milk an important public health issue in the country [305]. Milk is important part of the diet in Amazonas State and it is consumed both pasteurized and unpasteurized in many ways. Unfortunately, consumption of unpasteurized milk and its derivatives is very popular in the area and these products and activities include; drinking milk after milking (without boiling), consuming clotted milk (coalhada), adding raw milk to coffee (without boiling), and making cheese from unpasteurized milk (coalho).

Although essential for human nutrition, the unique composition and properties of milk and dairy products represent excellent growth media for many pathogenic microorganisms [306]. Raw milk and products made from unpasteurized milk can harbor several pathogens including the *M. tuberculosis, M. bovis,* and others non-tuberculosis *Mycobacterium* (NTM) [11]. In Turkey, PCR-RFLP analysis of 145 raw milk samples from cattle, found 11 NTM isolates (*Mycobacterium genavense, Mycobacterium simiae, Mycobacterium szulgai,* and *Mycobacterium fortuitum*) and 1 isolate was identified as *M. bovis* by spoligotyping.[307]. In Argentina, *M. bovis* was detected by PCR during milk screening in bulk tanks [308]. In Pakistan, a high prevalence rate of *M. bovis* in milk was found in cattle (6.4%), and buffaloes (6.2%), while the prevalence of *M. tuberculosis* was higher in goats (6.3%) [309]. In Nepal, *M. bovis* was also detected in buffalo milk samples [310]. Moreover, in USA, *M. bovis* was recovered from cheese originating from Mexico [311]. In common, the studies have shown that cattle shed Mycobacterium spp. into milk and that the risk of transmission of *M. bovis* to humans is present.

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In Brazil, milk contamination with *M. bovis* has been described in several states and geographic regions: Alagoas [312], Paraiba [313], and Pernambuco [314] from Northeast; São Paulo [315] from Southeast; and Rio Grande do Sul [316] from Southern region. *M. bovis* was isolated in milk samples collected directly from animals [314], from individual 50 liters cans [317], and bulk tanks [165]. Besides *M. bovis*, 21 different species of NTM had been isolated (*M. avium*, *M. flavescens*, *M. fortuitum, M. smegmatis*, *M. kansasii*, *M. gordonae*, *M. lentiflavum*, *M. nonchromogenicum*, *M. peregrinum*, *M. neoaurum*, *M. chelonae*, *M. scrofulaceum*, *M. ultracellulare*, *M. duvalii, M. haemophilum, M. immunogenum, M. mucogenicum, M. novocastrense, M. parafortuitum, M. terrae, and M. vaccae*) from raw and pasteurized milk, and cheese from dairy cattle [164], [165], [315], [317], [318]. In dairy buffalo, opportunistic pathogens such as *M. kansasii, M. simiae* and *M. lentiflavum* were isolated from raw milk but *M. bovis* has not been reported in milk thus far [319].

Speciation within the *Mycobacterium Tuberculosis Complex* (MTC) is conventionally made by biochemical or microbiological techniques although these tests are cumbersome, unreliable, and consume more time than molecular techniques [320]. Molecular techniques have the additional advantage of providing investigators with epidemiological data such as pathogen population, evolution, and geographical distribution as well as identification of new species [319]. The Whole genome sequencing (WGS) is a laboratory procedure that determines the order of all the nucleotides in an organism's DNA and can determine variations in any part of the genome [321]. WGS is useful at clarifying sources of infection, genotypes indistinguishable by other methods, and potentially estimating when a new strain was

introduced [125]. Another approach in genomic analysis is the Shotgun Metagenomic Sequencing (SMS) which is a method that enables evaluation of bacterial diversity and the detection of the abundance of microbes in various environments. SMS allows comprehensive sampling of all genes in all organisms present in microbiomes such as milk samples without some of the limitations imposed by culture methods [139]. A few studies on milk microbiota using SMS approach are now available [137], [138]. These studies not only allowed exploration of bacterial communities but also archaeal, fungal, and viral communities. They also gave access to a functional profiling of these microbial communities, including data on microbial metabolism, virulence, or antibiotic resistance [138].

The identification of the *Mycobacteria* that can cause TB especially in areas where bTB and human TB cohabit is epidemiological information necessary to eliminate the disease both from livestock and human population [59]. In Amazonas state, our previous studies demonstrated an overall bTB apparent prevalence (AP) of 5.4% and AP within species of 3.0% in cattle and 11.8% in buffalo [242]. Additionally, the state's annual incidence of human TB is persistently high with 64.8 cases per 100 thousand inhabitants in 2020 [322]. There are no actions or policies to address the possible spread of *Mycobacteria* from animals to humans in the region. As the cost of the omics analysis decreases and practical applications increase, the use of the SMS for surveillance of *M. tuberculosis* and *M. bovis* in milk has the potential to provide information needed to rapidly detect contaminated food and herds infected by *Mycobacteria* organisms, thereby preventing zTB and accelerating bTB eradication, respectively.

There is no epidemiological study regarding milk contamination by *Mycobacteria* in Brazilian Amazon. Therefore, we hypothesize that unpasteurized milk as consumed in Amazonas state can pose a potential risk of transmission of *M. bovis* to humans and causes zTB. To test this hypothesis, the goal of this study is to use SMS to assess the contamination in raw milk, from cattle and buffalo, by *M. bovis* and *M. tuberculosis* in Amazonas state, Brazil.

MATERIALS AND METHODS

Study design

A cross-sectional study was performed from February to August 2019. The study was based on a convenience sampling of raw milk from 50-liter (13.25 gallon) milk cans/containers sent for processing at three major milk plants and from dairy herds with unknown bTB history, in Amazonas State, Brazil. Two samples of 50 ml each were aseptically collected per can; samples were transported from the milk plant on ice and then frozen until the analysis. Although all epidemiological information from the herds was not available, it is known that in the area of study the average number of cows in milking per herd is about 100 animals.

Study population

A total of 250 samples were collected, 91 milk samples from cattle and 159 from buffalo (Figure 8.1). Due to logistical challenges the samples were put in pools according geographic region and species. A total of 21 pools were formed as follows: 91 samples from dairy cattle were put in 8 pools and 159 samples from buffalo milk were put in 13 pools (Figure 8.2)

DNA extraction

Aliquots of 50ml of milk were centrifuged at 3,000 rpm for 20 min, at 4ºC. After that, 250 µl of the pellet and 250 µl of the supernatant (grease cap) were dispensed in a 2.0 ml microtube and 100 µl of 10% Sodium Dodecyl Sulfate (SDS) and 20 µl of Proteinase K were added to each microtube. The samples were incubated at 65ºC in a thermoblock for 40 min and another 10 min at 100ºC. An equal volume (620 µL) of phenol/ chloroform/ isoamyl solution (24: 24: 1) were added and the microtubes were carefully turned 30x. The microtubes were then centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatants were transferred to new microtubes and an equal volume of chloroform/ isoamyl alcohol solution (24:1) was added. The microtubes were carefully turned 30x and then centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatants were transferred to new microtubes and 100 µl of 5M NaCl and 600 µl of isopropanol were added. The microtubes were then incubated at -20°C overnight and centrifuged at 12,000 rpm for 15 min at 4ºC. The supernatants were discarded and two washes with cold 70% ethanol were performed. The microtubes were then centrifuged for 10 sec at 4 ºC, the excess alcohol removed and then dried at 65°C in a thermoblock for 5 minutes. The pellets were resuspended in 20 µl of type I ultrapure water or more if the solution was too viscous (50 or 100 µl). The samples were diluted in ultrapure water type I in a 1/20 ratio and 2 µl were used for PCR analysis.

Milk Shotgun Metagenome Sequencing (SMS)

SMS was performed at the Next-Generation Sequencing multi-user platform of Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. Briefly, DNA quantification was performed using the QubitTM dsDNA HS Assay Kit (Thermo Fisher

Scientific, Waltham, USA) and the Agilent High Sensitivity DNA Kit (Agilent, California, USA). Milk WMS was carried out on a HiSeq instrument (Illumina, San Diego, California, USA) using HiSeq Rapid SBS Kit v2 (200 cycles) chemistry and the Nextera DNA Flex Library preparation kit (Illumina, San Diego, California, USA) according to the manufacturer's instructions.

Microbial taxonomy

As to identify *M. bovis* genomes in milk samples, *Mycobacterium tuberculosis* variant *bovis* AF2122/97 (ASM19583v2) and *Mycobacterium tuberculosis* H37Rv (ASM19595v2) were used as references. Taxonomic classification of high-throughput sequencing reads from SMS was by Metawrap pipeline [323], using Kraken-2 [324] and MegaBLAST [325], both visualized using Krona tools.[326]. Additionally, Nucleotide-Nucleotide (BLASTN) v. 2.11.0+ with Evalue 1e-5 as parameter was performed targeting specific *M. bovis* TbD1 and RD1 region and flanking RD4 region.

Statistical analysis

The descriptive statistics of the data, represented by the means of MTC reads, both between cattle and buffalos pool samples, was obtained by Freq procedure of the SAS program (SAS® 9.2, SAS Institute Inc., Cary, North Carolina, USA). To determine whether MTC genomic materials were significantly different between cattle and buffalo, we applied the T-test using the JMP program.

RESULTS

Fourteen of 21 samples (5 cattle and 9 buffalo) were found to be of acceptable quality for analysis using Kraken 2. In all samples, MTC genomes were identified (Figures 8.3 and 8.4 and Table 8.1). Similarly, 16/21 samples (5 cattle and 11 buffalo)

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were found to be of acceptable quality for use in MegaBLAST; the presence of MTC genomes was confirmed in all samples (Table 8.2).

Kraken-2 analysis of the SMS showed that within the *Mycobacterium* genus MTC genetic material ranged from 32% to 70% in cattle and from 66% to 95% in buffalo samples (Table 8.1). On the MegaBLAST analysis, MTC genetic material within *Mycobacteria* ranged from 59 to 89% in cattle and from 67 to 97% in buffalo (Table 8.2). On a most closely related species-level comparison, BLASTN results confirmed the presence of *M. bovis* genomes in 7 pools of milk (1 cattle and 6 buffalo), see Table 8.3.

DISCUSSION

The results are relevant due to the common practice of consuming unpasteurized milk in Brazil and particularly within the rural population of the Amazon region where drinking raw milk and consumption of dairy products made from raw milk is very popular.

Qualitative systematic literature review revealed a reported prevalence of human zTB ranging from 0% to 28% in several countries, with cattle and raw dairy products as the primary exposures [162]. Additionally, *M. bovis* was the agent in all confirmed zTB cases [162]. Occupational exposure, history of living in a rural area, and consumption of unpasteurized milk are the most frequent risk factors associated with zTB. In developed countries, where effective pasteurization is a reality, there is low risk of *M. bovis* transmission by milk [327]. Similarly, in developed regions of Brazil (such as Southern, Southeast, and Midwest) consumption of raw milk and its products is very rare, if at all. However, in other regions of Brazil (Northern and Northeastern) the consumption of raw milk and its products is quite regular. Policies of pasteurization of milk should be

enforced. Moreover, active surveillance of milk contamination by *M. bovis,* to identify

and eliminate bTB from herds and developing educational programs on the risk of

contracting zTB from the use of raw milk and its products, should be considered

important approaches.

Additionally, traditional microbiological detection techniques focus on culturable

bacteria. Culturing on selective media remains the gold standard for *M. tuberculosis* and

M. bovis detection. However, this process is extremely laborious and time consuming,

with low sensitivity inconclusive [328], and will not capture viable but nonculturable

bacteria or non-readily-culturable bacteria [329]. Molecular techniques have facilitated

the rapid detection and identification of foodborne pathogens, which has been crucial for

current surveillance and outbreak control [328]. For these reasons, the adoption of

molecular diagnostic methods based on DNA analysis by TB programs in countries with

widespread infection in human population and livestock herds, to complement or

replace the traditional microbial culture procedures is reasonable.

For surveillance purposes, to distinguish *M. bovis* from *M. tuberculosis* strains is

important for monitoring the spread of *M. bovis* among cattle and from cattle to humans

[63]. These data can generate valuable information for TB control programs.

Laboratory-based surveillance provides essential data for monitoring trends, detecting

outbreaks, and initiating the public health response to control many infectious diseases

[320]. In 2013, WGS was introduced as epidemiological tool to investigate bTB field

outbreaks by the USDA [125]. In its turn, SMS provides a pathway to watch the

microbial genetic diversity, deep sequencing enables the detection of individuals of the

microbial community that are not detectable by other conventional methods, and creating the possibility for SMS to be used as a screening tool in milk samples.

Collecting samples from bulk tank milk (BTM) is easy and fast, and such samples provide a good representation of the lactating herd. PCR has been used on BTM samples as a surveillance tool to identify the herd-level prevalence of *Mycoplasma* species across different regions and countries [330], [331]. However, this approach has limitations for bovine TB, because *Mycobacterium bovis* shedding is not continuous throughout the infection and can range intermittently, with bacteria shed in individual bursts or episodes [308], [317]. Therefore, to obtain a positive result, infected cows in the herd must be contributing to the bulk tank and must be shedding the pathogen at the time the BTM sample is collected. Thus, there is great potential to miss the identification of infected herds when relying on high-throughput technologies for screening of BTM samples alone. The results of the present study demonstrate the ability of SMS to detect MTC contamination in pools of milk samples representing 700 to 1,700 milking cows or buffalo. The adoption of omics technologies in active screening for pathogens in milk is a new and potentially useful tool.

Although next generation sequencing technologies can identify MTC and NTM, it is difficult to determine the MTC species because of the high sequence conservation, and in the case of insufficient coverage, targeted PCR should be performed to further identify the specific TB strain. To address the limitation, we obtained further evidence targeting the regions TBd1, RD1, and the region flanking RD4 in *M. bovis* using BLASTN. Our similarity results confirmed the presence of genetic material compatible

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with *M. bovis* in 1/5 milk samples of cattle and in 6/11 milk samples of buffalo. However, because SMS reads obtained varied from 100bp to 200bp it was not possible to obtain a full alignment with the whole 3 target regions, notwithstanding that in Brazil there is no report of other *MTC* members in cattle or buffalo besides *M. bovis*. The area of study has the highest prevalence of *M.* bovis described in the country [242]. All the previous studies performed in the area confirmed by the PCR presence of *M. bovis* in specimens from cattle and buffalo [201], and no other members of the *MTC* group have ever been described in milk in the country. Therefore, based on the above set of factors and logical reasoning our results strongly suggest considering the contamination by *M. bovis* on the above-mentioned samples.

In this study, SMS was able to detect genetic material consistent with *M. bovis,* in samples of raw milk agreeing with former studies [308], [309], [310]. Samples of raw milk from buffalo presented significantly higher contamination by genetic material compatible with *M. bovis* ($p > 0.04$) than samples from cattle. All of the above, demonstrates the urgency for measures to enforce the regulation for milk pasteurization to prevent the spread of *M. bovis* from animals to humans and the need of an epidemiological strategy to eliminate bTB, considering the particularities of buffalo species in Brazilian Amazon.

Isolation of clinically relevant *MTB sp*. from raw milk represents potential risk of mycobacterial infection and TB. With such entities circulating in milk and its derivatives, the public becomes susceptible by intake of both raw, and occasionally pasteurized milk, if the initial dose of these agents is high and enough bacilli survives the process.

Amazonas state shows the highest prevalence of AIDS in Brazil, which predisposes the population to such opportunistic infections. Due to the regional demand for raw products and the conditions (i.e., herd health status, transport temperature and humidity) between the producing and consumption of dairy products, the scale-up of heat treatment of milk will require not only technical capacity but also a better understanding of the social, cultural, and economic factors that may influence its implementation.

Ultimately, this study improved the scientific evidence base of the risk of zTB in Brazil and also represents a successful case of an intersectoral and collaborative approach where veterinary services, food safety authorities, academia, and agricultural and public health research institutes worked together towards the goal of ending TB.

CONCLUSIONS

From our study, we can make the following conclusions:

1. Our results support the hypothesis that unpasteurized milk as consumed in Amazonas state poses a potential risk of transmission of *M. bovis* to humans.

2. Raw milk, sampled in farms and in milk plants before pasteurization, were contaminated by *Mycobacterium tuberculosis complex* species, in Amazonas, Brazil.

3. Genetic materials compatible with *M. bovis* were for the first time described in milk in Amazonas, Brazil.

4. Urgent measures to enforce the regulation for milk pasteurization are needed in Amazonas, Brazil.

5. Great efforts and resources should be directed towards controlling bTB in cattle and buffalo.

6. SMS demonstrated potential as a screening tool for active surveillance of MTC in milk from cattle and buffalo.

APPENDIX

Table 8.1: Relative abundance of *Mycobacterium tuberculosis complex* (MTC) in milk samples from cattle and buffalo obtained by Kraken2 analysis of the Shotgun Metagenome Sequences.

Organism	Cattle			Buffalo		
	Reads	%*	%**	Reads	%*	%**
MTC	91 - 339 (n=5)	32 - 70	.002 - .007	—	—	—
MTC	—	—	—	20 - 5296 (n=9)	66 - 95	.0003 - .1

* Percentage of the genetic material within the *Mycobacterium* genus. The pool samples ranged from 700 to 1700 milking cows/ buffalo from different farms. ** Percentage in the whole genetic material of the pool sample. MTC = *Mycobacterium tuberculosis complex.* SMS = Shotgun Metagenomic Sequencing

Table 8.2: Relative abundance of Mycobacterium tuberculosis complex (MTC) in milk samples from cattle and buffalo obtained by MBLAST analysis of the Shotgun Metagenome Sequences.

Organism	Cattle			Buffalo		
	Reads	%*	%**	Reads	%*	%**
MTC	168 - 637 (n= 5)	59 - 89	.002 - .008	--	--	--
MTC	--	--		36 - 10342 (n= 9)	67 - 97	.01 - .1

* Percentage of the genetic material within the *Mycobacterium* genus. The pool samples ranged from 700 to 1700 milking cows/ buffalo from different farms. ** Percentage in the whole genetic material of the pool sample. MTC = *Mycobacterium tuberculosis complex*. MBLAST = Mega BLAST. SMS = Shotgun Metagenomic Sequencing.

Table 8.3: Results of analysis using Kraken 2, MegaBLAST, and BLASTN from the Shotgun Metagenomic Sequencing of raw milk from cattle and buffalo in Amazon region, Brazil.

Sample	Host	Kraken2	MegaBLAST	BLASTN
1	Cattle	*MTC* and *M. bovis*	*MTC*	*MTC*
2	Cattle	*MTC* and *M. bovis*	*MTC*	*M. bovis*
3	Cattle	*MTC* and *M. bovis*	*MTC*	*MTC*
6	Cattle	*MTC* and *M. bovis*	*MTC*	*MTC*
7	Cattle	*MTC*	*MTC*	*MTC*
1	Buffalo	*MTC* and *M. bovis*	*MTC*	*M. bovis*
3	Buffalo	*MTC*	*MTC*	*MTC*
4	Buffalo	*MTC* and *M. bovis*	*MTC*	*M. bovis*
5	Buffalo	*MTC* and *M. bovis*	*MTC*	*M. bovis*
7	Buffalo	*MTC* and *M. bovis*	*MTC*	*MTC*
8	Buffalo	*MTC* and *M. bovis*	*MTC*	*MTC*
9	Buffalo	*MTC*	*MTC*	*MTC*
10	Buffalo	*MTC*	*MTC*	*M. bovis*
11	Buffalo	*MTC* and *M. bovis*	*MTC*	*MTC*
12	Buffalo	*MTC* and *M. bovis*	*MTC*	*M. bovis*
13	Buffalo	*MTC* and *M. bovis*	*MTC*	*M. bovis*

MTC = *Mycobacterium tuberculosis complex*; MBLAST = Mega BLAST; BLASTN = nucleotide - nucleotide BLAST.
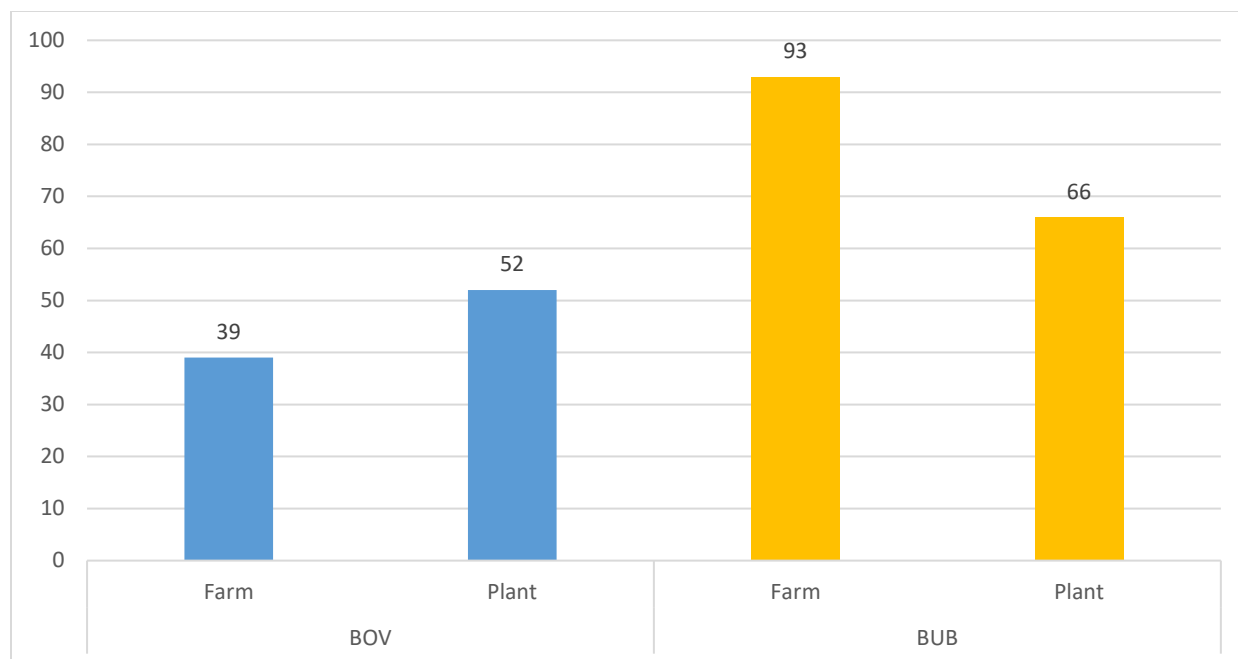
Figure 8.1: Milk sample distribution according to species and collection site, Amazonas, Brazil, 2019.
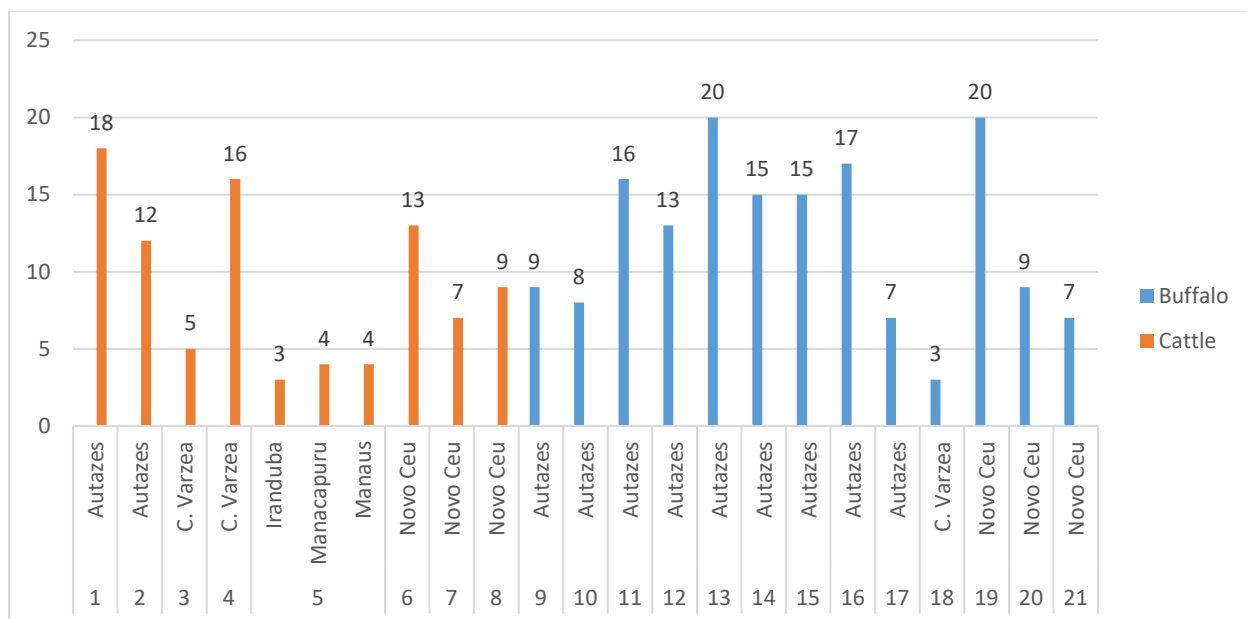
Figure 8.2: Milk sample distribution by pool and species, Amazonas, Brazil, 2019.
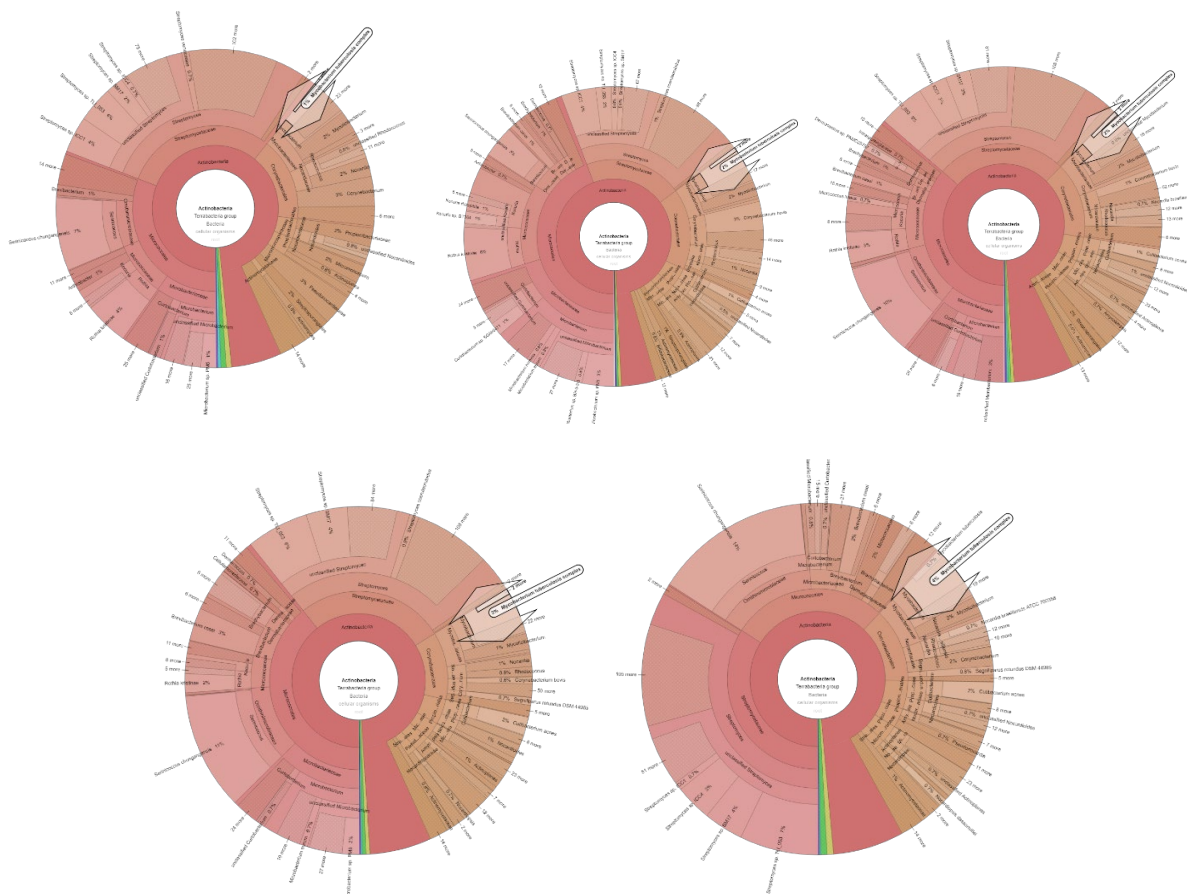
Figure 8.3: Shotgun Metagenome Sequencing of cattle's milk pools by Kraken 2
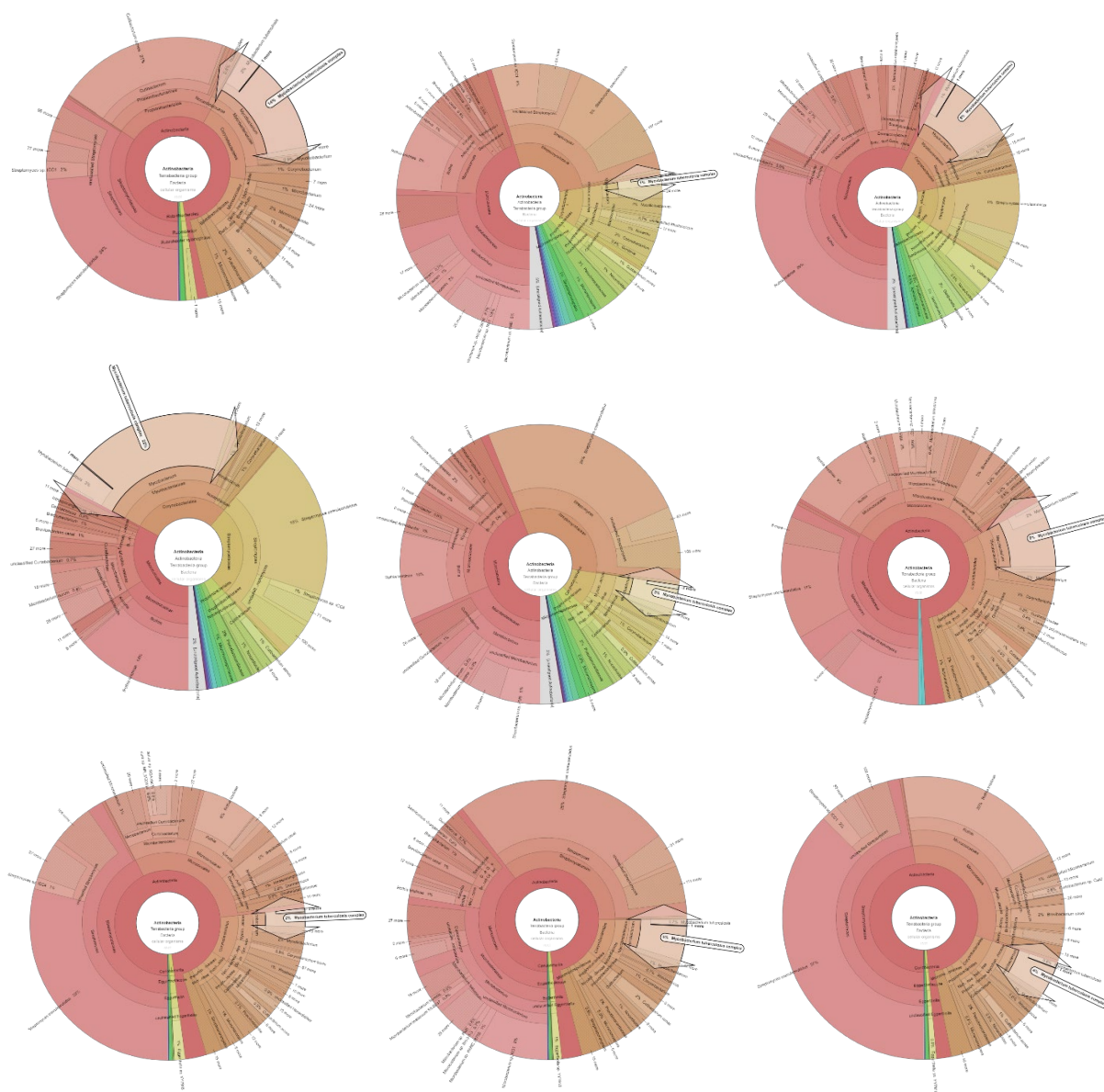
Figure 8.4: Shotgun Metagenome Sequencing of buffalo's milk pools by Kraken 2

CHAPTER IX – OVERALL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

Tuberculosis (TB), the major infectious disease in the world, has plagued humankind since the stone ages thanks to the ability of the causal agents to adapt to different environments and hosts. The causative agents of TB are known as the *Mycobacterium tuberculosis complex* (MTC) which evolved from an environmental organism to an obligate pathogen mastering the ability to grow inside host cells and to transmit from host to host [332].

Obligatory pathogens of the genus *Mycobacterium* comprise species belonging to the MTC, which includes: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. leprae*, *M. canetti* and recently described *M. orygis*, *M. mungi*, and *M. suricattae* [305]. Currently, infection by *M. bovis* is the standard for zTB burden and mortality estimation but this practice essentially ignores the contribution of other MTC species [333]. ZTB can also be caused by *M. caprae*, *M. canetti, M. mungi, M. orygis*, *M. pinnipedii*, and *M. microti* [334].

The current COVID-19 pandemic more than ever has raised the public's consciousness regarding the close and complex interrelationship between the health of humans, wildlife species, and domestic animals. Like COVID-19, zTB is evolving in an ever-changing global landscape and as a transboundary disease can cause serious socio-economic and public health consequences [333]. Moreover, TB caused by *M. bovis,* both in animals and humans, is poorly monitored due to country-by-country

variation in surveillance strategies and diagnostic capacities. As such, *M. bovis* remains an important, yet poorly addressed global problem.

The goal of this project was, through a One Health Approach, to perform a comprehensive epidemiologic study to clarify the burden of the *M. bovis* in Amazonas State. From July 2016 to February 2018, a total of 151 animals (45 buffalo and 106 cattle) were considered suspect of bTB and had tissues collected for laboratory confirmation using routine microbiological and molecular techniques. From February to August 2019, a total of 250 samples of raw milk were collected, 91 milk samples from cattle and 159 from buffalo for analysis using high throughput sequencing technologies. Additional valuable epidemiological information was collected that will be useful in planning programs to eradicate TB caused by *M. bovis* in animal and human populations in Amazonas state.

The study pictured a scenario, where the Amazon River basin presents not only the highest *M. bovis* prevalence ever reported in cattle and buffalo in Brazil but also the highest diversity of *M. bovis* in the country as determined by phylogenetic classification by spoligotypes and the novel linage classification. The study supported the evidence that *M. bovis* CCs classification was not sufficient to represent the species in Amazon region. The use of lineages classification was found to provide better information on the diversity of *M. bovis*, suggesting that the true global diversity of *M. bovis* strains remain to be discovered.

The presence of unusual spoligotypes (SB0822 and SB1608) and presence ancient strains (LB1), all of which were never described in the country demonstrates that *M. bovis* definitively presents a different genetic pattern in Amazon region

compared with the rest of Brazil. Moreover, our results confirmed that SB0822 is the predominant spoligotype in the Amazon region which differs from what has been reported is the rest of the country [113]. The spoligotype has been previously reported in cattle in Portugal [225] and in buffalo in Colombia [226]. Overall, our findings agree with the history of cattle in the region. The first livestock introduced came from Cape Verde (a former Portuguese colony in Africa) initially to Marajo Island and from there expanded to the floodplains of the Lower Amazon, on the banks of the Amazon River [271]. Finally, the presence of Lb1, infecting 2 buffalos, is surprising and requires further investigation into the actual origin of this strain. One hypothesis that could be tested is that this strain was a result of the introduction of cattle and buffalo by Portugal from her colonies in Africa to the Amazon region during the formation of Brazil.

To achieve the United Nations goal of ending the global TB epidemic by 2030, elimination of bTB is essential. For that reason, there is a need for evidence-based local strategies to eliminate the transmission of *M. bovis* within and between herds. To help to solve this challenge our study addressed three fundamental questions on the bTB epidemiology: 1) What is the bTB prevalence in Amazonas State? 2) What are the major risk factors for bTB transmission in the area? and 3) How can the detection of animals (cattle and buffalo) and herds infected by *M. bovis* be improved?

Our study revealed a prevalence of 3.0% in cattle and 11.8% in buffalo. The numbers represent significantly higher bTB prevalence than ever before reported in Brazil and may be explained by the characteristics of the local livestock. The local livestock industry in Amazonas state is characterized by family-run farms which are often made of relatively extensive bovine livestock systems, whether dairy, beef or

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mixed production. Herds with more than 100 animals, often have both cattle and buffalo. This of great importance since we found that buffalo *per se* were the major risk factors for bTB in the area of study. Due to the large territory, a good measure to help eradicate bTB should be the implementation of evidence-based control measures accordingly. Specific control strategies that are recommended include: 1) Establish zones according to the bTB prevalence, 2) Develop tuberculosis test requirements by zone, 3) Establish strict movement regulations between the zones, 4) Stablish a government subsidy for negative herd or a certification that would lead to a higher market price for milk; 5) Develop information/extension programs to discourage practices that promote mixing of cattle and buffalo.

To address the poor detection of cattle and herds infected by *M. bovis* using the traditional TST, we assessed the adoption of an antibody assays (IDEXX ELISA) to detect *M. bovis* infection in cattle. The IDEXX ELISA multiplex presented significantly higher sensitivity than the official comparative cervical test CCT with similar specificity results. Additionally, the parallel use of TST and ELISA increased the sensitivity and the feasibility of screenings for *M. bovis* infection diagnosis, and decreased time needed to cleanse the herds. Ultimately, the antibody assay was able to detect infected animals and herds missed by the CCT. Therefore, we provided evidence to support the validation of the IDEXX ELISA as a supplemental test for use by the Brazilian eradication program.

In 2020, of 10 million people with new active TB, 140,000 (range, 69,800–235,000) are estimated to be new cases of zTB (1.4%) of which an approximately 11,400 (8.1%, range 4,470-21,600) died due to the disease worldwide [4]. The situation

is more critical in countries with endemic bovine Tb, where populations consume raw milk, and where laboratory capacity is restricted [173]. Consumption of unpasteurized milk and dairy products, especially soft and hard cheeses have been identified as the primary risk factor of *M. bovis* infection in humans [11]. The real burden of *M. bovis* infection is unknown due to the absence of surveillance data from animal and human populations from most countries [68], [173]. Active surveillance and control of bTB in most parts of the world relies on the test-and-slaughter policy which is expensive and societally and/or economically unacceptable in many countries [335]. Our study sheds light on a potentially more feasible and efficient tool for active surveillance; the use of SMS in milk plants. SMS was able to detect milk contamination by MTC species including *M. bovis* and other pathogens in samples representing a range from 700 to 1700 cows in milking. Therefore, we recommend adoption of programs based on surveillance and traceback of tuberculous animals to herds of origin either by veterinary inspection in slaughterhouses or by screening of raw milk in milk plants.

Lastly, one particularly important finding from our study was the identification in raw milk of genetic material consistent with *M. tuberculosis H37rv*, and *M. africanum*, for the first time in Brazil. Coincidentally, in November 2020, the first case of pulmonary TB in Brazil, caused by *M. tuberculosis var. africanum*, in a 33-year-old Brazilian woman, African-America, native and resident of Marabá, Pará, northern Brazil, was reported [336]. The patient had no habits such as smoking, alcoholism and other comorbidities or previous surgeries, however apparently no questions regarding food habits were asked or disclaimed. The above information combined with our reporting of the circulation of a M. bovis Lb1 strain in the Amazon region [337] enforce the need for further studies to

clarify the circulation of ancient MTC strains in animals and humans in Brazilian Amazon.

OVERALL CONCLUSIONS

Overall, the study's findings revealed that *M. bovis* presents a distinct genetic profile in Brazilian Amazon when compared with the rest of the country.

The *M. bovis* prevalence rates in cattle and buffalo and its presence in raw milk from both species represents an eminent risk to public health in Brazilian Amazon (where the consumption of raw milk and its derivates is popular) and reveals an urgent need for evidence-based effective intervention aiming to reduce *M. bovis* prevalence in cattle and buffalo herds and to prevent its spread to the human population.

The current strategy of PNCEBT, based on voluntary adhesion and focused primarily on the identification of animals and herds infected by the *M. bovis* using TST protocols is likely to be inadequate to reach the goal of bTB eradication from the country. Adoption of supplemental serological tests for field detection and molecular technologies for postmortem and milk screening are strongly recommended. Modern epidemiological tools, such as identification of regional major risks, modelling, zooning, and traceability need to be considered for developing an efficient bTB disease surveillance and control program in Amazonas state.

RECOMMENDATIONS FOR NEW STUDIES

Ultimately, this study can stimulate a discussion about the many factors potentially impacting TB eradication schemes in animals and humans in Brazil and possibly stimulate new research in the areas identified. We suggest that new studies on the matter should be focused on the following:

1) A better understanding of the genetic profile of the *M. bovis* in the region. To clarify the origins of *M. bovis* ancient strains infecting cattle and buffalo in the area and the possible historical link with original cattle and buffalo introduced in the region coming from Africa.

2) Understanding why buffalo are more likely to be infected by *M. bovis* than cattle in Amazon. To investigate if factors linked to the host or to the pathogen or the combination host-pathogen factors and or local environmental factors can lead to higher infection rates in buffalo than in cattle.

3) Studies with active surveillance for *M. bovis* in TB patients are urgently required to assess the direct impact of the pathogen on human health in Amazonas state.

4) Following evidence from other studies in other countries, we suggest investigating the influence of wild animals on the epidemiology of bTB in the area.

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