

Diagnosis of mycobacteria in bovine milk: an overview

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ABSTRACT

Tuberculosis remains as the world's biggest threat. In 2014, human tuberculosis ranked as a major infectious disease by the first time, overcoming HIV death rates. Bovine tuberculosis is a chronic disease of global distribution that affects animals and can be transmitted to humans by the consumption of raw milk, representing a serious public health concern. Despite the efforts of different countries to control and eradicate bovine tuberculosis, the high negative economic impact on meat and milk production chains remains, given the decreased production efficiency (approximately 25%), the high number of condemned carcasses, and increased animal culling rates. This scenario has motivated the establishment of official programs based on regulations and diagnostic procedures. Although *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the major pathogenic species to humans and bovines, respectively, nontuberculous mycobacteria within the *Mycobacterium* genus have become increasingly important in recent decades due to human infections, including the ones that occur in immunocompetent people. Diagnosis of mycobacteria can be performed by microbiological culture from tissue samples (lymph nodes, lungs) and secretions (sputum, milk). In general, these pathogens demand special nutrient requirements for isolation/growth, and the use of selective and rich culture media. Indeed, within these genera, mycobacteria are classified as either fast- or slow-growth microorganisms. Regarding the latter ones, incubation times can vary from 45 to 90 days. Although microbiological culture is still considered the gold standard method for diagnosis, molecular approaches have been increasingly used. We describe here an overview of the diagnosis of *Mycobacterium* species in bovine milk.

KEYWORDS: Milk. Mycobacteria. Bovine. Microbiology. Molecular.

INTRODUCTION

The genus *Mycobacterium* includes a set of species with public health relevance, which has been divided into three groups according to clinical importance: (1) obligatory human and animal pathogens, (2) potentially pathogenic microorganisms (opportunistic) for animals and humans, and (3) saprophytes or ubiquitous microorganisms¹⁻³. Obligatory pathogens include species belonging to the *Mycobacterium tuberculosis* complex (*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*⁴⁻⁶). The potentially pathogenic species are represented by the *M. avium intracellulare* complex (*M. avium* subspecies *avium*, *M. avium paratuberculosis*, *M. avium hominissuis*, *M. avium silvaticum*, *M. colombiense* and *M. indicus pranii*), *M. marinum*, *M. fortuitum*, *M. scrofulaceum*, *M. kansasii*, *M. xenopi*, among others. Saprophytic mycobacteria are represented

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by *M. gordonae*, *M. terrae*, *M. smegmatis*, and *M. phlei*, also called atypical, “nontuberculous”, or environmental mycobacteria^{1,7-9}.

In the public health context, *M. tuberculosis* and *M. bovis* have the greatest impact, causing tuberculosis, an infectious disease of global distribution that shows chronic evolution and affects humans, domestic and wildlife animals⁹⁻¹². The World Health Organization (WHO) considers tuberculosis a re-emerging disease and a major threat due to the increased number of reports in humans, particularly in people living with HIV/AIDS, as well as the rise of multidrug-resistant isolates^{11,13}. In 2015, the WHO global report revealed about 9.6 million new cases of human tuberculosis, and 1.5 million deaths. The same organization reported that, in 2014, tuberculosis ranked by the first time as a major infectious disease¹⁴. It is estimated that *M. bovis* is the causal agent of human tuberculosis in about 10-15% of the cases in developing countries, and in about 1-2% of the cases in developed countries^{11,15}. In Brazil, 70,000 new cases are officially reported each year, resulting in about 4,600 deaths¹⁶. Moreover, human cases caused by nontuberculous mycobacteria have increased¹⁷. The significance of these bacteria as primary pathogens have been considered after the 21st century, given the emergence of pulmonary infections in patients with pre-existing pulmonary disease¹⁸, and the increase in the number of people living with HIV/AIDS.

Mycobacterium species are transmitted to humans predominantly by the inhalation of contaminated aerosols and consumption of contaminated water^{11,19}, as well as consumption of raw dairy products or those not heat-treated by pasteurization, boiling, or UHT (ultra-high temperature) processing. It is estimated that, in Brazil, about 20-30% of cow's milk is marketed without health inspection²⁰. This fact enhances the potential zoonotic risk of the microorganism transmission through milk, especially pathogenic species of *Mycobacterium*^{11,21}. Furthermore, *Mycobacterium* sp. can remain viable in cheese and yogurt made from raw milk for up to 14 days and up to 100 days in butter²². In humans, extrapulmonary manifestations (bone, joints, liver) are the most commonly reported in infections caused by *M. bovis* from cattle, whereas cases of pneumonia in rural and slaughterers workers have been reported, reflecting an occupational disease behavior²³.

Currently, according to the Brazilian Institute of Geography and Statistic (IBGE), Brazil has the largest commercial cattle herd in the world, with about 212 million animals²⁴. It is estimated that approximately 1.3% of the Brazilian cattle herd is affected by tuberculosis²⁵, particularly dairy cattle²⁶. In the public health context, mycobacterial infections in cattle cause substantial economic losses to

owners, mainly involving the condemnation of carcasses in slaughterhouses, reduction in milk and meat yield, animals slaughtered because of positive tuberculin tests carried out in control/eradication programs, commercial value depreciation of tuberculin-positive animals and international embargoes on the export of products from cattle origin²⁷.

In 2001, because of the negative impact of tuberculosis on the meat and milk production chain, the need to promote cattle product competitiveness in a highly demanding international market, as well as the zoonotic impact of the pathogen, Brazil created the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT), which established the rules and procedures for the diagnosis of the disease in cattle and buffaloes. In general, the program requires culling of reactor animals, standardized diagnostic tests and equipment (automatic antigen applicator and callipers), and training of veterinarians in diagnostic procedures²⁶. In 2011, the Brazilian Normative Instruction (IN) 62²⁸ was established (replacing IN 51) standardizing procedures from production to the marketing of different types of milk in the country, with emphasis on the quality control of raw milk.

In raw milk, the identification of *M. bovis* and atypical mycobacteria has been described in Tanzania²⁹, *M. bovis* and *M. africanum* in Nigeria³⁰, *M. bovis* in Tunisia,³¹ and atypical *Mycobacterium* species in Turkey³² and Brazil²⁵. Particularly in Brazil, Pardo *et al.*³³ in 52 cows showing positive or inconclusive results in Stormont test, identified *M. bovis* by phenotypic methods, and other atypical mycobacteria in 78 (10%) milk samples from 19 animals. In Tunisia, Ben Kahla *et al.*³¹, based on phenotypic methods and spoligotyping detected five (4.9%) isolates of *M. bovis* obtained from 306 milk samples from 102 cows that were positive in a single cervical tuberculin test. More recently, Pandey *et al.*³⁴, in Zambia, investigated the presence of *M. bovis* in milk of 16 tuberculin-positive cows and identified three (18.7%) positive samples using phenotypic methods, which were confirmed by Polymerase Chain Reaction (PCR). All these studies have shown that cattle shed the pathogen into milk, displaying the public health impact of these findings, because of the risk of consumption of raw milk or milk products that did not undergo official health inspection and/or adequate heat treatment.

The isolation of *Mycobacterium* species has extremely demanding requirements, based on selective and rich medium (usually egg-based)^{9,35,36}. Mycobacteria are classically divided into fast- and slow-growth species. Slow-growth species can take up to 90 days for isolation. Due to the long time required for microbial isolation combined with phenotypic diagnosis, the development of molecular

techniques allowing fast, accurate and reliable diagnosis of *Mycobacterium* species was stimulated. Indeed, Polymerase Chain Reaction (PCR) techniques associated with restriction enzyme analysis pattern (PRA), Multiplex Chain Reaction (Multiplex PCR) and Nested Polymerase Chain Reaction (Nested PCR) have been used. Other studies have reported increased diagnostic sensitivity and specificity by combining phenotypic techniques and molecular markers for the diagnosis of *Mycobacterium* sp. in individual animals, the identification of mycobacterium species, the geographical distribution of allelic diversity in outbreaks, and for epidemiological surveillance studies of bovine tuberculosis³⁷⁻⁴⁰ with spoligotyping (spacer oligonucleotide typing), and in the last decade, mycobacterial interspersed repetitive unit variable number of tandem repeats typing (MIRU-VNTR), and repeated identical sequences of DNA tandem (Exact tandem repeat-ETR)⁴¹.

Considering the threat bovine tuberculosis poses to animal health, the habit of consuming raw or non-heat-treated milk in Brazil, the Public Health concern in eliminating mycobacteria in dairy cows and the identification of mycobacteria genetic markers in molecular techniques that enable valuable diagnosis in short time, we describe here an overview of the diagnosis of *Mycobacterium* species in bovine milk.

BOVINE TUBERCULOSIS: GENERAL ASPECTS OF THE DISEASE

Tuberculosis is one of the most important zoonotic diseases in cattle, particularly in Latin America, Africa and Asia. The disease is caused by *M. bovis*, although other species of the genus *Mycobacterium* may affect cattle^{11,12,42}.

Although some European countries have reached a tuberculosis-free status, such as Switzerland⁴³, the increase in the number of human infections by *M. bovis* was noticed in the last decade, as a probable reflex of the endemic status of the disease in cattle. The increasing prevalence of bovine tuberculosis was recorded in the UK (3.27%) and Ireland (4.37%)⁴⁴. In Africa, Sudan reported 0.18% prevalence of tuberculosis in slaughterhouse cattle⁴⁵, whereas in Ethiopia the records on the occurrence of the disease have varied widely (1.5% to 78%)⁴⁶. The US fights the disease since 1900 with a control program based on tuberculin testing and culling of positive animals. However, despite the significant reduction in prevalence in the last decades, the disease was observed to reemerge in the States of Texas (1995), Minnesota (2005), and more recently, California (2008)²⁷.

In Latin America, a subdivision of the prevalence of bovine tuberculosis into categories was proposed: < 0.1%, from 0.1 to 1%, and > 1%; however, this classification

is influenced by the number of animals raised in each country⁴⁷. In Brazil, the disease is present throughout the country since 1920, but few actions with significant impact had been taken to fight the disease until 2001, when the PNCEBT²⁷ was instituted.

It is estimated that 1.3% of the Brazilian cattle herd is affected by tuberculosis²⁵. A recent study (2012-2013) of bovine tuberculosis prevalence in Brazil was conducted by the Ministry of Agriculture, Livestock and Supply (MAPA), based on statistical estimates of samples. Data from this study - not officially published yet - were preliminarily reported by Ferreira Neto (personal communication) in the V Brazilian Milk Quality Congress, which took place from 10th to 12th June, 2013, in *Águas de Lindóia, São Paulo*. In the State of *São Paulo*, 2,009 farms were investigated and 173 (8.6%) tuberculin-positive animals were diagnosed. In these farms of the State of *São Paulo*, 20,278 animals were evaluated, from which 320 (1.6%) were positive. In the same study, in the State of *Paraná*, 1,419 farms were investigated, from which 33 (2.3%) animals were positive in the tuberculin test. In the farms from the State of *Paraná*, 15,982 animals were evaluated, from which 63 (0.39%) were positive. A summary of these data on the bovine tuberculosis prevalence in some States of Brazil were reported by Paes and Franco⁴⁸ (Table 1).

Table 1 - Prevalence of bovine tuberculosis in some States of Brazil

State	Prevalence in the farms	Prevalence of positive animals
<i>Rondônia</i>	2.3%	0.1%
Bahia	1.0%	0.1%
Mato Grosso	1.2%	0.1%
<i>Paraná</i>	2.32%	0.39%
<i>São Paulo</i>	8.61%	1.57%

Tuberculosis occurs in cattle regardless of age and gender, although due to the chronic evolution, adult animals are more often diagnosed and they are more commonly symptomatic. *Bos taurus* is usually more affected than Zebu cattle (*Bos indicus*)⁴². The introduction of infected animals in a herd is probably the main risk factor for the spread of the disease among dairy animals⁴⁹. Mycobacteria are eliminated by respiratory tract secretions, milk, colostrum, feces, urine and, occasionally, semen. Cattle-to-cattle transmission occurs mainly by contact with aerosols or consumption of contaminated water, food and milk⁴⁹⁻⁵¹.

After invasion by oral or respiratory routes, the pathogen is actively phagocytosed by macrophages and multiplies upon entry, but it is not eliminated in the phagolysosome

due to the peculiar characteristics of the cell wall structure. Then, it is captured in regional lymph nodes and the response of CD4 + lymphocytes (6-8 weeks) starts, which is very important in the ante-mortem diagnosis, because these cells are recruited in the tuberculin test. The microorganism induces a typical granulomatous reaction, with caseous and/or necrotic lesions and a peculiar architecture formed by the free mycobacteria or the pathogen within macrophages, macrophages modulated in epithelioid cells and giant cells, and presence of caseum, all surrounded by a fibrous capsule. Chronic disease progression is characterized by formation of large caseous nodules, particularly in the lungs (airborne infection) and intestinal tract (oral infection). In immunocompromised or debilitated animals, early generalization may occur with spread of small nodules to several organs, including the mammary gland^{27,52-54}.

Bovine tuberculosis causes production efficiency losses ranging from 10 to 25% in infected animals, leading to severe losses related to reduced milk and meat production, condemnation of carcasses in slaughterhouses, sanitary slaughter of tuberculin-positive animals, and restrictions on the export of animals and meat products of bovine origin^{26,27}.

Bovine tuberculosis was fought in other countries and control or eradication of the disease was reached after systematic tuberculin testing, slaughtering of positive animals in officially inspected slaughterhouses, control of the animal movement, adoption of quarantine and testing of newly acquired animals, certification of farms free of the disease, health education, and heat treatment of milk^{26,27,42}.

Brazilian regulations do not allow the treatment of cattle with tuberculosis and recommend the culling and slaughtering of animals with positive tuberculin tests in inspected abattoirs, or even mass killing in the farm of origin in the presence of large number of positive animals as well as other sanitary actions planned for positive herds²⁶.

MYCOBACTERIA IN DAIRY CATTLE

Bovine tuberculosis affects various organs and tissues, including the mammary gland. It is usually caused by *M. bovis*, although other species can infect the udders, particularly atypical mycobacteria^{42,55}. Infections with *M. bovis* in cattle are progressive²⁷. In contrast, although *M. tuberculosis* can affect the mammary gland, infection is usually not progressive. Dairy cattle are more predisposed to tuberculosis probably due to the proximity of the animals in the milking and feeding environment⁵¹.

Mastitis

Unlike the most frequent agents of bovine mastitis,

represented by staphylococci, streptococci and enterobacteria, which commonly cause an ascending infection of the teat canal, mycobacteria reach the mammary gland by systemic dissemination, probably after the spread of the primary infection by respiratory or digestive route^{42,56}. Occasionally, infection can come from the environment of farms or by contamination of cannulas used in intramammary treatments. Mycobacteria as primary causes of bovine mastitis are considered of low occurrence, although prevalence can be high in farms endemic for tuberculosis or where outbreaks were observed^{42,57}. In cattle, the disseminated form of tuberculosis occurs mainly in immunocompromised animals or those showing general weakness, with early generalization resulting in small nodules (granuloma) in the mammary gland and other organs²⁷.

The occurrence of clinical or subclinical mastitis and the frequency of mycobacteria shedding into milk is controversial⁵⁸⁻⁶⁰. Cows with tuberculosis show reduced milk production, hardened masses in the udder, enlargement of regional lymph nodes and watery milk, although probably less than 1% of cows with tuberculosis show mammary signs.⁴² Paes²⁷ reported that tuberculous mastitis causes hardening of mammary quarters and enlargement of supramammary lymph nodes. In the early stages of the disease, signs of clinical mastitis may not be seen in milk (subclinical mastitis). In chronic cases, milk can show fine lumps at the end of milking and color may be change to amber, with the elimination of bacilli in milk.

Raw milk

In raw milk, the risk of mycobacteria elimination in lactating cows was investigated in Tanzania using phenotypic and molecular methods. Among 805 milk samples, there was a predominance of atypical mycobacteria such as *M. flavescens* (n=13), *M. terrae* (n=7), *M. smegmatis* (n=4), *M. fortuitum* (n=2), *M. gordonae* (n=1), whereas only two isolates were identified as *M. bovis*. Despite the low prevalence of *M. bovis*, the presence of atypical mycobacteria in the milk of animals is a public health concern, because of the habit of consuming raw milk by these people, besides the high incidence of people living with HIV/AIDS²⁹.

In Turkey, 35 samples of raw milk were evaluated for the presence of mycobacteria using phenotypic methods and confirmed by Polymerase Chain Reaction - Restriction Pattern Analysis (PCR-PRA), resulting in the detection of *M. terrae*, *M. kansasii*, *M. agri*, and *M. haemophilum*³². In another study in the same country, *M. genavense*, *M. simiae*, *M. szulgai*, *M. bovis* and *M. fortuitum*⁶¹ were identified.

M. bovis (n=4) and *M. africanum* (n=1) were identified among 400 samples of unpasteurized cow milk in Nigeria using phenotypic and molecular methods³⁰.

Tuberculin test

Few studies have focused on the identification of the presence of mycobacteria eliminated by dairy cows that were positive in tuberculin tests. In this context, the presence of mycobacteria was investigated in 306 samples of raw milk collected from 102 positive cows using the single cervical tuberculin test, from herds with tuberculosis history in Tunisia. Five isolates of *M. bovis* were identified by phenotypic methods and spoligotyping, underscoring the risks of raw milk consumption³¹. Pandey *et al.*⁴³, in Zambia, investigated the presence of *M. bovis* in 16 tuberculin-positive dairy cows and three (18.7%) positive samples were detected using phenotypic methods, confirmed by PCR. This finding confirmed that the animals were eliminating the pathogen in milk and it reinforced the public health impact, given the high prevalence of people living with HIV/AIDS in the country, as well as all over the African continent.

In Egypt, 46 cows presenting clinical signs of tuberculosis, including low production, emaciation, anorexia, intermittent diarrhea (unresponsive to anthelmintic drugs), cough and labored breathing were subjected to the single cervical and caudal fold test. From 23 milk samples, one (4.35%) was positive for *M. bovis* using the Lowenstein-Jensen medium with sodium pyruvate, evidencing typical colonies and compatible biochemical results⁶². Likewise, Nasr *et al.*⁶³ collected samples from 50 cows reactors to the single cervical tuberculin test and 50 cows that were nonreactors. Results showed that samples from two reactor animals (4%) were suggestive of *M. bovis* based on growth in Lowenstein-Jensen medium with pyruvate; another sample was identified as *Mycobacterium* spp. by growth in Lowenstein-Jensen medium with glycerol.

Another study in India investigated mycobacteria in 181 raw milk samples, from which one sample was classified as *M. tuberculosis* by molecular methods. It is noteworthy that humans are the sources of *M. tuberculosis* infection in animals⁶⁴. In Pakistan, Jalil *et al.*⁶⁵ analyzed 1,000 milk samples obtained from 185 bovines and 815 buffalos and reported 20 positive samples to *M. bovis* using Lowenstein-Jensen and Stonebrink media with subsequent inoculation in rabbits and Guinea pigs. Qamar and Ashar⁶⁶, between January and June 2013, collected 210 bovine milk samples [UHT (n=35), pasteurized (n=33), retail (n=92), and bulk tank (n=60)], among which nine positive samples for *Mycobacterium* were identified, three in bulk tank milk. In Iraq, seven of 68 raw milk samples grown in mycobacteria

medium showed colonies compatible with *M. bovis*, which were confirmed by biochemical and molecular methods⁶⁷.

In Brazil, Pardo *et al.*³³ analyzed 52 cows with positive or inconclusive results to Stormont's test, and identified *M. bovis* and atypical mycobacteria using phenotypic methods, in 78 (10%) milk samples from 19 animals. More recently, Franco *et al.*²⁵ in the same country, investigated the presence of mycobacteria in 300 milk samples, 100 from collective bulk tanks, 100 from bulk tanks of individual farms, and 100 samples marketed without sanitary inspection, popularly called "informal milk" in the State of *São Paulo*. These samples were assessed by phenotypic methods, confirmed by PCR-PRA. The following species were isolated in pure cultures: *M. bovis*, *M. gordonae*, *M. fortuitum*, *M. intracellulare*, *M. flavescens*, *M. duvalii*, *M. haemophilum*, *M. immunogenum*, *M. lentiflavum*, *M. mucogenicum*, *M. novocastrense*, *M. parafortuitum*, *M. smegmatis*, *M. terrae*, and *M. vaccae*. There were no significant differences between different types of milk, since mycobacteria were recovered and identified in 9% of the bulk tanks of individual farms, 7% of collective bulk tanks, and 8% of "informal" milk samples. The authors pointed out to the Public Health the threat posed by these results, because of the risk of milk consumption containing the pathogen, particularly from "informal milk", which does not undergo any heat treatment and/or the official health inspection.

MICROBIOLOGICAL DIAGNOSIS

Microbiological culture remains the gold standard test for *Mycobacterium* sp. detection in clinical samples⁶⁸, due to its sensitivity and specificity. After culture, other tests are carried out, such as species identification, antimicrobial susceptibility profile, genotyping, as well as monitoring of human patients response to treatment⁶⁹. Rich selective media are required for mycobacteria species isolation, especially containing egg³⁵, which is commonly used in veterinary microbiology³⁶. Among these media, Lowenstein-Jensen, made with egg and glycerol^{70,71}, favors the isolation of *M. tuberculosis* and *M. avium*, although glycerol inhibits the isolation of *M. bovis*. Lowenstein-Jensen medium has a green color due to the addition of malachite green to inhibit contaminants, and mycobacteria colonies show a yellowish hue with a creamy appearance (Figure 1). Stonebrink is another media, rich in pyruvate, favoring the multiplication of *M. bovis* (Figure 2), *M. africanum* and *M. microti*, since these species are unable to use glycerol as a source of carbon^{48,72,73}.

Nevertheless, clinical samples have to be decontaminated to allow *Mycobacterium* spp. isolation. The greatest difficulty in selecting a reagent and determining its concentration



Figure 1 - Yellowish and creamy appearance of *M. avium* colonies in Lowenstein-Jensen medium after 20 days of incubation.

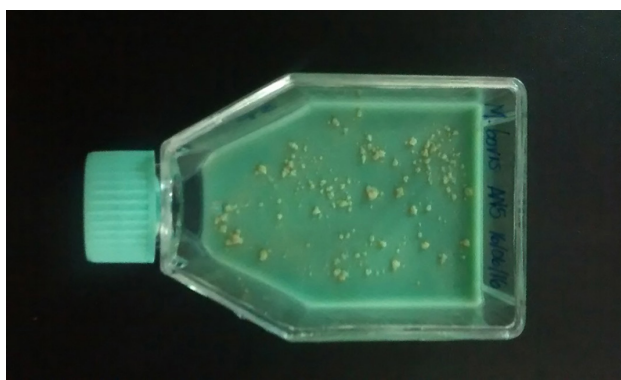


Figure 2 - Colonies of *M. bovis* in Stonebrink media.

to decontaminate a sample is the adverse effect that most substances have on mycobacteria. The ideal reagent should have minimal toxicity to the genus *Mycobacterium* but maximum toxicity to other contaminating microorganisms⁷⁴. The most commonly substances used are alkalis (8%) and acids (8%) (Petroff method) before microbiological culture^{75,76}. The sample decontamination procedure consists of placing an aliquot of milk (about 8 mL), previously centrifuged at 7,280 g to concentrate the cells and fat, followed by the discard of serum, in a sodium hydroxide 8% (alkali) solution. The sample is homogenized by vortexing and incubated for 30 minutes at 37 °C. Afterwards, sulfuric acid 8% is added to stabilize pH and, as a last step, the pellet (material resulting from the centrifugation) is washed two times to eliminate any sodium hydroxide or sulfuric acid residue²⁵. Samples are grown under aerobic conditions at 37 °C and evaluated weekly for up to 90 days. Phenotypic diagnosis is classically based on isolation time, morphologic characteristics of the colonies and production of pigments in the presence or absence of light^{77,78}.

MOLECULAR DIAGNOSIS

The long time required in microbial isolation (starting

after six weeks)²⁷ and biochemical tests for mycobacteria phenotypical identification has stimulated the development of molecular techniques in the diagnosis of *Mycobacterium* species from human and animal origin. The advent of new molecular techniques has enabled the investigation of epidemiological data, diagnosis of outbreaks, virulence aspects, clonal structure of the pathogen population and distribution of species from different geographic areas. It has also enabled the investigation of pathogen evolution, taxonomic reclassification, and identification of new species. These data can support control measures, prophylaxis, and/or treatment of human and animal disease^{37-40,79}.

M. tuberculosis complex species have genetic homology of 99.9% at nucleotide level and identical 16S rRNA sequences, which allows a certain adaptability of molecular techniques developed for the diagnosis. In this context, the genome of *M. tuberculosis* has more than 99.95% similarity with *M. bovis* genome. However, they have differences in virulence and host susceptibility⁸⁰. Despite the low incidence of mutations, there are diverse loci among species that have been targets in the development of molecular techniques to be used in the diagnosis of the pathogen in different clinical specimens, leading to the recognition of new species and changes in mycobacterium taxonomy³⁷.

Within the approaches used in molecular diagnosis, there are different techniques that can be applied to mycobacteria diagnosis with variable sensitivity and specificity. **Table 2** shows studies around the world involving different methods of mycobacterial diagnosis in bovine milk samples, presented in chronological order, and **Table 3** lists the non-tuberculous mycobacteria that have been identified in bovine milk.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a popular molecular biology technique in which DNA is replicated enzymatically without necessarily using a living organism, such as bacteria or yeasts. This technique enables the extensive amplification of a small amount of the DNA molecule in an exponential manner⁸¹.

DNA amplification via PCR techniques have become tools for epidemiological studies of bovine tuberculosis transmission, and have given prominence to a modern research field. The agility and speed in detecting mycobacteria can be decisive in the choice of these methods, which discriminate species and different isolates of the same species at the DNA level⁸². For example, a conventional PCR technique is used aiming at the amplification of a

Table 2 - Chronological list of studies involving mycobacterial diagnosis in cow milk samples

Number of animals	Number of samples	Number of positive animals	Type tuberculin test	Culture media	Molecular assay	Serology	Staining method	NTM	<i>M. bovis</i>	<i>M. tuberculosis</i>	Country	Reference
805			Non-related	LJ; ST	M-PCR		ZN	27	2		Tanzania	29
52	780		Stormont	LJ; ST			ZN	3	1		Brazil	33
1000		73		ST			ZN		20		Pakistan	65
78			Non-related	LJ; ST	PCR-RFLP			10	1		Brazil	87
144	753	37	CSIT	LJ; ST	N-PCR				20	19	India	86
35			Non-related	LJ	PCR-PRA		ZN	15	0		Turkey	32
23			CSIT; CFSIT	ST		ELISA		0	1		Egypt	62
102	306		CSIT	LJ; Coletsos	Spoligotyping; VNTR		ZN	6	5		Tunisia	31
145				LJ	PCR		ZN	11	1		Turkey	61
1.025	16	21	SICCT	LJ; ST	M-PCR; LAMP			0	3		Zambia	34
100	100	50	CSIT	LJ; ST		ELISA	ZN	1	2		Egypt	63
60				LJ; ST			ZN	3			Pakistan	66
104	150				PCR				0		Israel	98
32			Non-related					13	0		Brazil	88
82					PCR		ZN		10		Iraq	90
150	150	150	SICCT	LJ; ST	PCR				75		Brazil	99
50	50	34	CSIT		M-PCR			4	1		Brazil	100
181				LJ; ST	PCR; M-PCR		ZN; Auramin			4	India	64

CSIT: Single Cervical Intradermal Test, **CFSIT:** Single Caudal Fold Intradermal Test, **SICCT:** Single Intradermal Cervical Comparative Test, **LJ:** Löwenstein-Jensen, **ST:** Stonebrink, **PCR:** Polymerase Chain Reaction, **M-PCR:** Multiplex Polymerase Chain Reaction, **PCR-RFLP:** Restriction Fragment Length Polymorphism, **N-PCR:** Nested Polymerase Chain Reaction, **PCR-PRA:** Polymerase Chain Reaction of Restriction Pattern Analysis, **VNTR:** Variable Number Tandem Repeat, **LAMP:** Loop-mediated Isothermal Amplification System, **ELISA:** Enzyme-Linked Immunosorbent Assay, **ZN:** Ziehl-Neelsen, **NTM:** Nontuberculous Mycobacteria.

Table 3 - Nontuberculous mycobacteria detected in milk samples by molecular and microbiological methods

Total number of isolates	<i>M. terrae</i>	<i>M. peregrinum</i>	<i>M. smegmatis</i>	<i>M. Kansasii</i>	<i>M. flavescens</i>	<i>M. neoaurum</i>	<i>M. scrofulaceum</i>	<i>M. fortuitum</i>	<i>M. nonchromogenicum</i>	<i>M. gordonae</i>	<i>M. marinum</i>	<i>M. agri</i>	<i>M. haemophilum</i>	<i>M. genavense</i>	<i>M. simiae</i>	<i>M. szulgai</i>	<i>M. avium</i>	<i>M. paratuberculosis</i>	Reference
17		2	3	1	3	1	1	1	5										88
27	7		4		13			2		1									29
6					1														31
15	6			3								3	1						32
10								4		5	1								87
11								1						6	2	2			61
3								2									1		33
265																		80	102
70																		2	103
220																		63	104

500-base pair fragment of the RvD1-Rv2031c region, found only in *M. bovis* strains^{83,84}.

Nested Polymerase Chain Reaction (Nested PCR)

Nested Polymerase Chain reaction is a modification of PCR with the purpose of reducing the binding of non-specific products due to non-expected amplification of primer binding sites. It runs with two sets of primer pairs used in two continuous PCRs. The second run targets the final product⁸¹.

In Egypt, the Nested PCR technique was used to investigate *M. bovis* in cattle and buffalo milk samples of animals that were positive (n=190) and negative (n=225) to the tuberculin skin test and in commercial milk samples (n=95). In that study, *Mycobacterium* was detected in 33 samples (6.35%), 22 belonging to the *M. tuberculosis* complex, whereas the Ziehl-Neelsen microscopic evaluation indicated the presence of BAAR in 23 samples, 14 positive samples in bacteriological culture, and 12 positive samples in mice inoculation⁸⁵. In the same study, PCR was used to diagnose *M. bovis*, and 16 positive samples were obtained. Nested PCR performed better in terms of sensitivity and specificity, because it was able to identify a greater number of samples compared to traditional techniques, including conventional PCR⁸⁵. In India⁸⁶, the same technique was used to detect *M. tuberculosis* and *M. bovis* in different types of samples, including raw milk from cattle positive to the tuberculin test. The results on the correlation of the association of the standard technique (bacterial culture)

with Nested PCR indicated a higher sensitivity (97.3%) compared to the cultures of the standard method (29.7%), but specificity was higher for culture (77.5%).

Multiplex Polymerase Chain Reaction (Mutiplex PCR)

Multiplex is a kind of PCR that uses several primer sets and amplifies many regions simultaneously. Bhanurekha *et al.*⁶⁴ used Multiplex PCR for the differentiation of *M. tuberculosis* and *M. bovis* in 181 raw milk samples from small farms to monitor the presence of zoonotic tuberculosis, identifying four positive samples for *M. tuberculosis* (n=3) and *M. bovis* (n=1).

Polymerase Chain Reaction with Pattern Restriction Analysis (PCR-PRA)

PCR-PRA is based on the segregation of DNA sections by restriction endonucleases, generating fragments used in the classification of mycobacteria species. This method has focused in the nucleotide sequence⁷⁹ of locus *hsp65*.

Particularly in Brazil, raw bovine milk and pasteurized milk were screened for the presence of mycobacteria by PCR-PRA^{87,88}. The following species were reported: *M. nonchromogenicum*, *M. peregrinum*, *M. smegmatis*, *M. fortuitum*, *M. marinum*, and *M. kansasii*. *M. bovis* was identified only in one raw milk sample⁸⁸. Although most mycobacteria do not belong to the *M. tuberculosis* complex, it is important to identify failures in the process of milk pasteurization that pose risks to humans.

Restriction Fragment Length Polymorphism IS6110 (RFLP IS6110)

The insertion sequence IS6110 is considered specific to the members of the *M. tuberculosis* complex and the difference in location and number of copies of the insertion sequence is a source of polymorphism between isolates⁸⁰. That polymorphism is detected by RFLP.

Vitale *et al.*⁸⁹, in Italy, evaluated the possible application of PCR IS6110 (specific technique for the detection of *M. tuberculosis* complex) in the diagnosis of bovine tuberculosis. Milk, nasal swabs, and lymph nodes aspirates were sampled from animals subjected to skin test (n=100), and lymph nodes and lungs were sampled in animals with positive results (n=60). From the results of the analyses of all milk samples (n=54), 47 (87%) were positive in PCR. The sensitivity and specificity was 100% when compared with tissue samples. Other authors have also used IS6110 PCR in the diagnosis of bovine tuberculosis⁹⁰.

A study carried out in Argentina by Zumárraga *et al.*⁹¹, searching for the presence of *M. bovis* in bulk milk from 117 tuberculosis-free certified farms and 80 non-certified farms using IS6110 PCR milk technique, found that 67 (38%) samples from certified-free herds were positive for *M. bovis*, and 35 (44%) samples from uncertified herds were positive for *M. bovis*. The study also compared the results of tuberculin tests in terms of sensitivity. It was found that in certified-free herds, 67 samples were positive in PCR IS6110 and only three were positive in the tuberculin test, while in uncertified farms, 17 of the 35 positive samples were negative in the tuberculin test. The authors attributed these results to the slaughtering of animals that were positive in the tuberculin test, before the official inspection could be able to issue the certificates on disease-free status.

GENETIC FINGERPRINTING

Spacer Oligonucleotide typing (Spoligotyping)

Typing of mycobacteria species can be performed by spoligotyping, which is based on DNA amplification of direct repeat (DR) loci, found only in the genome of the *M. tuberculosis* complex. The DR regions are repeated, with two types of continuous identical sequences (DR sequences) and different sequences (spacers). Polymorphism of mycobacteria is evaluated by the presence or absence of spacers at each locus, as well as by the number of DR regions in the isolates. The advantage of this technique is its speed, reliability, and species differentiation of *M. tuberculosis* and *M. bovis*. It also enables the molecular study of spoligotypes geographical dispersion^{80,82}.

Variable number tandem repeat (VNTR) and mycobacterial interspersed repetitive units (MIRU) typing

Tandem repeats are allelic variations that occur as repetitions of sequences of nucleotides found in intergenic regions of the genome. These repeated sequences are known in eukaryotic cells. The hyper variability of these repetitions in human and animal cells generate the Variable number tandem repeat (VNTR). In the diagnosis of mycobacterial isolates, the polymorphism may be analyzed by repetitions of sequences of nucleotides, or even variation in the number of repeating units^{39,40}. The identification of 24 VNTR loci of *M. tuberculosis* genome received the name Mycobacterial Interspersed Repetitive Units (MIRU)⁹², and it has been used as a molecular marker for diagnosis of mycobacterial infections. Identification and classification by MIRU-VNTR method has proven to be fast, sensitive and affordable, particularly for isolated mycobacteria⁹³.

Spoligotyping and MIRU-VNTR were carried out in Tunisia by Kahla *et al.*³¹ in milk samples (n=306) of cows (n=102), positive to the single cervical intradermal tuberculin test and with or without clinical signs of tuberculosis and mastitis. From 102 cows, five were positive to *M. bovis*. Spoligotyping and VNTR revealed the heterogeneity of the isolates, because the first technique determined three types, and the second one identified four *M. bovis* profiles.

DNA sequencing

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases (adenine, guanine, cytosine and thymine) in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. The knowledge of DNA sequences has become indispensable to basic biological research and to numerous applied fields, such as medical diagnosis, biotechnology, forensic biology, virology, and biological taxonomy. The high speed of sequencing achieved with modern DNA sequencing technology has been critical in the knowledge of complete DNA sequences, or genomes of numerous types and species, including the human genome and other complete DNA sequences of many animal, plant, and microbial species⁹⁴.

Although some authors have applied DNA sequencing to classify *Mycobacterium* species based on the *hsp65* gene⁹⁵⁻⁹⁷, to date there is no evidence on the application of DNA sequencing for the diagnosis of *Mycobacterium* spp. in bovine milk.

Although there is a diversity of molecular methods for mycobacteria diagnosis, sequence analysis has been the most used method in clinical laboratories. The identification of specific signs in variable regions of highly conserved genes has simplified PCR protocols and the analysis of amplified products⁹⁷⁻¹⁰². For NTM diagnosis, the *hsp65* gene is the most widely target used to classify this group of mycobacteria. When the target is *M. bovis* or *M. tuberculosis*, the MIRU-VNTR is a highly discriminatory method and an important tool in epidemiological research⁹⁹.

THE MYCOBACTERIUM BOVIS PUBLIC HEALTH RISK

Despite the fact that *M. bovis* is not the major cause of human tuberculosis, human infection by this species of mycobacteria has been increasingly reported. Humans become infected mainly by consumption of raw milk from infected cows¹⁰³. In 2015, 149,000 cases of zoonotic TB occurred worldwide, with a global mortality rate of 13,400 cases¹⁰⁴. Particularly in Brazil, despite the existence of a national eradication plan, milk is still marketed without sanitary inspection, which is a threat to public health²¹. Furthermore, there is a need to strengthen the surveillance of zoonotic TB in this country for better knowledge on the impact of the disease in dairy animals¹⁰⁴.

CONCLUSIONS

Despite advances and control programs, tuberculosis remains as a major human and animal health threat. In Brazil, bovine tuberculosis is still a major infectious disease. Due to implications on public health and negative effects on milk production chain, demands on the research of bovine tuberculosis and other infections caused by mycobacteria have been increasing. The development and application of methods and more efficient techniques are critical tools in the diagnosis of the disease to determine control measures and effective eradication.

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