Diagnosis and risk factors of *Mycobacterium avium* subsp. *paratuberculosis* **(MAP) in dairy herds of the Northern Region of Antioquia, Colombia**

Graduate Student

Nathalia María del Pilar Correa Valencia, DMV

Director

Jorge Arturo Fernández Silva, DVM, MPH, Dr. Med. Vet.

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Recuerda que cualquier momento es bueno para comenzar
y que ninguno es tan terrible para claudicar.
No olvides que la causa de tu presente es tu pasado
así como la causa de tu futuro será tu presente.
Aprende de los audaces, de los fuertes,
de quién no acepta situaciones, de quién vivirá a pesar de todo,
piensa menos en tus problemas y más en tu trabajo
y tus problemas sin alimentarlos, morirán.
Aprende a nacer desde el dolor
y a ser más grande que el más grande de los obstáculos.

Pablo Neruda

A mi familia, siempre.

Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

AFB	Acid Fast Bacteria
AGID	Agar Gel immunoDiffusion
bp	Base Pairs
CD	Crohn´s Disease
CF	Complement Fixation
CFU	Colony Forming Units
CI	Confidence Intervals
CIE	Counterimmunoelectrophoresis
CMI	Cell-Mediated Immunity
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunoabsorbent Assay
e.g.	exempli gratia (for example)
et al.	Et alii (and others)
FC	Fecal Culture
FISH	Fluorescent *in situ* hybridization
g	gram
h	hour
HE	Hematoxylin and Eosin staining
HEYM	Herrold´s Egg Yolk Medium
HPC	Hexadecyl Pyridinium Chloride
IAC	Internal Amplification Control
i.e.	id est (that is)
IF	Indirect Immuno-Fluorescence
INF	Interferon
IS	Insertion Sequence
JD	Johne´s Disease

L	Liter
M.	Mycobacterium
MAA	*Mycobacterium avium* subsp. *avium*
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
min	Minute
ml	Milliliter
mm	Millimeter
OD	Optical Density
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivate
PTB	Paratuberculosis
qPCR	Quantitative (real-time PCR)
S/P	Value of the sample / Value of the positive control
SD	Standard Deviation
Se	Sensitivity
Sp	Specificity
subsp.	subspecies
U	Unit
µl	Microliter
µM	Micromolar
w/v	Weight/Volume
%	Percentage

General Summary

Introduction: paratuberculosis is a slow-developing infectious disease, characterized by chronic granulomatous enterocolitis. This disease has a variable incubation period from 6 months to over 15 years, and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Some studies have been conducted in cattle during the last decades in Colombia. However, those studies were designed using a relatively small population, were not aimed to establish prevalence, and were limited to the assessment of risk factors. **Objective:** to determine the prevalence of MAP, confirmed by real-time PCR, and to explore the main risk factors associated with ELISA and/or real-time PCR positive results in animals of some dairy herds of the Northern region of Antioquia, Colombia. **Methods:** serum and fecal samples, and related data were taken from 696 randomly selected bovines in 28 dairy herds, located in 12 different districts in one of the main dairy municipality in Colombia (San Pedro de los Milagros). The samples were analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) kit, Herrold´s egg yolk medium (HEYM) culture, an end-point IS*900*-specific nested PCR protocol, and a commercial *F57*-real-time PCR kit. The information on risk factors was analyzed by means of descriptive tatistics and logistic regression. **Results:** the seroprevalence obtained was 3.6% (1/28) at herd-level and 2% (14/696) at animal-level. Days in milk between 100 and 200 days and over 200 days, and daily milk production between 20 to 40 L/cow and over 40 L/cow, with Odds Ratios of 4.42, 3.45, 2.53, and 20.38, respectively, were associated with MAP seropositivity. None of the fecal samples from the seropositive herd resulted positive by duplicate to HEYM culture. None of the samples was found to be positive by *F57*-real-time PCR. Seven of the 27 samples were found to be positive by end-point IS*900* -specific nested PCR. Agreement was found between ELISA and end-point IS*900* -specific nested PCR in one of the samples. **Conclusion:** this study demonstrates MAP presence in dairy herds from Antioquia and the relationship between MAP seropositivity and milk yield and

lactation stage. It also gives information about limitations of the different MAP-diagnostic techniques to be considered for the determination of an infected animal and herd.

Resumen General

Introducción: la paratuberculosis es una enfermedad infecciosa de desarrollo lento, caracterizada por una enterocolitis granulomatosa crónica. Esta enfermedad tiene un periodo de incubación que varía entre 6 meses y 15 años, y es causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Algunos estudios se han desarrollado en el ganado bovino durante las últimas décadas en Colombia. Sin embargo, estos estudios fueron diseñados utilizando una población relativamente pequeña, no buscaban estimar la seroprevalencia y presentaron limitaciones al momento de definir factores de riesgo. **Objetivo:** determinar la prevalencia de MAP, confirmada por PCR en tiempo real, y explorar los principales factores de riesgo asociados con los resultados positivos de ELISA y/o PCR en tiempo real en animales de algunos hatos lecheros en la región norte de Antioquia, Colombia. **Métodos:** se tomó muestras de suero y materia fecal, así como información relacionada de 696 bovinos selccionados aleatoriamentre, en 28 hatos localizados en 12 veredas diferentes de uno de los principales municipios lecheros en Colombia (San Pedro de los Milagros). Las muestras fueron analizadas utilizando un kit comercial de ensayo por inmunoabsorción ligado a enzimas (ELISA), un medio de cultivo Herrold´s egg yolk medium (HEYM), un protocolo de PCR convencional anidado para IS*900*, y un kit comercial de PCR en tiempo real para *F57*. La información sobre factores de riesgo fue analizada por medio de estadística descriptiva y regresión logística. **Resultados:** la seroprevalencia obtenida fue de 3,6% (1/28) a nivel hato y del 2% (14/696) a nivel individual. Las variables días en leche, entre 100 y 200 diás y más de 200 días, y la producción diaria de leche, entre los 20 y 40 L/vaca y más de 40 L/vaca, con Odds Ratios de 4,42, 3,45, 2,53 y 20,38, respectivamente, estuvieron asociados a la seroprevalencia de MAP. Ninguna de las muestras fecales del hato seropositivo resultó positiva al cultivo en HEYM. Ninguna de las muestras resulto positiva por PCR en tiempo real para *F57*. Siete de las 27 muestras resultaron positivas por PCR convencional anidado para IS*900*. Se encontró concordancia entre los resultados de ELISA y de PCR convencional anidado para IS*900* para una de las muestras. **Conclusión:** este estudio

demuestra la presencia de MAP en hatos lecheros de Antioquia y la relación existente entre la seropositividad a MAP con la producción de leche y el estadio de lactancia. También aporta información sobre las limitaciones de las diferentes técnicas diganósticas de MAP a considerar para la determinación de un animal y un hato como infectados.

General Introduction

Paratuberculosis (PTB) is a severe enteritis that affects cattle and other domestic and wild ruminants (Harris and Barletta, 2001). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causal agent of PTB, a Gram-positive, facultative, mycobactin-dependant, slow growing and acid–fast bacillus (Chiodini *et al.,* 1984; Sweeney, 1996). MAP is very resistant both environmental and chemical changes, and can persist in the environment, including soil, stream water, and manure slurry storage, for up to a year (Sweeney, 1996; Eppleston *et al.,* 2014; Kaevska *et al.,* 2014; Salgado *et al.,* 2015), It has been detected in food, especially in milk and dairy products (Bülte *et al*., 2005; Sweeney *et al.,* 2012; Atreya *et al.,* 2014; Hanifian, 2014; Liverani *et al*., 2014; Botsaris *et al.,* 2016; Galiero *et al.,* 2016).

PTB is a slow-developing infectious disease characterized by chronic granulomatous enterocolitis and regional lymphangitis and lymphadenitis (Clarke, 1997). Incubation period may range from less than 6 months to over 15 years and clinical disease is the terminal stage of a slow chronic subclinical infection (Chiodini *et al.,* 1984).

Four categories or stages of disease have been determined for PTB (Whitlock *et al.,* 2000; Tiwari *et al.,* 2006; Fecteau and Whitlock, 2010). In the first stage or “silent” infection, animals present no clinical signs, but are possibly shedding infectious organisms undetectable with any diagnostic test. In the second stage or subclinical disease, animals do not show visible clinical signs, but they may have detectable antibodies to MAP. During this long preclinical period (2-5 years), it persists and multiplies in sub-epithelial macrophages leading to a chronic trans-mural inflammatory reaction (Clarke, 1997; Manning and Collins, 2001). In the third stage or clinical disease, most animals test positive on fecal culture and have increased antibody detectable by enzyme-linked immune-assay (ELISA).

In the advanced clinical disease or fourth stage, animals are diarrheic, lethargic, weak, and emaciated, being culled from the herd due to decreased milk production and severe weight loss (Whitlock and Buergelt, 1996).

The fecal–oral route, especially at early life stage of animals, is the main way to contract PTB in dairy cattle at the individual level. Cows become infected as calves soon after birth, by oral ingestion of the organism probably from the udder, from an animal that was shedding the organism, or from contaminated utensils. Consequently, the major sources of MAP infection are infected animals (Manning and Collins, 2001), and therefore the contamination of udder of the dam, pasture, feedstuff or utensils with feces is described as the principal factor to avoid when a control of the infection in the herd is desired (Sweeney, 1996).

The majority of herds acquire MAP through purchase of infected animals (Sweeney, 1996). Economic losses are higher in PTB infected herds compared to PTB–non infected herds, due to reduced milk production, increased cow replacement, lower cull–cow revenue and greater cow mortality (Hutchinson, 1996; Ott *et al.,* 1999; Johnson *et al.,* 2001; Kudahl *et al*., 2004; Kostoulas *et al.,* 2006; Weber, 2006; Beaudeau *et al.,* 2007; Gonda *et al.,* 2007; Marce *et al.,* 2009; Nielsen and Toft, 2009; Richardson and More, 2009; Djønne, 2010; Donat *et al*., 2014; McAloon *et al.,* 2016).

It has been suggested that MAP could be involved as part of the causal structure or as an opportunist in Crohn´s disease of humans (Chacon *et al.,* 2004; Uzoigwe *et al.,* 2007; Nacy and Buckley, 2008; Lowe *et al*., 2008; Atreya *et al.,* 2014). This potential zoonotic role, the human exposure to MAP via milk, and the fact that this relation cannot be proved or disproved (Waddell *et al*., 2008; Das and Seril, 2012; Davis and Madsen-Bouters, 2012; Gitlin *et al.*, 2012; Kuenstner, 2012; Atreya *et al.,* 2014; Liverani *et al.,* 2014; McNees *et al.,* 2015; Sechi and Dow, 2015), are reasons for great concern. It is also considered that PTB has a global distribution (Manning and Collins, 2010).

Therefore, PTB belongs to the List of Diseases of the World Organization for Animal Health (OIE) due to its international spread and zoonotic potential, which drives not only to public and animal health disease risks, but also to commercial restrictions (Anonymous, 2015).

For the *ante-mortem* diagnosis of PTB in cattle, several types of test are available and recommended. These include tests to detect antibodies against MAP, detection of MAP genes, and bacterial culture (Collins *et al.,* 2006; Nielsen and Toft, 2008; Stevenson, 2010a; 2010b; 2015). Sensitivity and specificity of tests to *ante-mortem* diagnosis of PTB vary significantly depending on MAP infection stage (Nielsen and Toft, 2008).

Several commercial ELISA kits for PTB diagnosis are currently available, and multiple studies have compared their accuracy (Buendía *et al.,* 2013; Sonawane and Tripathi, 2013; Donat *et al.,* 2014; Lavers *et al*., 2014; 2015; Nielsen and Toft, 2014). ELISA test is also the most widely used to establish PTB status of herds, but it has shown limitations in some extend relating low sensitivity, primarily because of the slow progression of MAP infection, that does not ensure an adequate detection capacity of animals in an early stage of infection. On the contrary ELISA is highly specific, with a low presentation of false positive results (Harris and Barletta, 2001; Fry *et al.,* 2008). Sensitivity of ELISA is the highest for animals with lepromatous lesions, those with clinical symptoms, or those that shed large number of bacteria. For these reasons, the test itself supports a probability of infection (Nielsen and Toft, 2008).

Detection of MAP genes by polymerase chain reaction (PCR) have shown advantages (speed, identification of agent, lack of contamination) and disadvantages (moderate sensitivity, high cost, special equipment and skilled personal required; Collins, 1996). The limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, the matrix tested, and the PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, or multiplex PCR; Möbius *et al.,* 2008; Bölske and Herthnek, 2010; National Advisory Committee on Microbiological Criteria for

Foods, 2010; Stevenson, 2010a; 2010b; 2015). However, due to recent developments of PCR, it is being suggested for herd screening (Collins *et al.,* 2006; Anonymous, 2010), and it has been recently brought to discussion as a possible new golden standard for PTB (Stevenson, 2010a; 2010b; 2015).

Microbiological diagnosis still remains as the golden standard, but its sensitivity for infected and affected animals lies around 70%; in infected cattle is around 30% (Nielsen and Toft, 2008), mainly because of the intermittent shedding of microorganisms and diverse features of the culture techniques (Whitlock *et al.,* 2000). FC has been used as an acceptable standard technique for detecting the infection status of animals –related to elimination rate-, for estimating the sensitivity of other diagnostic tests (e.g. ELISA, PCR), and as an excellent confirmatory test for animals that tested positive with immunological tests (Motiwala *et al.,* 2005; Aly *et al.,* 2012). Disadvantages of culture are slow detection, generally 12 to 16 weeks or longer and detection of only animals shedding MAP in feces (Collins, 1996). Therefore, it is considered that none of the diagnostic tests is capable of detecting all sub-clinically infected animals (Chacon *et al.,* 2004; Lavers *et al.,* 2013). Literature suggests that sampling all adult cattle in every herd, environmental sampling, serial testing and the use of two to three diagnostic tests is recommended for herd screening and to increase the accuracy of MAP infection diagnosis (Collins *et al*., 2006; Stevenson, 2010; Serraino *et al.,* 2014). A low agreement between direct and indirect MAP-diagnostic techniques has been also reported (Muskens *et al.,* 2003; Glanemann *et al.,* 2004; Dreier *et al.,* 2006; Fernández-Silva *et al.,* 2011a; 2011b).

Paratuberculosis is a common disease in all countries with a significant dairy industry, especially in areas with a moderate and humid climate (Barkema *et al.,* 2010). True prevalence of MAP infection among cattle in Europe appeared to be approximately 20% and is at least 3-5% in several countries; herd prevalence appeared to be >50% (Nielsen and Toft, 2009).

In the United States, results from serologic testing revealed that 3.4% of cows and 21.6% of dairy herds showed probability of being infected with MAP (Wells and Wagner, 2000).

The presence of PTB and the circulation of MAP among dairy herds and wild animals has been already demonstrated by clinical, pathological, serological, microbiological and molecular procedures (Paolicchii *et al.*, 2003; Holzmann *et al.*, 2004; Ristow *et al.*, 2007; Fernández-Silva *et al.,* 2012; Fritsch et al., 2012; Shaughnessy *et al.,* 2013; Salgado *et al.*, 2014;). During the 1990´s herd level prevalence of MAP infection in countries with a significant cattle industry was calculated at approximately 10%, while more recently it has been estimated to be 30-50% based in several studies (Barkema *et al.,* 2010). In South America and the Caribbean, few studies have reported consistent sero-prevalences. Animal level and herd level from this region range from 2.7 to 72%, and from 18.7 to 100%, respectively (Fernández-Silva *et al.*, 2014).

Many and different individual animal and management herd factors have been identified to influence the PTB infection status in dairy cattle (Collins *et al.,* 1994; Goodger *et al.*, 1996; Cetinkaya *et al.,* 1997; Obasanjo *et al.,* 1997; Johnson-Ifearulundu and Kaneene, 1998; 1999; Jakobsen *et al.,* 2000; Wells and Wagner, 2000; Daniels *et al*., 2002; Hacker *et al*., 2004; Dieguez *et al.,* 2008; Nielsen *et al,* 2008; Ansari-Lari *et al.,* 2009; Tiwari *et al*., 2009; Barrett *et al.,* 2011; Sorge *et al.,* 2012; Elliott *et al.,* 2014; Pieper *et al.,* 2015; Vilar *et al*., 2015; Sun *et al.,* 2015; Wolf *et al.,* 2016). Most of these studies have been conducted at the herd level and have used mainly serological results to establish the PTB diagnosis of animals and the subsequent identification of risk factors; some studies, however, have used other methods (e.g. PCR; Ansari-Lari *et al.,* 2009; Wolf *et al.,* 2016) or more than one method (Kobayashi *et al.,* 2007) for the determination of risk factors.

In Colombia, the existence of MAP was first reported in 1924 by the Cuban veterinarian Ildefonso Pérez Vigueras in cattle with clinical signs of the disease (Plata-Guerrero, 1931; Góngora and Villamil, 1999).

This documentation was the first confirmation of PTB in the country and occurred in the municipality of Usme (Cundinamarca) in a herd of imported cattle (Vega-Morales, 1947; Góngora and Villamil, 1999). Some studies were carried during the following years, but the majority of studies on MAP and PTB were carried out during the present decade (2010-2020; Zapata *et al.,* 2010; Fernández-Silva *et al.,* 2011a; Fernández-Silva *et al.,* 2011b; Ramirez-Vásquez *et al.,* 2011; Fernández-Silva, 2012; Del Río *et al.,* 2013; Ramírez-García and Maldonado-Estrada, 2013). These latter studies were very useful to confirm the presence of MAP in local cattle. However, the studies were performed in a relative small dairy cattle population and were limited in delivering information on risk factors.

Despite of these investigative efforts, no official control or eradication program for PTB carried out in Colombia and it is considered that its control is a farmer responsibility. According to Correa-Valencia *et al.* (2016) the disease and the agent are present in Colombia and partial epidemiological information is available, but there is still missing information about the whole situation of PTB and MAP infection in the country. Consequently, the Colombian official control office has announced that PTB is a mandatory notifiable disease (ICA, 2015), being this the first step for the disease control in the country.

The understanding of this important animal disease, that affects cattle production and public health —since the zoonotic potential of this infection is widely accepted, and lacks of officially established control program by the Colombian animal health authorities— should be a research main objective for the scientists, industry, and academy. The knowledge of its prevalence at the herd and animal level, and the risk factors assessment, are the key issues when decision or policy makers determine whether the infection should be considered important or not, and what measures to apply (Nielsen and Toft, 2009).

This topic is of major interest of the proposing line of research, because it investigates phenomena that relate animal and human health, using immune-based, culture, and molecular diagnostic tests, and epidemiology as basis to achieve its goals on health improvement.

The hypotheses considered for this research included and expected MAP sero-prevalence in the study herds around 60% at herd level and 10% at animal level, and, that at least, one individual animal feature, one herd characteristic and one herd management practice are potential risk factors for MAP ELISA positive results in the study herds.

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Objectives

General Objective

Determine the prevalence of MAP, confirmed by real-time PCR, and to explore the main risk factors associated with ELISA and/or real-time PCR positive results in animals of some dairy herds of the Northern region of Antioquia, Colombia.

Specific Objectives

1. Determine MAP sero-prevalence at an individual and herd level using serum ELISA.

2. Confirm ELISA positive results using fecal real-time PCR.

3. Explore the main risk factors associated to MAP ELISA and/or real-time PCR positive results at animal and herd level.

Literature Reviews

Many information has been published to describe the diagnosis alternatives available for the detection either the agent (MAP) or the disease (PTB). Although the literature covers a wide variety of such alternatives, this review focuses on four major points of view, which emerge repeatedly throughout the literature reviewed. These themes are clinical diagnosis and post-mortem findings, serological, microbiological, and molecular diagnosis. Conclusions and general recommendations are given explaining the main characteristics of each diagnosis alternative, its limitations as well as advantages. Even if the literature presents these themes in a variety of contexts, this review (published in Revista ACOVEZ) focuses on their application in decision-making, a primary step to control this important disease.

Diagnóstico de la paratuberculosis bovina: Revisión.

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Nathalia M. Correa Valencia. MV, MSc(est). Universidad de Antioquia.
mariadelp.correa@udea.edu.co
Nicolás F. Ramírez Vásquez. MV, MSc, Dr. An Sci. Universidad de Antioquia.
nicolas.ramirez@udea.edu.co
Jorge A. Fernández Silva. MV, MSP, Dr. Med Vet. Universidad de Antioquia.
jorge.fernandez@udea.edu.co

Resumen

La paratuberculosis (PTB) o enfermedad de Johne (EJ) es una enfermedad infecciosa causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP), el cual afecta rumiantes domésticos y salvajes, además de otras especies. Se caracteriza por diarrea y caquexia progresiva, la cual conduce a la muerte del animal. La PTB es una enfermedad endémica a nivel mundial, con altos niveles de prevalencia, fuerte impacto económico en la producción de carne y leche e importancia en salud pública, debido a su posible asociación con la enfermedad de Crohn. Aunque la prueba de referencia es la identificación de MAP en cultivo bacteriológico, existen diferentes pruebas diagnósticas para detectar animales o hatos infectados. La correcta elección y aplicación de cada una de estas pruebas asegura el éxito del diagnóstico y permite establecer un programa de control. La presente revisión pretende exponer las alternativas diagnósticas disponibles actualmente para la detección del agente y de la enfermedad, definiendo sus características, aplicaciones, ventajas y desventajas.

Palabras claves: *Mycobacterium avium subsp. paratuberculosis, paratuberculosis, pruebas diagnósticas.*

Abstract

Paratuberculosis (PTB) or Johne's disease (JD) is an infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), affecting domestic and wild ruminants and some other species. It is characterized by diarrhea and progressive cachexia, which may cause the death of the animal. The PTB is endemic worldwide, with high prevalence levels, strong economic impact in meat and milk production, and public health relevance because of its possible association with Crohn's disease. Although the current reference diagnostic test is the identification of MAP in the bacterial culture, there are different diagnostic tests to identify infected individuals or herds. The correct choice and application of each of these diagnostic tests will ensure their success and may allow

establishing a control program. The aim of the present review is to expound the currently available diagnostic alternatives for the detection of the agent and the disease, describing their characteristics, applications, advantages, and disadvantages.

Keywords: *diagnostic tests, Mycobacterium avium* subsp. *paratuberculosis, paratuberculosis*.

Introducción

La paratuberculosis (PTB) o Enfermedad de Johne (EJ), causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP), es considerada como uno de los problemas más serios que afectan la población mundial de rumiantes, además de su efecto en la economía global y la controversia que existe alrededor de su efecto patógeno en humanos (Whittington *et al.,* 2011; Chiodini *et al.,* 2012). La EJ se describe como una enfermedad intestinal crónica, contagiosa y fatal, caracterizada por una enterocolitis granulomatosa crónica con linfadenitis y linfangitis regional (Clarke, 1997). Los signos cardinales de la PTB bovina incluyen pérdida crónica y progresiva de peso, acompañado de diarrea crónica o intermitente refractaria al tratamiento. La enfermedad clínica puede ser precipitada por el parto, la lactancia u otro tipo de estrés (Carvalho *et al.,* 2009). La PTB pertenece a la lista de enfermedades de la Organización Mundial de Sanidad Animal (OIE), dada su distribución internacional y su potencial zoonótico, lo cual representa no solo riesgo para la salud pública y animal, sino también restricciones comerciales (Nielsen y Toft, 2009; Turenne y Alexander, 2010). Existe evidencia de la presencia de MAP en alimentos de origen animal para consumo humano y en tejidos afectados de pacientes con la Enfermedad de Crohn, confirmándose su asociación a la enfermedad, mas no su causalidad (Cirone *et al.,* 2007).

Las vacas infectadas con MAP eliminan la micobacteria principalmente en heces, las cuales contaminan directamente la leche y las canales en el pos-sacrificio (Carvalho *et al.,* 2009; Chiodini *et al.,* 2012). En Europa, Estados Unidos y en algunos países de Suramérica se ha demostrado que el 3-5% de las vacas y alrededor del 50% de los hatos lecheros están infectados por MAP (Wells y Wagner, 2000; Paolicchi *et al.,* 2003; Holzmann *et al.,* 2004; Ristow *et al.,* 2007; Fry *et al.,* 2008; Nielsen y Toft, 2009; Fernández-Silva *et al.,* 2012). Estudios en Latinoamérica y el Caribe revelan una prevalencia del 16,9% y del 75,8% en el ganado a nivel animal y hato, respectivamente (Fernández-Silva *et al*., 2014). En la industria lechera, las pérdidas económicas por PTB no sólo están definidas por los costos de los medicamentos y la atención veterinaria, sino también por las pérdidas en producción de leche, descarte prematuro de animales clínicos o infectados, susceptibilidad a otras enfermedades y a problemas reproductivos, pérdida de peso en ganado joven, aumento de la tasa de reemplazos y el bajo costo de la canal del animal descartado (Nielsen y Toft, 2008; Lombard, 2011; Over *et al.,* 2011). El control de la enfermedad se basa en la detección y remoción de los animales, lo cual está fundamentado en el diagnóstico de los individuos infectados, afectados y/o eliminadores de MAP. Los métodos disponibles para el diagnóstico incluyen la aplicación de técnicas clínicas, serológicas, microbiológicas y moleculares. La presente revisión pretende exponer las alternativas diagnósticas disponibles actualmente para la detección del agente y de la enfermedad, definiendo sus características, aplicaciones, ventajas y desventajas.

Diagnóstico clínico y hallazgos *post-mortem*

Los bovinos son más susceptibles a la infección por MAP antes del nacimiento o tempranamente después de nacer (Sweeney *et al.,* 1992; Clarke, 1997). Sin embargo, no se observan signos clínicos antes de los dos años de edad, siendo más frecuentemente observados entre los 2 y 6 años de edad (Blood y Radostis, 1992).

Factores como la nutrición deficiente, el estrés de transporte, lactancia, parto e inmunosupresión son detonantes de la fase clínica de la infección (Salem *et al.,* 2013). La fase clínica inicial puede ser imperceptible para el productor, ya que se limita a una pérdida de peso leve, disminución en la producción láctea, con apetito normal.

A medida que el microorganismo prolifera en la mucosa intestinal las lesiones se hacen más extensas y se desarrolla el síndrome de mal absorción, durante el cual el animal inicia la diarrea intensa (Dirksen *et al.,* 2005; Tiwari *et al.,* 2006). Varias semanas después del inicio de la diarrea se nota un edema submandibular, en la mayoría de los casos, debido a la pérdida de proteínas desde el torrente sanguíneo hacia el tracto intestinal (Manning y Collins 2001). Más tarde el edema puede desaparecer y la sed se incrementa, como resultado de la pérdida de fluidos por la diarrea. Normalmente los animales no presentan fiebre, tienen apetito normal, mientras que las heces son acuosas, homogéneas y sin olor ofensivo, ni sangre, debris epitelial o moco. El animal afectado llega a una deshidratación severa y caquexia (Fotografía 1; Blood y Radostis, 1992; Tiwari *et al.,* 2006).

De acuerdo a Andrews *et al.* (2004) cualquier enfermedad debilitante que resulte en emaciación se puede confundir con PTB. Sin embargo, en ésta enfermedad la diarrea profusa contiene frecuentemente burbujas lo cual la diferencia de otras enfermedades emaciantes como *Fasciola hepatica* (mariposa del hígado), enfermedades metabólicas, reticuloperitonitis traumática o desnutrición (Butler, 1993). En relación al diagnóstico post-mortem, las lesiones en bovinos quedan restringidas a la parte posterior del aparato digestivo, principalmente íleon, y linfonodos mesentéricos e ileocecales (Blood y Radostis, 1992; Gasque, 2008; Manning y Collins 2001).

La mucosa del íleon, ciego y algunas veces el colon esta congestiva y blanda a la manipulación, y usualmente se observa una superficie rugosa y unos linfonodos agrandados y prominentes (Manning y Collins 2001; Andrews *et al.,* 2004).

En Colombia varios estudios han reportado casos clínicos de PTB en bovinos (Huber, 1954; García, 1957; Ramírez-Vásquez *et al.,* 2011; Ramírez-García y Maldonado-Estrada, 2013).

Figura 1. Vaca con diarrea crónica y pérdida progresiva de la condición corporal.

Diagnóstico serológico

Entre los métodos para el diagnóstico indirecto de la EJ se incluye el diagnóstico serológico, el cual se basa en la detección de anticuerpos tipo IgG producidos por el animal como respuesta a la exposición a MAP y usando diferentes antígenos de esta micobacteria (Nielsen, 2010). Dentro de las pruebas de diagnóstico serológico el ELISA (del inglés Enzyme-Linked ImmunoSorbent Assay), el cual detecta anticuerpos en suero sanguíneo o leche, es una de las pruebas serológicas más usadas para el diagnóstico de la PTB (Harris y Barletta, 2001).

En el mercado mundial se dispone de varios kits comerciales de ELISA para el diagnóstico de PTB, los cuales han sido usados y evaluados en diferentes estudios independientes mostrando importantes variaciones en su sensibilidad y especificidad (Kohler *et al.,* 2008; Fry *et al*., 2008; Nielsen y Toft, 2008). En general, la sensibilidad del ELISA para la detección de anticuerpos contra MAP es baja, pero aumenta con la edad del animal.

En general, la especificidad se estima por encima del 95% para la mayoría de los kits comerciales disponibles (Nielsen y Toft, 2008). La habilidad del ELISA para detectar animales infectados depende de la frecuencia de aplicación de la prueba, de la prueba como tal, y del punto de corte escogido con el fin de determinar si la prueba es positiva o negativa (Nielsen, 2010; Stevenson, 2010). La sensibilidad del test en animales con cuadro clínico y/o excretando grandes cantidades de MAP en la materia fecal es alta (Kohler *et al.,* 2008; Nielsen y Toft, 2008). Algunas vacas infectadas producen anticuerpos muchos años antes de comenzar con la excreción de una cantidad detectable de MAP en materia fecal; por el contrario, en algunos animales los anticuerpos contra MAP pueden no ser detectables durante las fases tempranas cuando la excreción fecal de MAP es mínima (Nielsen, 2010).

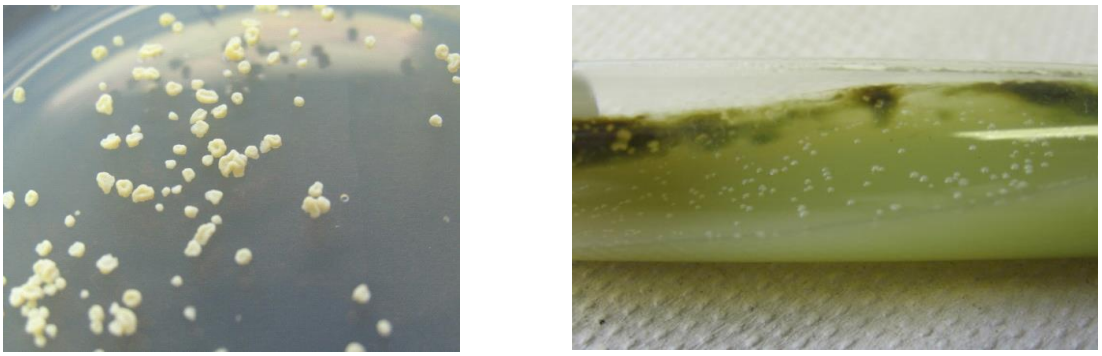
Dentro de las principales ventajas de las pruebas serológicas están sus bajos costos, se adaptan fácilmente al trabajo rutinario de alto volumen de pruebas y los resultados pueden estar disponibles en pocos días o semanas (Nielsen, 2010). Una de las mayores desventajas de este tipo de pruebas es que éstas no arrojan una medida directa de la infección por MAP, grado de infecciosidad o de estar afectado por una infección debida a MAP (Nielsen, 2010). En América Latina y el Caribe, ELISA ha sido la prueba diagnóstica más usada para determinar la frecuencia de PTB en bovinos, cabras y ovejas (Fernández-Silva *et al*., 2014). En Colombia varios estudios han empleado la técnica de ELISA para el diagnóstico de PTB en bovinos (Patiño y Estrada, 1999**;** Mancipe *et al.,* 2009; de Waard, 2010; Fernández-Silva *et al*., 2011a, Fernández-Silva *et al.,* 2011b).

Diagnóstico microbiológico

El cultivo y la identificación de MAP se considera como el diagnóstico definitivo de la EJ en el animal individual y en el hato (Whittington, 2010). Sin embargo, aunque el cultivo aún se considera como la *prueba de oro* (prueba de referencia), su sensibilidad puede ser 30% en animales subclínicos (Nielsen y Toft, 2008) debido principalmente a la intermitencia en la excreción de microrganismos y a algunas características de las técnicas de cultivo (Whitlock *et al.,* 2000).

Esto quiere decir que la sensibilidad del cultivo fecal es alta en animales sintomáticos, pero puede ser baja para la detección de animales subclínicos. Por otro lado, se considera que la especificidad es 100% si los aislamientos obtenidos efectivamente se confirman como MAP (Nielsen y Toft, 2008). Las principales desventajas del cultivo son la lenta detección -generalmente 12 a 16 semanas en muestras clínicas que contienen cepas de origen bovino-, la detección es posible únicamente en animales infectados que estén excretando MAP en materia fecal y el costo relativamente alto en comparación con otras pruebas, como por ejemplo las pruebas serológicas (Collins, 1996).

Para el cultivo se utilizan tanto medios líquidos como medios sólidos (Fotografía 2), pero no todos los medios soportan adecuadamente el crecimiento de los diferentes tipos de cepas (de Juan *et al.,* 2006; Cernicchiaro *et al*., 2008; Whittington *et al.,* 2011). En América Latina y el Caribe, el cultivo ha sido la prueba diagnóstica más usada después del ELISA para determinar la frecuencia de PTB en bovinos, cabras y ovejas (Fernández-Silva *et al.,* 2014). En Colombia varios estudios han empleado el cultivo para el diagnóstico de PTB en bovinos (Isaza, 1978; Góngora y Perea, 1984; Fernández-Silva *et al.,* 2011a, Fernández-Silva *et al*., 2011b; Zapata *et al*., 2010).



A B

Figura 2. (A) Colonias de la cepa de referencia K-10 (ATCC® BAA-968™) de MAP cultivada sobre agar Middlebrook 7H10 (Merck KGaA, Darmstadt, Alemania) suplementado con micobactina J (Allied Monitor, Inc. Fayette, USA). (B) Colonias de aislamientos colombianos de MAP de materia fecal bovina inoculada sobre agar Herrold con yema de huevo (Herrold's Egg Yolk Agar) suplementado con anfotericina, ácido nalidíxico, vancomicina y micobactina J (Becton Dickinson, Heidelberg, Alemania). La imagen permite apreciar las colonias de color blanco sobre el medio de cultivo y restos de la muestra fecal.

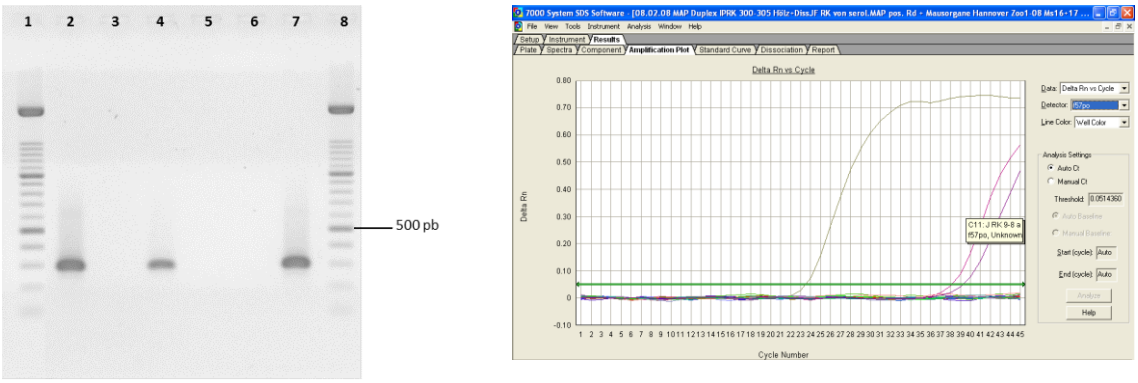
Diagnóstico molecular

La detección de genes de MAP por PCR (del inglés Polymerase Chain Reaction) ha mostrado ventajas: rapidez, identificación del agente, ausencia de contaminación, así como desventajas: sensibilidad moderada, alto costo, equipo especial y personal calificado requerido (Collins, 1996). Sin embargo, debido a los desarrollos recientes, la PCR se sugiere para el tamizaje de hatos (Collins *et al.,* 2006; Anonymous, 2010), y ha sido sugerida como una posible nueva *prueba de oro* para la PTB (Stevenson, 2010a; 2010b). Por otro lado, la técnica de PCR es rápida y específica y en contraste con el diagnóstico basado en cultivo, no es necesario aplicar otro tipo de pruebas para confirmar la identidad del microorganismo detectado (Collins, 1996).

El gen más comúnmente utilizado para la detección de MAP es el elemento multicopia secuencia de inserción 900 (IS*900,* Bull *et al.,* 2003; National Advisory Committee on Microbiological Criteria for Foods, 2010; Bolske y Herthnek, 2010; Stevenson, 2010a; 2010b; Gill *et al.,* 2011). Sin embargo, otras micobacterias diferentes a MAP han sido reportadas con elementos similares a IS*900* con secuencias de nucleótidos que son idénticas a la secuencia IS*900* de MAP hasta un 94% (Englund *et al.,* 2002). Algunos sistemas de PCR que están dirigidas a IS*900* pueden dar resultados falsos positivos con ADN de micobacterias diferentes de MAP y con ADN de otro tipo de organismos (Möbius *et al.,* 2008). En respuesta a la incertidumbre sobre la especificidad de los sistemas de PCR que se dirigen a la IS*900* para la identificación de MAP, se han propuesto otras secuencias para la identificación de MAP por PCR: IS*Map02*, IS*Mav2*, hspX, locus 255 y *F57*.

La PCR se desempeña muy bien como prueba confirmatoria en cultivos, pero su aplicación a muestras clínicas ha sido problemática debido principalmente a problemas asociados con la extracción de ADN de matrices complejas como leche, heces y sangre y por la presencia de inhibidores de la PCR (Stevenson, 2010). Los límites de detección, la sensibilidad y la especificidad varían con la secuencia blanco y la elección de los cebadores o primer, la matriz evaluada y el formato o tipo de PCR utilizado, como son convencional (Fotografía 3), transcriptasa reversa, PCR en tiempo real y PCR múltiple (National Advisory Committee on Microbiological Criteria for Foods, 2010).

Los diferentes formatos de PCR y las técnicas para el enriquecimiento o concentración de MAP son variables presentando ventajas y desventajas dependiendo de las matrices utilizadas para la detección de MAP y la forma como se aplican las técnicas (Möbius *et al.,* 2008; Bolske y Herthnek, 2010; Stevenson, 2010). En Colombia varios estudios han empleado la PCR para el diagnóstico de paratuberculosis en bovinos (Zapata *et al.* 2010; Ramírez-García y Maldonado-Estrada, 2013; Fernández-Silva *et al.* 2011a, Fernández-Silva *et al.* 2011b).



A B

Figura 3. (A) Resultados de una PCR convencional anidada para la detección de IS*900* de MAP en muestras de materia fecal bovina. Carril 1 y 8: marcador de peso molecular (escalera de ADN de 100 pares de bases, pb). Carriles 2, 4 y 7: muestras positivas y control positivo, respectivamente, mostrando el producto de 294pb obtenido con los cebadores TJ1 a TJ4 según Bull *et al.* (2003). (B) Gráfico de amplificación de una PCR en tiempo real para la detección de *F57* e ISMav2 de MAP en muestras de materia fecal bovina. La curva de la izquierda muestra el control positive (valor ciclo umbral, del inglés Cycle threshold value o Ct-value= 23.48), la curva de la mitad muestra un resultado débil positivo a *F57* ("JRK 9-8a *F57* po, Unknown", valor Ct= 38.18), la curva de la derecha muestra un inesperado resultado débil positivo del control negativo (Ct= 39.90). Las curvas planas muestran las muestras negativas. La línea verde muestra el umbral. La interpretación de los valores de Ct es <37 positivo, ≥40 negativo, 37-40 débil-positivo, control positivo <28. Delta Rn (ΔRn) corresponde a la magnitud de la señal generada por los fluorocromos de la sonda VIC (*F57*) o FAM (ISMav2) en el sistema de PCR en tiempo real según Schönenbrücher *et al.,* 2008.

Conclusiones y recomendaciones generales

El diagnóstico clínico definitivo ante y *post-mortem* es realizado según los signos encontrados en el animal y en el tracto gastrointestinal al momento de la necropsia, lo cual requiere la experticia y conocimiento por parte del clínico. Otra alternativa diagnóstica es la evaluación de la respuesta humoral frente a MAP, cuya sensibilidad y especificada va a depender a su vez del estadio de la enfermedad.

La respuesta humoral contra MAP en animales subclínicos puede variar considerablemente a través del tiempo, incluso día a día, probablemente por fluctuaciones en la producción de anticuerpos. La sensibilidad de estos tests aumenta a medida que aumenta la magnitud de la eliminación fecal de MAP y el grado de afección clínica. Por su parte, la detección de MAP por medio del cultivo en medio sólido es aún la prueba de referencia o *prueba de oro*, dado que permite categorizar los animales según el grado de eliminación fecal de la micobacteria. Sin embargo, éste método es lento y poco sensible, especialmente en los estadios tempranos de la enfermedad, lo cual podría afectar la toma de decisiones frente a la remoción de animales infectados de los hatos, permitiendo la entrada y circulación en los mismos.

La detección de MAP por PCR es rápida y específica y no requiere viabilidad de la micobacteria, lo cual es un factor de ventaja si se le compara con el cultivo, sin embargo, requiere personal y equipo especializado, y aun se discute sobre su sensibilidad analítica.

Existe aún un profundo vacío en la definición de un test único que permita diagnosticar efectivamente la PTB bovina dada la complejidad inmunológica y la duración -aunque larga- variable, del periodo subclínico de la enfermedad, especialmente si se requiere una alta especificidad y una alta sensibilidad, además son necesarios los mecanismos que permitan una interpretación adecuada de los métodos ya disponibles. Las limitaciones de cada test diagnostico determinará el uso combinado de dos o tres de ellos, repetidos a lo largo del tiempo y sobre el mismo animal, definiendo así el estadio de la infección y de la enfermedad en los animales individuales y en los hatos.

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It was back in 1895 when Johne and Frottingham described the disease for the first time. The literature also recalls us what Bang described back in 1906, referring to a non-tuberculosis related disease, and using the "Johne´s disease" denomination for the first time. In addition, by 1902 and 1908 the disease was reported in the most of the world. Twort isolated the causative agent in 1910 and started the trip that finally named the agent as Mycobacterium paratuberculosis. Exploring the basis of the investigation of the disease and the agent that causes it, we wanted to search for the history of paratuberculosis in our country, because it deserves the intensive investigation its importance demands, being this review (under peer reviewing, submitted in 2015) the first approximation.

Mycobacterium avium* subsp*. paratuberculosis in Colombia,

1924-2015: 90 years in the presence of an absent

***Mycobacterium avium* subsp. *paratuberculosis* en Colombia, 1924-2015: 90 años en la presencia de un ausente**

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Nathalia Correa-Valencia, MV, MCV(c), DCV(c); Yadi Marcela García-Tamayo, MV; Jorge A. Fernández-Silva, MV, MSP, Dr. med. vet.

Epidemiología y Salud Pública Veterinaria, Centauro, Escuela de Medicina Veterinaria, Facultad de Ciencias Agrarias, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia

Summary

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an acid-fast, Gram-positive bacillus. MAP is the causal agent of paratuberculosis (PTB) or Johne's disease, an infectious disease affecting domestic ruminants and some wild species. Its importance as a zoonotic agent due to its relation to Crohn's disease (CD) of humans is still under debate and investigation. The aim of the present review is to summarize original studies on MAP carried out in Colombia as from 1924, as well as to highlight their strengths, weaknesses, and future research opportunities with emphasis on the diagnostic and epidemiologic points of view. The initial search for existing publications reporting original studies on MAP, PTB, and the relation MAP and CD was carried out by searching the available databases and national libraries. After compilation of the available studies, the relevant data (year, department, specie studied, diagnostic tests used, study design, results, and authors) were extracted and recommendations about future research opportunities on MAP research were made.

Keywords: *Colombia, Mycobacterium avium* subsp*. paratuberculosis, Johne's disease*.

Resumen

Mycobacterium avium subsp. *paratuberculosis* (MAP) es un bacilo ácido resistente, Gram positivo. MAP es el agente causal de la paratuberculosis (PTB) o enfermedad de Johne, una enfermedad infecciosa que afecta rumiantes domésticos y algunas especies salvajes. Su importancia como agente zoonótico debido a su relación con la enfermedad de Crohn (CD) en humanos está aún en debate y bajo investigación. El objetivo de la presente revisión es exponer los estudios originales sobre MAP llevados a cabo en Colombia desde 1924, asi como resaltar sus fortalezas, debilidades y oportunidades de investigación futura con énfasis en los puntos de vista diagnóstico y epidemiológico.

La búsqueda inicial de las publicaciones existentes sobre estudios originales realizados acerca de MAP, PTB y la relación MAP y CD fue realizada en las bases de datos disponibles y en bibliotecas nacionales. Luego de la compilación de los estudios disponibles, los datos relevantes (año, departamento, especie estudiada, prueba diagnóstica, diseño del estudio, resultados y autores) fueron extraídos y se realizaron recomendaciones sobre futuras oportunidades de investigación sobre MAP.

Palabras claves: *Colombia, Mycobacterium avium subsp. paratuberculosis, enfermedad de Johne*.

***Mycobacterium avium subsp. paratuberculosis* (MAP)**

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an intracellular, obligate, weakly Gram-positive, acid-fast bacterium that is 0.5-1.5 µm in length, which may cause a persistent infection in intestinal macrophages in host tissue leading to immune and inflammatory reactions (1-3). MAP belongs to the *Mycobacterium avium* complex (MAC), which comprises different subspecies that exhibit varying grades of adaptation to a particular host frame and are characterized by different pathogenicity profiles (4). As in other members of the Mycobacteriaceae genus, MAP´s cell wall structure is rich in complex lipids as a unique characteristic of this genus. Some reports lead to the thought that this peculiar cell wall composition is responsible for the persistence of this type of bacteria both in the environment and in the host (4,5). Nevertheless, MAP can be distinguished from other closely related mycobacteria by its unique requirement for the addition of mycobactin J in artificial culture media, mainly for primary cultures (6).

MAP also has the slowest growth rate among injurious mycobacteria. After inoculation with samples from infected animals and incubation under optimal conditions, MAP colonies usually do not appear for 3 months or longer, and, ironically, the pathogenic

potential of mycobacteria intensifies as their growth rate decreases. Thus, slow-growing mycobacteria, such as MAP, are more pathogenic than fast growing mycobacteria (5).
MAP has a remarkable tropism for the intestine, which is not seen in any other mycobacterial species (7). Once ingested, MAP advances to the subepithelial macrophages, its host cells, by invasion of the lamina propia (8-10) and can survive in macrophages using a wide range of not fully described mechanisms to evade immune system responses (11).

The post-genomic era of MAP research began in 2005 with the publication of the complete genome sequence of isolate K-10, which was isolated from a Wisconsin dairy herd in 1990 (12) and has 4350 predicted open reading frames and 4.8 Mb. Subsequent automated analyses of the genome sequence have annotated a total of up to 4587 genes (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntma03). This finding has opened the door to many new research opportunities.

MAP infections have been reported to impact a wide group of domestic and wild species, including ruminants (13-16) and humans (17-20). Recent data from whole-genome comparison studies support the classification of MAP isolates into the two major strain types, I (Sheep type; S) and II (Cattle type; C; 21). These strains show differences related to the ease of primary isolation, incubation time for primary growth on solid and liquid media, and host preference or range, among others (22), which should be considered when epidemiologic assays are designed and analyzed.

MAP and Johne's disease

MAP causes paratuberculosis (PTB), or Johne's disease (JD), a slow-developing and incurable infectious animal disease characterized by chronic granulomatous enterocolitis. This disease has a variable incubation period from 6 months to over 15 years (7). PTB is transmitted between animals by an oral-fecal route, but intrauterine and trans-mammary

pathways have also been considered (23-25). Animals from 0 to 6 months of age are thought to be most susceptible (26-28).

Nevertheless, experimental infection studies have demonstrated that goats are naturally less resistant to PTB compared to sheep and cattle (29). Chronic, progressive weight loss and chronic or intermittent diarrhea are the primary clinical signs of bovine PTB (7, 30), but in goats and sheep, the symptoms are vague and unspecific, and like many other diseases, only characterized by weight loss (31,32). Diarrhea is not a relevant symptom in small ruminants, as it occurs in cattle (7,33,34). The clinical disease is most frequent among cattle 2-5 years old, although younger and older cattle (0-13 years old) can be affected (35). In other domesticated and wild ruminants, the clinical disease development and course has been difficult to establish (32). In sheep, the clinical signs are limited to weight loss, which can occur from 2 years of age, with an important period of succumbing to the disease from 3-5 years of age (33,36). In goats, the clinical development of the disease is similar to that in sheep (32,34,37). Parturition, lactation, or other stresses may provoke clinical manifestations (7,30,38).

Treatment for PTB is infrequently indicated or undertaken; however, treatment using therapeutic agents may be used to alleviate clinical signs and specifically may be considered for animals of genetic value. Some studies about chemoprophylactic treatments of animals in various stages of the disease and ages have been conducted with variable results (39-41).

There are reports of infections with MAP and clinical cases of JD from all continents that have ruminant populations in whatever levels of farming (42,43).

Johne´s disease causes important economic losses in infected flocks and herds (31) and produces a 6-19 % decrease in the production of meat, milk, or both (32,44,45). Ovine and caprine PTB has been linked to losses related to death, early culling, and reduced milk production (46-48).

Control of PTB in farm ruminants by testing, culling, and herd/flock management are important for limiting the economic impacts and losses related to MAP infection and have been the methodologies used in control measure programs in the USA, Australia, and Europe (49-52). Regardless of the mechanism, the notion that infection is age dependent is so widely held that control programs to block infection transmission are primarily focused on the neonatal period, which seem more susceptible to MAP, such that even adults may readily become infected (53). Control programs focused on blocking primary infection in the neonatal period consider the avoidance of colostrum, milk, water, or feed intake for neonates with MAP-contaminated manure facilities (51,52,54).

MAP and Crohn's disease

Crohn´s disease (CD) was named after Burrill B. Crohn, an American physician who published a paper in 1932 that clearly distinguished CD from intestinal TB (55). CD is a human chronic inflammatory disease of still unresolved etiology which primarily causes ulcerations of the small and large intestines, although it can affect the digestive system from the mouth to the anus. Common symptoms of CD include severe and watery or bloody diarrhea, abdominal pain, fever, weight loss, and bloating (56,57).

The histopathological characteristics of JD resemble CD (58,59). One of the first documented descriptions of CD was in 1769 by Giovanni Battista, an Italian physician. He described the results of an autopsy on a man who had suffered from chronic bowel movements throughout his life and subsequently died from diarrhea and fever (60).

In 1913, Dalziel (61) described the clinical and pathologic similarities between PTB in cattle and CD in humans, which were both chronic inflammatory bowel diseases. This report initiated the controversy about the etiological role of MAP in CD and implied a potential zoonotic behavior for MAP (18,20,62,63). In agreement with this, MAP has been detected in the tissues of CD patients (64-67).

The source, the route of infection, the persistence mechanisms, and the consequences of MAP infection in humans are known factors; a number of findings also point to a zoonotic capacity for MAP (59,68,69).

Nacy and Buckley (70) referred to the epidemiological similarities between JD and CD, including that the triggering event occurs in early in life, a prolonged period—the incubation period—exists between the trigger and clinical disease, the onset of clinical disease commonly occurs after sexual maturity, both diseases follow a defined onset distribution pattern, the main target organ is the ileum, and the host response for both is a chronic granulomatous inflammation.

Other facts that support the existence of a link between MAP and CD include the clinical and pathological similarities between JD and CD causing a fatal gastrointestinal disease (71,72), its presence in the food chain (milk, meat) and water supplies, signifying a possible route of exposure to MAP for the general public (73,74), an increased detection of MAP in CD tissues by culture, PCR, and FISH (fluorescent *in situ* hybridization; 71,75,76), positive blood cultures for MAP in CD patients (77), an increased serological response to MAP in CD patients (78-80), detection of MAP in human breast milk by culture and PCR (81), progression of cervical lymphadenopathy to distal ileitis in a patient with MAP infection (82), and therapeutic responses to combination antituberculosis therapy (83,84).

However, some authors have reported facts that do not support a link between MAP and CD. These include dissimilarities in the clinical and pathological responses in JD and CD (85), lack of epidemiological support for transmissible infection (86), dairy farmers and others who may have greater exposure to MAP than the general population not experiencing higher rates of CD in one study (87), dissimilar genotypes of CD and bovine MAP isolates (86), variability in the detection of MAP by PCR (0-100 % in CD and ulcerative colitis tissues; 74) and serological testing (88), cell-mediated immune responses to MAP or MAP antigens not being demonstrated in CD patients, no evidence

of a mycobacterial cell wall by histochemical staining, no worsening of CD with immunosuppressive agents or HIV (human immunodeficiency virus) infection, no documented cell-mediated immune response to MAP in patients with CD (79,80), and no therapeutic response to traditional antimycobacterial antibiotics (89).

Therefore, an association between CD and PTB has been shown, but a causal relationship remains to be demonstrated (59,62,63,90-93).

MAP in food and in the environment

Animals infected by MAP, whether affected clinically or subclinically, can shed live bacteria in both feces and milk (94-96). If these animals are farmed for food production, the safety of foods derived from them becomes important because of its impact on public health (14,59,93). Although infected animals represent the main source for MAP contamination, this bacterium can also be found in the environment (97-99). MAP resists extreme environmental conditions and can survive for months or years in soil and water (100,101). Thus, in addition to animal products, ground-waters and rivers contaminated with MAP are suggested as risks for MAP transmission to humans (99,102-104).

Accordingly, MAP or MAP DNA has been detected in raw milk, in bulk milk containers, in pasteurized milk, and in infant food formula (59,105-107), as well as in cheese, ice cream, and flavored milk drinks (108-111). Additionally, it has been found in carcasses (112), muscle and organ tissues (113-117), and retail meat (118). There is evidence indicating that MAP is not killed by the standard food processing techniques, such as cooking and pasteurization (73,119,120). The partial resistance of MAP to pasteurization has been investigated intensively (121-125), and has revealed its presence as a food contaminant in pasteurized (post-exposure to 70 °C) milk, cheeses, and yogurt. Thus it is proposed that MAP survival in pasteurized dairy products may have served as a vehicle for MAP infection in a subset of CD patients (111,126,127).

Diagnosis of MAP

Several tests have been used to diagnose MAP in cattle, sheep, goats, and humans. The most common are clinical and histopathological, immune-based (enzyme-linked immunoassay—ELISA, interferon gamma [IFN-γ] assay, and intradermal Johnin test [IJT]), microbiological (from tissues, feces, and environmental samples), and the detection of MAP-DNA by polymerase chain reaction (PCR) (feces, milk, tissue, and blood; 128). Descriptions of these tests and their advantages and disadvantages follow.

Clinical and histopathological diagnosis: Animals do not usually show clinical signs before two years of age, and this is more common between 2-6 years of age in all species (7,129-131). Factors such as nutritional deficiency, transport stress, nursing, calving, and immunosuppression are common triggers of the clinical stage of the disease in all susceptible species (132). The beginning of the clinical stage can be imperceptible for the producer because it is usually referred to mild weight loss or diminished milk production with normal appetite. As the more extensive the lesions are associated with the more severe signs, the animal develops a malabsorption syndrome, including intense diarrhea (9,133).

In cattle, several weeks after the onset of diarrhea a submandibular edema appears as a consequence of protein loss from the bloodstream to the intestinal tract (97). Weeks later the edema disappears and thirst increases as a result of fluid loss. In all cases related to PTB infection, fever is absent and appetite is normal, and aqueous, homogeneous, non-offensive smelling, and bloody mucus and debris-less feces are observed. The affected animal develops a severe dehydration and cachexia (9,129).

The clinical signs of JD in sheep are restricted to progressive weight loss with occasional edema. In advanced cases, hypoalbuminemia and hypocalcemia may be observed (36,131). Most sheep that die of PTB have normal feces. Diarrhea is not considered to be a feature of this disease in small ruminants, except in the terminal stages of disease (134).

Goats in advanced clinical disease develop a rough skin and a poor coat, and eventually emaciation, dehydration, and anemia with submandibular edema. At this stage of the infection, diarrhea, or more typically, massy feces, can be seen (135).

In all affected species, the necropsy findings are commonly restricted to the ileum and the mesenteric and ileocecal lymph nodes. In most of the cases congestive and “wrinkled” surfaces of the ileum, cecum, and colon are observed (97,136).

The main histopathological lesions consist of epithelioid cell granulomas of various sizes with differing numbers of acid-fast bacilli observed in Ziehl Neelsen (ZN)-stained sections. All sections can show granulomas, but their distribution and intensity varies from case to case. Most of the small to large granulomas have minimal to medium lymphocyte infiltration. However, no destructive changes (desquamation of the epithelium, erosion, ulceration, fissure formation, edema, hemorrhage, severe lympho-plasmacytic infiltration, or lymphoid follicle formation) are observed. In severe cases, the lymphoid follicles of Peyer’s patches are replaced by granulomas and, in some cases, these structures are mainly composed of giant cells (48,137,138).

Immune-based diagnosis: Immune-based diagnostic tests for PTB rely on the occurrence of an immune response to infection by MAP (54,139,140). ELISA is the most popular test for detecting an immune response to MAP. Several commercial ELISA kits for PTB diagnosis are currently available and multiple studies have compared their accuracy (141-146). The main advantage of ELISA tests is that they are relatively inexpensive, easy to perform, and quantitative results can be obtained in 1–2 hours in routine circumstances (53,140,147). False-positive reactions may occur due to cross-reacting antibodies, laboratory errors, or in vaccinated animals without them necessarily having a MAP infection, and false negatives may occur due to the low sensitivity of the test (140,148). A major disadvantage of ELISA is that it does not provide a direct measure of the degree of MAP infection, infectiousness or how the animal is affected by a MAP infection (139,149,150). This disadvantage can impact the communication of test results.

Nevertheless, with the appropriate interpretation, ELISA tests may be more reliable than microbiological tests, depending on the intent of testing (144-146).

However, some studies have shown that antibodies are produced much earlier than they can be detected by ELISA, and it has generally been believed that the early immune response to a MAP infection consists primarily of a cellular immune response characterized by IFN-γ production (141,142,150).

Animals entering stage II of the disease (unapparent carrier adults) have higher concentrations of MAP in their intestinal tissues (38).
However, these animals do not manifest weight loss or diarrhea but may have an altered immune response with increased IFN-γ production by T cells sensitized to specific mitogens and/or increased antibody response to MAP (151-153).

According to Nielsen and Toft (35), the results of these studies may not be representative of the populations in general and could be difficult to extrapolate. PPD antigens used in the IFN-γ test are crudely steam-sterilized mycobacterial culture extracts containing many cross-reacting antigens with other related bacteria (154). Therefore, previous sensitization to PPD and vaccination interfere with the specificity of the test and make the interpretation of results difficult (148).

The skin test for the diagnosis of PTB, the IJT (intradermal Johnin test), is carried out by the intradermal inoculation of the antigen into a clipped or shaven site, usually on the side of the middle third of the neck. The skin thickness is measured with calipers before and 72 hours after inoculation. Increases in skin thickness more than 2 mm should be regarded as indicating the presence of delayed-type hypersensitivity (DTH). However, sensitization to the *Mycobacterium avium* complex is widespread in animals, and neither avian tuberculin nor Johnin are highly specific. The IJT specificity is close to 80 %, but sensibility cut-off values have not been established (155,156).

The comparative intradermal tuberculin test is used to differentiate between animals infected with *Mycobacterium bovis* and those responding to bovine tuberculin as a result of exposure to other mycobacteria. The test involves the intradermal injection of bovine PPD (purified protein derivate) and avian PPD into different sites, usually on the same side of the neck, and measuring the response 3 days later. This sensitization can be attributed to the antigenic cross-reactivity among mycobacterial species and related genera (157).

Microbiological diagnosis: Cultivation of MAP from feces and tissues is the most reliable method of detecting infected animals (31,35,38). Usually, the specificity of fecal culture (FC) is considered to be almost 100 % if the isolates obtained are confirmed to be MAP by molecular methods such as PCR (35,158,159). FC has been used as an acceptable standard procedure for detecting the infection status of animals, for estimating the sensitivity of other diagnostic tests, and as an excellent confirmatory test for animals that tested positive with immunological tests (160,161).

Although the FC has many limitations, such as a long incubation period, high costs, risk of contamination with other mycobacteria or fungi, and time required to report the results, it is still considered to be the “gold standard” for the detection of MAP (35,162,163).

Special aspects of MAP can affect the FC accuracy, for example, MAP´s elimination through feces is intermittent and occurs in an advanced stage of the disease, mainly when the animals have clinical symptoms (7,132,163). The cultivation of MAP from environmental samples, including soil, water and pasture, using methods based on those for feces is not technically difficult, is inexpensive, and is useful for detecting infected herds (159,162-165). However, the bacteriological culture of pooled fecal samples and environmental sampling are cost-effective methods for classifying herds as MAP infected (162,166-170).

Infection can be established if a thorough microbiological examination of the animal is made at the slaughter process, but it is insufficient to sample tissues only from the ileum and ileocecal lymph nodes because this will fail to detect many infected animals (171).

In tissue cultures the lesions induced by the organism in the intestinal tract is specific, but even where the microorganisms are visualized in tissues associated with the granulomatous infiltrate, specificity is not certain because other mycobacterial species sometimes infect the gut (163).

Molecular diagnosis: The detection of MAP genes by PCR has shown advantages (rapidity, identification of agent, lack of contamination) and disadvantages (moderate sensitivity, high cost, special equipment and skilled personnel required (172). However, due to recent developments, PCR has been suggested for herd screening (157,173), and it has been recently discussed as a possible new gold standard for PTB (128). The PCR technique is rapid and specific, and in contrast to a culture–based diagnostic, no additional tests are required to confirm the identity of the organism detected (172). The most popular target gene for the detection of MAP is the multi-copy element IS*900* (98,128,174,175). However, mycobacteria other than MAP have been found to carry IS*900*-like elements with nucleotide sequences that are up to 94 % identical to the nucleotide sequence of MAP IS*900* (176). Some PCR systems that target IS*900* also can give false-positive results with DNA from mycobacteria other than MAP and with DNA from other types of organisms (177,178).

In response to the uncertainty about the specificity of PCR systems that target IS*900* for the identification of MAP, the use of several other target sequences for MAP identification systems have been proposed: ISMap02, ISMav2, hspX, locus 255, and *F57*. The *F57* sequence appears to have been the most widely used of these targets. Both single-round and nested PCR systems that target the *F57* sequence have been reported to be highly specific for MAP (179-182).

PCR performs well as a confirmatory test on cultures, but its application to clinical samples has been problematic, mainly due to the problems associated with DNA extraction from complex matrices such as milk, feces, and blood and the presence of PCR inhibitors (128,183). The limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, the matrix tested, and the PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, or multiplex PCR; 174).

Its formats and techniques for the enrichment or concentration of MAP are variable and have advantages or disadvantages depending on the matrices used for MAP detection and the way they are performed (128,175,177). The lack of a 100 % accurate reference diagnostic test and a variable incubation time seem to be the main obstacles in the perfect test evaluation application (171).

When a test combination is considered, it must be taken into account that some infected cows produce antibodies for several years prior to the continuous fecal-shedding of detectable quantities of MAP. However, in other animals, antibodies may not be detectable during the early stages of infection when MAP fecal-shedding is minimal (140,171,184). Current diagnostic tests cannot completely discriminate between infected and uninfected animals, which emphasizes the need for an appropriate test (31).

Epidemiology of MAP

Both MAP infections and clinical cases of JD have been reported from all continents that have ruminant populations in any degree of husbandry, and cross-country infection must be considered (42,185). Multiple studies on the determination of the within-herd and between-herd prevalence of MAP infections around the world have been carried out (31,132,186).

Intensive farming systems, acid soils, low dietary intake, stress related to transport, lactation and parturition, and immunosuppression by agents such as bovine viral diarrhea virus (BVDV) are reported as risk factors worldwide (7,30,35,54,187-193).

This disease is a problem in cattle in Australia, Canada, Argentina, the USA, Mexico, Brazil, New Zealand, Denmark, Belgium, Norway, Switzerland, Netherlands, Hungry, Austria, France, Spain, Germany, England, Scotland, Ireland, Italy, Czech Republic, Slovakia, Norway, Greece, Thailand, India, Japan, Saudi Arabia, Iran, Egypt, Morocco, and South Africa. Sweden and some states in Australia are the only regions that claim to be free from the disease (194-199). The current herd- and animal-level prevalences are unknown for many countries. The prevalence of infection is increasing in some countries that do not have mandatory control programs (132,186).

The true prevalence among cattle appears to be approximately 20 % and is at least 3-5 % in several European and Asian countries. Between-herd prevalence guesstimates appear to be >50 % (7,31).

According to Manning and Collins (37), over 50 % of dairy cattle herds in Europe and North America are infected.

Critical issues were identified in the majority of the prevalence studies analyzed by Nielsen and Toft (31) in Europe, primarily due to lack of knowledge about the accuracy of the diagnostic test used or to studies in which the study population did not reflect the target population. According to Fernández-Silva *et al.* (186), prevalence studies in Latin American and Caribbean countries revealed an overall prevalence of 16.9 and 75.8 % in cattle at the animal and herd levels, respectively. In the same report the prevalence was 16 % in sheep at the animal level, and 4.3 % and 3.7 % in goats at the animal and flock levels, respectively.

The prevalence reported in small ruminants in several other countries is 73.7 % in sheep in Italy (200), 46.7 % in sheep in Portugal (201), 52 % in sheep and 50 % in goats in Cyprus (202), and 48-57 % in goats and 42.4 % in sheep in Brazil (203).

To comprehend the genetic epidemiology, the genetic variation in MAP contributing to the host's susceptibility to infection must be one of the main objectives for all animal improvement programs involved in PTB control to reduce susceptibility to transmission and to gain a better understanding of the mechanisms of disease (132,204).

Interest in and the application of strain-typing methods for a better identification of genetic diversity within MAP isolates has increased since the beginning of the 21st century, (132,205,206).

MAP isolations have been classified into two groups according to culture characteristics: host preference and the pathogenic capacity of the strain. Types I/III (sheep-type) and Type II (cattle-type) strains have been widely described (207). Nevertheless, MAP strains can be isolated from a wide range of species.

Strains I/III had been predominantly, but not exclusively, isolated from sheep and goats, suggesting a host preference for these species. Type II strains have a wider range of hosts and tare most commonly isolated from domestic and wildlife species, including cattle and non-ruminants (22). Goats can be infected by both strain types, and it has been discovered that the isolates from goats are less pathogenic than those from cattle (208).

Several typing techniques targeting different structures in the genome have been reported including pulsed-field gel electrophoresis (PFGE; 207), multiplex PCR of the IS*900* loci, mycobacterial interspersed repetitive units (MIRU; 206), multilocus variable-number tandem-repeat analysis (VNTR; 209), randomly amplified polymorphic DNA analysis (RAPD; 210), and amplified fragment length polymorphisms (AFLP; 160).

Restriction fragment length polymorphism (RFLP), mycobacterial interspersed repetitive unit- (MIRU)–VNTR, and mixed liquor suspended solids reaction (MLSSR) methods can be used as markers for further subdivision among MAP and are the most commonly used markers (159,211-216).

More recently, after finishing the complete genome sequence of the MAP ATCC® 19698 strain (217), new systemic procedures targeting more discrimination between isolates were developed, such as the multilocus short sequence repeats sequencing (MLSSR; 218), and new loci containing the VNTR of specific MIRUs (219). Most recent epidemiological studies for typing MAP isolates are based mainly on these genomic repetitive sequence methods (220).

Results

The investigation of MAP in Colombia

The review of MAP investigations in Colombia was carried out by searching all available reports published in scientific and informative journals, as well as in theses or degree works. The main characteristics (year of publication, country department, species, diagnostic test, study design, and summary of results) of the MAP original studies (n = 17) were reviewed (Table 1). The existence of MAP in Colombia was first documented in 1924 by the Cuban veterinarian Ildefonso Pérez Vigueras in cattle with PTB (221,222). This documentation was the first confirmation of PTB in the country and occurred in the municipality of Usme (Cundinamarca) in a herd of imported cattle (222-224).

All original studies on MAP in Colombia refer to PTB. No original studies of the zoonotic potential of MAP have been carried out thus far in Colombia. MAP has been reported in neither food nor in humans in the country.

One publication by Albornoz (225) comparing bovine PTB with human leprosy was not available. Its significance as an original study could not be evaluated, therefore it was not taken into account in this review.

The majority of studies on MAP (PTB, 41.2 %, 7/17) were carried out during the present decade (2010-2020). No more than two studies on MAP (PTB) in Colombia were published in previous decades (Table 1). The majority of studies were carried out in the departments of Antioquia (52.9 %; 9/17) and Cundinamarca (35.3 %; 6/17) and in the departments of Caldas and Tolima (5.8 %; 1/17), as well as in Nariño (5.8 %; 1/17; Table 1). The original studies concerning MAP in Colombia only reported the results from cattle and sheep. Studies on cattle were the most common (88.2 %; 15/17) compared to sheep (11.8 %; 2/17). Other relevant species in the country (wild mammals, goats, buffaloes, or humans) were not found or cited in any original study reviewed (Table 1).

The most common diagnostic test used to investigate MAP in Colombia is ZN-staining (in feces, tissues, and/or rectal mucosa scrapings; 24.3%; 9/37), followed by nested q-PCR (16.2 %; 6/37), ELISA (16.2 %; 6/37), intradermal bovine and/or avian-PPD (13.5 %; 5/37), histopathological studies (8.1 %; 3/37), fecal and tissue culture (individual or pooled; 5.4 %; 2/37), CF (complement fixation; 5.4 %; 2/37), coprologic examination (feces and/or rectal mucosa scrapings; 2.7 %; 1/37), IF (indirect immuno-fluorescence; 2.7 %; 1/37), CIE (counter immuno-electrophoresis; 2.7 %; 1/37), and clinical and hematological evaluations (2.7 %; 1/35; Table 1).

The studies reviewed include descriptive studies (case reports, series of case reports, surveys), observational studies (cross-sectional studies), diagnostic test evaluations, risk factor analyses, and experimental studies (clinical trials) testing treatments. Thus far, no cohort or case and control studies have been published in Colombia (208).

Discussion

This review summarizes for the first time the original studies on MAP carried out in Colombia since 1924. In recent years the presence and distribution of MAP in the country, especially in farmed animals and humans, have been reviewed (222,226,227). However, no review of the original studies has been undertaken. According to several anecdotal reports, opinions about the distribution of MAP (PTB) in cattle and small ruminants are not homogeneously defined or conclusive. Some academics and producers consider MAP (especially PTB) as a significant problem, while others claim the absence or very low prevalence of MAP in farmed animals. The relationship between MAP and CD has been essentially not discussed in academic fields in the country, except for some sporadic reviews (222,226).

The number of publications reporting original studies on MAP, especially PTB, in recent years is relatively low compared to other countries in Latin America (186), but is higher than expected for Colombian conditions.

This finding suggests a growing interest about MAP research in the country, as well as an increasing preoccupation about this microorganism and its negative effects on animal health, animal production, and its zoonotic potential (public health impact). In addition to the original studies reported here, several literature reviews, case reports, and editorials about MAP, PTB, and the relationship between MAP and CD have been published in Colombia by several authors and institutions, demonstrating the national academic and productive concern about this microorganism and its effects (222,226-233). These publications were not considered to be original studies and are not further discussed in this review, but they are of great value for the national knowledge base about MAP.

PTB is not a notifiable disease In Colombia and other Latin American countries (186,229) and is not of major concern to animal health authorities. This could explain the low number

of initiatives for the research, prevention, and control in animals, as well as for the detection of the microorganism in food, the environment, and humans. In South America, only one country-wide PTB review has been published in Brazil (234). According to this review, 35 studies have been carried out in Brazil since its first report in 1915, including on cattle, sheep, goats, and buffaloes and using the same diagnostic tools that have been used in Colombia according to the present report (234). An increasing number of original studies concerning MAP (PTB) during the last decade is related to the growing interest of academics (as seen in the present review) and producer associations about the disease, including the federación colombiana de ganaderos (Fedegán; 235). According to the latter, PTB is a disease without official control by the state, which delegates this responsibility to the producer.

The locations of the majority of studies do not follow a clear trend but could be related to the high concentrations of cattle in some of the departments (i. e., Antioquia and Cudinamarca; 236), or to the particular interests of academics, scientists, or cattle producers. Since the first report in 1924, Cundimamarca has been a department with common reports of PTB (222-224,237-241). This could be explained by the long tradition of the Faculty of Veterinary Medicine of the Universidad Nacional de Colombia in Bogotá, the oldest faculty of veterinary medicine in the country, where the first studies in the early 20th century were carried out, most of them being degree works.

More recently, Antioquia has been publishing the majority of original studies, all of them from academics at the Universidad de Antioquia and the Universidad CES (242-249 Correa-Valencia *et al.,* 2015, personal communication).

As expected, studies on cattle were the most common, most likely due to the size of the population in the country (236) and to the production systems related to milk and meat. In contrast, studies on sheep populations are less common in the country and could be due to their smaller populations (250).

The common use of ZN-staining, intradermal bovine and/or avian-PPD, and ELISA is not surprising given their relatively low cost and availability of materials, qualified personnel, and infrastructure for these types of tests (172). However, the use of fecal and tissue cultures and nested q-PCR are becoming more common and could be related to the recent development of the diagnostic capacity in universities compared to national laboratories more than to animal health authorities and to the expansion of the reagents and equipment supplies for such diagnoses in the country.

The absence of cohort and case-control studies is a common element in animal diseases in Colombia. Observational and experimental studies are more complex, laborious, demanding, and expensive. In addition, the microbiological and physiopathological characteristics of MAP make these studies complicated under Colombian conditions. Nevertheless, the current MAP situation in Colombia demands additional observational studies in addition to surveys and case reports to enhance our comprehension of the epidemiological situation and to assess the true zoonotic threat.

In general, much progress has been made on MAP research in the areas of diagnosis and epidemiology as is reported by the studies included in this review. However, many unanswered questions remain and research opportunities in the country are plentiful. One of the main research opportunities concerns the epidemiologic and biological behavior of MAP (PTB) under local agro-ecological conditions, local wildlife distributions, and productive and cultural conditions, which must be considered in the analysis as possible sources of molecular and epidemiological diversity.

The zoonotic potential of MAP has been debated for almost a century because of similarities between JD in cattle and CD in humans. Nevertheless, evidence for the zoonotic potential of MAP is not demonstrated, but should not be ignored because of the genetic, environmental, immunological, and microbiological influences in several combinations that have been proposed (124). A similar situation concerning the lack of studies to detect MAP in CD patients has been reported in Chile (251).

However, previous national reviews have concluded that the zoonotic potential of MAP should not be ignored. CD has been known in the country since the 1950s and the incidence rate and prevalence are increasing (77,000 cases of extrapolated prevalence), and no national consolidated information about the disease is available (222,226). According to some of these authors, efforts should be made to correlate both diseases in areas with a high prevalence or incidence of both. In addition, the laboratory infrastructure—mainly developed for foot-and-mouth disease control—should cover other entities with relevance for public health and international trade such as PTB (222).

Conclusions

In Colombia 17 original studies about MAP, including reports of clinical cases, diagnostic test evaluations, surveys, observational (cross-sectional) and experimental studies (clinical trials) testing treatments have been carried out so far in two different species (bovine and ovine), mainly using ZN-staining, and predominantly in Antioquia and Cundinamarca. No original study on the zoonotic potential of MAP or its detection in food or in humans has thus far been carried out in the country.

In general, the results reported by the original studies included in this review are still insufficient to accurately reflect the epidemiologic situation about MAP or its economic and public health impact in Colombia. Although the existence of MAP in Colombia has been confirmed for almost a century, the small number of studies, as well as several flaws in the published studies, limit the evidence about the magnitude of MAP circulation in animals, humans, the environment, and food in Colombia.

It is imperative that we improve the laboratory diagnostic capability for MAP in the near future and increase the number of studies dealing with the microbiologic, immunologic, epidemiologic, and economic aspects of MAP in several domestic and wild animal species.

The determination of at least regional prevalences in domestic animal populations is of high priority. It is advisable to initiate studies on the detection of MAP in humans, the environment, and in food for human consumption.

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Ethical standards

The manuscript does not contain clinical studies or patient data.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Summary of published original studies on *Mycobacterium avium* subsp. *paratuberculosis* in Colombia, 1924-2015.

	Year of publication	Country department	Species	Diagnostic test	Study design	Summary of results	Reference
1	1947	Cundinamarca	Bovine	Intradermal avian – PPD; coprologic examination	30 animals with different coprologic examination results (6 negative, 8 suspicious, and 16 positive) were inoculated with intradermal avian – PPD. Temperature measurements were done three times before tuberculin inoculation. The next day tuberculin results were determined and temperature was measured every 2 hours.	The 6 animals negative to the coprologic examination were also negative by intradermal avian – PPD. One animal out of the 8 suspicious and 6 animals out of the 16 positive by coprologic examination were positive by intradermal avian – PPD, respectively.	224
2	1954	Cundinamarca	Bovine	Intradermal avian – PPD; ZN-staining in tissues and feces	9 medical cases were reviewed looking for PTB. Administration of isonicotimilhidrazina (orally) and cortisone (intramuscular) in the treatment of AFB- related diseases was performed.	The AFB were confirmed in all the animals which were also negative to intradermal avian – PPD test. 40 % (4/9) of the cases reported an improvement in body temperature and weight after treatment.	237
3	1957	Nariño	Bovine	ZN-staining in rectal mucosa scrapings and	Medical reports of 11 cases were reviewed, with ages between 3 to 12 years.	Reports positive and negative results for PTB, with different diagnosis tools, and describes	252

				fecal samples; histopathological studies		clinical signs and treatment approaches related or not to the disease.	
4	1978	Cundinamarca	Bovine	ZN-staining to rectal mucosa samples; CF; IF (bovine anti-gamaglobuline);	Two groups of adult cattle were described (67 Holstein and Normando, tested twice with 6 months of difference, clinically normal, and 65 animals, older than 2 years of age, clinically compatible with PTB).	3.51 % (7/199) were positive to ZN-staining. 2.02 % (4/199) serums were positive to CF. 5.52 % (11/199) serums reacted positively to IF The authors concluded an important confidence of the IF test compared to baciloscopy and CF to diagnose PTB in cattle.	238
5	1983	Cundinamarca	Ovine	CF; ZN-staining to fecal samples	Blood and fecal samples were taken from 480 adult sheep.	11.25 % (54/480) serums were positive to CF. 5.62 % (27/480) fecal samples were positive to 6ZN-staining. A necropsy was performed during the study to a male adult sheep. The FC, baciloscopy, and histopathology results confirmed the diagnosis of PTB.	239

6	1984	Cundinamarca	Bovine	Histopathological studies (ileocecal valve portions, and mesenteric lymph nodes post- slaughter); ZN-staining to tissue samples rectal mucosa scrapings; CIE test.	94 animals were sampled (Holstein, Normando, and cross-breed cows and bulls), all older than 3 years of age. The groups were designated according to the presence of diarrhea compatible to PTB: females with diarrhea (n=52) and without diarrhea (n=18), males with diarrhea (n=3), and without diarrhea (n=21).	11.70 % (11/94) animals were positive to histopathological studies with HE and ZN-staining. 6.38 % (6/94) were positive to ZN-staining in rectal mucosa scrapings. 9.57 % (9/94) were positive to CIE test in serum samples. The authors concluded that CIE test had the highest sensitivity, because it detected 81.1 % (9/11) histopathology positive animals, even higher than rectal mucosa scraping tests (54 %).	240
7	1999	Caldas and Tolima	Bovine	ELISA	177 Normando animals from three farms were tested.	The seroprevalence obtained for each farm was 3.4 % (2/59), 1.7 % (1/59), and, for the third farm no positive animals were found.	253
8	2001	Antioquia	Bovine	Intradermal avian and bovine-PPD	The study was carried out in three farms located in three different municipalities of the department: San Pedro de los Milagros, n=77; Gómez Plata, n=76, and Barbosa, n=78). The	11 % (19/176) of the animals were positive to intradermal bovine-PPD test, all of them of San Pedro de los Milagros, while 27.8 % were suspicious. This result lead to apply the comparative skin test and	242

					study population was 176 animals over 1 year of age.	some animals were considered suspicious (20 %) and some others positive to PTB (confirmed by the necropsy of 4 animals with compatible signs).	
9	2009	Cundinamarca	Ovine	ZN-staining to fecal samples; Intradermal bovine-PPD; ELISA	250 female sheep (*Ovis aries*) of the Black face, Cheviot, Corriedale, Hampshire, Merino rambouillet, Romney marsh, Mora, creole and cross-breeds, with ages between 1 and 9 years old were sampled	4% (10/250) of the fecal samples were positive to ZN-staining. Animals between 2-6 years old presented AFB in fecal samples, whereas the animals older than 8 years were suspicious. 4.9 % (16/250) were positive to the tuberculin test, and 1.1 % (3/250) was suspicious. 0.8 % (2/250) was positive to ELISA. Animals that resulted suspicious and positive to intradermal test were confirmed by ZN-staining in fecal samples. 62.5 % (10/16) of these animals were positive to both (ZN-staining and tuberculin tests), 18.8 % (3/16) were negative to both, and other 18.8 % (3/16) were	241

						positive to the tuberculin test only.	
10	2010	Antioquia	Bovine	ZN-staining to FC; FC (Middlebrook 7H9 Broth, with mycobactin-J); q-PCR (IS*900*)	The study was carried out in one herd considered enzootic for PTB. 15 Holstein and BON (Blanco-orejinegro) x Holstein cows were tested. The average age of sampled cows was 6.7 year. Fecal samples were individually taken of clinical healthy cows and cows with diarrhea.	56 % (9/15) were positive to ZN-staining in FC, whereas 20 % (3/15) gave a positive result by PCR applied to positive FC.	243
11	2011a	Antioquia	Bovine	Non-absorbed Indirect ELISA (A); Absorbed Indirect ELISA (B); nested PCR (IS*900*) and q-PCR (F5, ISMav2); FC	14 dairy herds of 9 districts were sampled. Only one herd had presented sporadic clinical cases compatible with paratuberculosis confirmed by PCR and histopathology.	10 % (31/315), 87 % (268/315), and 2.6 % (8/315) of the samples were positive, negative, and doubtful, respectively with ELISA A. 70 % (10/14) of the herds were considered positive when having at least one ELISA A-seropositive animal. 5.1 % (2/39) positive and doubtful samples in ELISA A were also positive with ELISA B, 94 % (37/39) were negative, and none was doubtful. 19.3 % (6/31) positive animals with ELISA A were positive to	244

						nested PCR. One positive animal to q-PCR were also positive to nested PCR. 16 and 6.5 % of the ELISA A-positive animals were positive to nested PCR and q-PCR, respectively. FC was negative in all samples.	
12	2011b	Antioquia	Bovine	Absorbed Indirect ELISA (C); Tissue and slurry pooled-FC (HEYM); q-PCR (*F57*, ISMav2) and nested PCR (IS*900*)	5 herds previously tested by the authors, referring to those who resulted ELISA and PCR positive but FC negative for MAP, and one additional herd not tested before were included in the study. The herds participated with 384 cows (≥2 years). Serum samples (n=329) and fecal samples (n=386) were taken from all animals in every herd. Slurry samples of one herd (n=3) and tissue samples (n=2) were also taken during this study.	1.8 % (6/329) results were positive to ELISA C, 97.5 % (321/329) were negative, and 0.6 % (2/329) was doubtful, as well as positive results in 40 % (2/5) of the herds. The FC and nested and q-PCR supported that 1/36 herds was positive to culture (at 5-6 weeks of incubation). The FC produced positive results after 17 weeks. ELISA C results were confirmed by FC in only one symptomatic animal of one of the herds. Eight MAP isolates were recovered including four isolates from fecal samples, one from a mesenteric lymph node, one from colon tissue	245

						sample, and two from pooled slurry samples.	
13	2011	Antioquia	Bovine	Clinical evaluation; hematology; Intradermal avian– PPD; histopathological studies; ZN-staining	This was a retrospective study that included clinical and diagnostic tests applied to 5 cows with clinical signs compatible with PTB (ages between 1.8 and 7 years) in one herd considered enzootic for the disease. The study considered all the information from 2000 to 2008.	All 5 animals reported chronic diarrhea and weight loss, physiological constants and coprology studies were normal. Hypoproteinemia with atypical lynfocitosis was also found. In the necropsy compatible PTB lesions were observed, including emaciation, thickening of intestinal mucosa related to ileum and cecal valve, mesenteric vessels dilation, and lymphatic nodes enlargement. ZN-staining positive findings and granulomatous enteritis and lymphadenitis were also reported.	247
14	2012	Antioquia	Bovine	ELISA	Risk factors assessment, related to seropositive results of a screening of 307 animals, in 14 dairy herds of 9 districts, was done.	Treatment of symptomatic animals (regarding the presentation of disease in the herd), feed type of calves before weaning (colostrums of their own dams or colostrum pools), and manure	246

						spread on pastures, were confirmed as risk factors.	
15	2013a	Antioquia	Bovine	q-PCR (IS*900)*	This study reported a case of a 6-year-old lactating Holstein cow diagnosed with stage IV of PTB (according to epidemiological and clinical findings). Blood and milk samples were processed directly for isolation of mononuclear cells by centrifugation from mesenteric lymph nodes, spleen, mediastinal lymph nodes, blood, and milk. The cells obtained by this protocol were identified as bovine macrophages.	The strongest q-PCR signal was observed in macrophages isolated from mesenteric lymph nodes and colon mucosa, whereas the lowest signal was obtained from mediastinal lymph nodes, milk, or peripheral blood macrophages.	248
16	2013	Antioquia	Bovine	q-PCR (IS*900)*	48 cows with compatible signs of PTB (chronic diarrea and weight loss) were euthanized. 3-5 lymph nodes (LNM) per cow were obtained and trasnported in sterile PBS medium. After culturing, DNA from macrophages was extracted.	The macrophages obtained from 4 cows presented infection by MAP. 8.51 % of the cultures were positive by q-PCR.	249
17	2015	Antioquia	Bovine	ELISA	Risk factors assessment, related to seropositive results of a screening of 696 randomly selected bovines in 28 dairy	1/28 (3.6 %) and 14/696 (2 %) of the herds and animals were seropositive, respectively. Days in milk between 100 and	Correa-Valencia *et al.* (personal communication)

		herds located in 12 different districts of San Pedro de los Milagros, was done.	200 days and over 200 days, and daily milk production between 20 to 40 L/cow and over 40 L/cow, were associated with MAP seropositivity with Odds Ratios of 4.42, 3.45, 2.53, and 20.38, respectively.

PPD: Purified Protein Derivate; ZN: Ziehl-Neelsen; PTB: Paratuberculosis; MAP: *Mycobacterium avium* subsp. *paratuberculosis;* MAA*: Mycobacterium avium* subsp. *Avium;* AFB: Acid Fast Bacteria; CF: Complement Fixation; IF: Indirect Immuno-Fluorescence; HE: Hematoxylin and Eosin staining; CIE: Counter Immuno-Electrophoresis; HEYM: Herrold's Egg Yolk Agar medium; FC: Fecal Culture; ELISA: Enzyme-Linked ImmunoSorbent Assay; PCR: Polymerase Chain Reaction.

Chapter one

The accomplishment of the specific objectives 1 (determine MAP sero-prevalence at an individual and herd level using serum ELISA) and 2 (explore the main risk factors associated to MAP ELISA and/or real-time PCR positive results at animal and herd level) originated the presentation of the talk "Correa-Valencia NM, Ramírez NF, Olivera M, Fernández-Silva JA. Milk yield and lactation stage are positively associated with ELISA results for Mycobacterium avium subsp. paratuberculosis in dairy cows from Northern Antioquia, Colombia", presented at the ENICIP, 2015. The abstract was published in Rev Colomb Cienc Pecu 2015; 28: Sup, 94. In addition, the original article "Milk yield and lactation stage are associated with positive results to ELISA for Mycobacterium avium subsp. paratuberculosis in dairy cows from Northern Antioquia, Colombia: a preliminary study" has been already published [Trop Anim Health Prod (2016) 48:1191–1200]. Both report the seroprevalence obtained in the study population according to ELISA results and case definition for animals and herds, and the risk factors detected using statistical data analysis for the ELISA positive animals.

Milk yield and lactation stage are associated with positive results to ELISA for Mycobacterium avium* subsp*. paratuberculosis in dairy cows from Northern Antioquia, Colombia: a preliminary study

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Nathalia María Correa-Valencia[1] & Nicolás Fernando Ramírez[1] & Martha Olivera[2] & Jorge Arturo Fernández-Silva[1,3]

[1]Grupo Centauro, Facultad de Ciencias Agrarias, Universidad de Antioquia, Medellín, Colombia.
[2]Grupo Biogénesis, Facultad de Ciencias Agrarias, Universidad de Antioquia, Medellín, Colombia.

Abstract

Paratuberculosis is a slow-developing infectious disease characterized by chronic granulomatous enterocolitis. This disease has a variable incubation period from 6 months to over 15 years and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Some studies have been conducted in cattle during the last decades in Colombia. However, those studies were designed using relatively small populations and were not aimed to establish prevalence. This study aimed to determine the MAP seroprevalence in selected dairy herds and to explore risk factors associated with the serology results. Serum samples and related data were collected from 696 randomly selected bovines in 28 dairy herds located in 12 different districts in one of the main dairy municipalities in Colombia (San Pedro de los Milagros). The samples were analyzed using a commercial ELISA kit. The information on risk factors was analyzed using a logistic regression. The apparent seroprevalence was 3.6% (1/28) at the herd-level and 2% (14/696) at the animal-level. The number of days in milk production between 100 and 200 days and over 200 days and the daily milk production between 20 to 40 L/cow and over 40 L/cow were associated with MAP seropositivity with Odds Ratios of 4.42, 3.45, 2.53, and 20.38, respectively. This study demonstrates the MAP seroprevalence in dairy herds from Antioquia and the possible relationship between MAP seropositivity, milk yield and lactation stage.

Keywords: *dairy cattle, Johne's disease, milk production, Mycobacterium avium subsp. paratuberculosis, seroprevalence, risk factors.*

Introduction

Paratuberculosis (PTB), also known as Johne´s disease (JD), is a severe slow-developing and incurable granulomatous enteritis (Clarke, 1997). This disease affects cattle and other domestic and wild ruminants (Nielsen and Toft, 2009; Sweeney *et al.*, 2012). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causal agent of PTB. It is a Gram-positive, facultative anaerobic, mycobactin-dependant, slow growing and acid-fast bacillus (AFB) that may cause a persistent infection in a host tissue´s intestinal macrophages and lead to immune and inflammatory reactions (Sweeney, 1996). MAP can resist environmental and chemical changes and persists in spoils, stream water, and manure slurry storages for up to a year (Sweeney, 1996). MAP has been associated with the human chronic enteritis known as Crohn´s disease (Sweeney *et al.*, 2012; Atreya *et al*., 2014; Liverani *et al.*, 2014).

MAP infections produce important economic losses related to cattle production in infected herds (Marce *et al.,* 2009; Nielsen and Toft, 2009). Economic losses due to reduced milk production, increased cow replacement, lower cull-cow revenue and greater cow mortality are higher in PTB-infected herds compared to PTB-negative herds (Johnson *et al.,* 2001; Kudahl *et al.,* 2004; Weber, 2006; Beaudeau *et al.,* 2007; Gonda *et al.,* 2007; Nielsen and Toft, 2009; Richardson and More, 2009; McAloon *et al.,* 2016). There are reports of infections with MAP and clinical cases of JD from all countries that have ruminant populations (Marce et al., 2009; Nielsen and Toft, 2009; Juste and Pérez, 2011). It is thought that this disease has a global distribution (Manning and Collins, 2010). Therefore, PTB belongs to the List of Diseases of the World Organization for Animal Health (OIE) because of its international distribution and zoonotic potential, leading to not only public and animal health risks but also commercial restrictions (Anonymous, 2000; 2015).

Parturition, lactation, or other stresses may provoke clinical stages of this disease (Clarke, 1997; Fecteau and Whitlock, 2010). The main transmission route at an individual level in natural conditions is the oral-fecal route, especially at early stages of life in animals. However, intrauterine and trans-mammary routes have also been considered (Lambeth *et al.,* 2004; Whittington and Windsor, 2009).

MAP infections occur in young animals, and it is generally assumed that some age-resistance takes place. Animals from 0 to 6 months of age are thought to be the most susceptible to MAP infections (McGregor *et al.,* 2012; Mortier *et al.,* 2013).

Consequently, the major sources of MAP infection are infected animals (Manning and Collins, 2001) and the contamination of the udder of the calf´s dam, the pasture, the feedstuff or the implements with feces. These are described as the principal factors to avoid when the control of the disease in the herd is desired (Sweeney, 1996; O'Brien *et al*., 2006).

For an *ante-mortem* diagnosis of PTB in cattle, several tests are available and recommended. These include tests to detect antibodies against MAP, the direct detection of MAP genes, bacterial cultures of fecal samples (individual, pooled, and environmental), and tests to detect MAP in tissue samples (Collins *et al.,* 2006). The sensitivity and specificity of tests for the ante-mortem diagnosis of PTB vary significantly depending on the MAP infection or clinical stage (Nielsen and Toft, 2008a). Therefore, it is considered that none of the diagnostic tests are capable of detecting all subclinically infected animals (Lavers *et al.,* 2013). In any case, sampling all adult cattle in every herd, environmental sampling, serial testing, and the use of two to three diagnostic tests has been recommended for herd screening and to increase the accuracy of MAP diagnosis (Collins *et al.,* 2006; Stevenson, 2010; Serraino *et al.,* 2014).

Different individual and herd-level factors related to within-herd contact have been shown to influence the PTB infection status in dairy cattle (Johnson-Ifearulundu and Kaneene, 1998; 1999; Hacker *et al.,* 2004; Dieguez *et al.*, 2008). Some of those risk factors include "not cleaning maternity pens after each use" (Johnson-Ifearulundu and Kaneene, 1998; Tiwari *et al.,* 2009), "more than one cow in a maternity pen" (Wells and Wagner, 2000; Tiwari *et al.,* 2009), "presence and percentage of cows born at other dairies" (Wells and Wagner, 2000; Chia *et al.*, 2002; Tiwari *et al.,* 2009), "contamination of udders of peri-parturient cows with manure" (Ansari-Lari *et al.,* 2009), "winter group-housing for pre-weaned calves" (Wells and Wagner, 2000; Tiwari *et al.,* 2009; Ridge *et al.,* 2010; Pithua *et al.,* 2013), "animals fed colostrum from multiple cows" (Nielsen and Toft, 2008b), "Bovine Viral Diarrhea Virus (BVDV)-seropositive herds" and "BVDV vaccination not done properly in calves" (Tiwari *et al.,* 2009), "housing replacement calves with adult cattle before they were six months old" (Collins *et al.,* 1994; Diéguez *et al.,* 2008), "suckling from foster cows" (Nielsen and Toft, 2008b), "feeding milk with antibiotics" (Ridge *et al.,* 2010), "exposure of calves 0–6 weeks to adults feces", "young stock contact with adult feces from same equipment used for cleaning" "feces spread on forage fed to any age group" (Goodger *et al.,* 1996; Obasanjo *et al.,* 1997), and "cows with more than 4 parturitions" Jakobsen *et al.,* 2000).

In South America and the Caribbean, few studies have reported consistent seroprevalence. Animal and herd-level prevalence of PTB from this region range from 2.7 to 72% and from 18.7 to 100%, respectively (Fernández-Silva *et al.,* 2014). In Colombia, PTB was first reported in cattle in 1924, probably from imported animals (Vega-Morales, 1947). After this, PTB research in cattle has been sporadic and has mainly focused on clinical, histopathological, serologic, microbiological, and/or molecular diagnosis (Vega-Morales, 1947; Isaza-Triviño, 1978; Góngora and Perea, 1984; Mancipe *et al.,* 2009; Ramírez-Vásquez *et al.,* 2001; Zapata *et al.,* 2010; Fernández-Silva *et al.,* 2011a, 2011b; Ramírez-Vásquez *et al.,* 2011; Ramírez-García and Maldonado-Estrada, 2013), treatment (Huber-Luna, 1954), prevalence (Patiño-Murillo and Estrada-Arbeláez, 1999; Fernández-Silva *et al.,* 2011a), and molecular characterization (Fernández-Silva *et al*, 2011b).

These studies were very useful in confirming the presence of MAP in local cattle. However, the studies were performed in a relatively small dairy cattle population.

Despite these investigative efforts, no official control or eradication program for PTB has been carried out in Colombia. Its control is considered a farmer's responsibility. The main objective of the current study was to determine the seroprevalence of MAP and explore the main risk factors associated with enzyme-linked immunoassay (ELISA) positive results in cows of dairy herds of one municipality of the Northern Region of Antioquia, Colombia.

Materials and methods

Ethical considerations

This research was approved by the Ethics Committee for Animal Experimentation of the Universidad of Antioquia, Colombia (Act number 88, from March 27, 2014).

Study design

Twelve districts (out of 37) of a municipality located in the Northern Region of Antioquia, Colombia that contribute 70% of the municipality´s cattle population were included in the study. Proportional allocation design of the herds to be sampled in each of the selected districts as well as an adjustment by cluster was considered. A sample of 28 dairy herds inside the selected districts without a previous PTB diagnosis and/or without known history of PTB was selected, according to its specific weight in the dairy population of the municipality. Accounting for a loss of 28% and an average adult population (≥ 2 years of age) per herd estimated to be 23, 696 animals were randomly sampled. According to the study design, 29 animals per herd were tested by ELISA.

In the study region, dairy production is the main economic activity. Dairy production takes place in all places within the region, and Holstein is the predominant dairy cattle breed.
In all the cases, the herds had to fulfill the following conditions to be enrolled in the study: security during sampling visits, geographical accessibility, and willingness of herd owner to participate in the study, allow sampling of all the necessary animals, and provide information regarding animal features and herd management practices. In addition, herds had to have the minimum facilities for the personnel to carry out the procedures safely on animals. All herds accomplishing these inclusion criteria were included in the random selection process.

Serum samples and information

All the herds were visited and tested once from May to July, 2014. In each herd, information and whole blood samples were taken from each animal over 2 years of age. The sample collection was conducted according to standard methods to avoid unnecessary pain or stress to animals. Blood samples were taken from the coccygeal or jugular vein, collected in red-top plastic Vacutainer® tubes and transported in a refrigerated cage until their arrival at the laboratory, where they were centrifuged at 1008 RCF for 5 minutes to obtain the serum for the ELISA test. The obtained serum was frozen for 30 to 45 days at -20°C. After this time, frozen samples were thawed at room temperature before being tested by ELISA. In each herd, the information on individual animal features, herd characteristics, and herd management practices were collected through questionnaires administered directly to herd owners or managers on every visit and by direct observation of the individual and herd characteristics, as well as management practices (questionnaires available upon request). The questionnaires were administered by one of the authors to ensure that recording was clear, complete, and consistent.

ELISA

A serum ELISA was performed using a pre-absorbed serum ELISA Parachek®2 (Prionics AG, Switzerland) following the manufacturer's instructions. This test included a pre-absorption step with *Mycobacterium phlei* to reduce cross-reactions. A herd was considered ELISA-positive if the herd had at least two serum ELISA-positive animals. This avoided the risk of confirming a herd as positive based on one single false positive result by the test, as it is defined by the manufacturer of the diagnostic test used. An animal was considered ELISA-positive if serum sample was above or equal to the cut-off of 15 Percent Positivity (%P), as it is defined by the manufacturer of the diagnostic test used.

Statistical analysis

All the information generated during the study was entered into Excel worksheets (Microsoft Corp., Redmond, WA, USA) and then exported to Stata 12.0 (StataCorp, 2011, Texas, USA) for statistical analysis. The data were examined for biologically implausible entries (those unlikely to be true). Any erroneous data (those incorrect, detected during the editing process of the database) were removed or corrected. Descriptive statistics were computed for all the variables of interest. Observations were stratified by district and sampling weights were computed based on the specific weight of the district on the reference population. Variables were checked for more than 30% missing values, case in which they should have been deleted from the analysis. None of the variables showed more than 30% missing values. Pearson and Spearman correlation analyses were used for continuous and categorical variables, respectively. A complex design analysis was conducted according to a cluster effect and the stratified nature of the study using the Survey command. Unconditional associations between each risk factor and the outcome of interest -ELISA positive- were computed. Associations with $p \leq 0.25$ were retained for consideration in a multivariable model. A complete multivariable logistic regression model was constructed considering a significance level of $p<0.05$.

The potential confounding effect of parturition was evaluated by refitting the final model with parturition omitted to see if the coefficients for other predictors changed substantially. The results from the final models are presented as odds ratios (OR) with 95% CIs. The model fit was assessed using a Hosmer-Lemeshow goodness of fit test.

Case definition

The case definition for a MAP-infected herd was the one with at least two seropositive animals determined by serum ELISA. The case definition for a MAP-infected animal was seropositivity of an individual serum ELISA.

Pre-test of the methodology

All testing procedures and questionnaires were pre-tested on a small scale to evaluate their effectiveness in order to accomplish the objectives of the study.

Results

Descriptive statistics

The study population was mainly composed of Holstein (77.6%) cows (99.6%), older than 3 years of age (74.9%), in lactation (83.3%), with more than 200 days in milk (57.1%) and less than three parities (67%) (Table 2). The individual daily milk production was predominately 20-40 L/cow (45.8%), and the percentage of animals not born in the herd was 69.7% (Table 2).

Table 2: Animal-level predictors in bovines from dairy herds of San Pedro de los Milagros, Antioquia, Colombia

Variable	Description	Unit/category	Observations	Distribution (%)
Breed	According to herd registers	Holstein	540	77.6
		Jersey	120	17.2
		Other*	36	5.2
		Total	696	
Sex	According to herd registers	Female	693	99.6
		Male	3	0.4
		Total	696	
Age	According to herd registers	2–3 years old	175	25.1
		> 3 years old	521	74.9
		Total	696	
Milk production state	According to herd registers	Heifer	68	10.5
		Milking cow	538	83.3
		Dry cow	40	6.2
		Total	646	
Days in milk	Days that had passed from the first day the cow started producing milk to the moment of the testing	< 100	158	22.7
		≥ 100–≤ 200	140	20.1
		> 200	397	57.1
		Total	695	
Parity	Times the cow had gave birth during its life to the moment of the testing	< 3	376	67
		≥ 3–≤ 8	188	32.4
		> 8	132	0.6
		Total	696	
Individual daily milk production	Total milk obtained during the previous day to the moment of testing	< 20	125	53.1
		≥ 20–≤ 40	312	45.8
		> 40	92	1.1
		Total	529	
Born in the herd	The cow had been born in the herd or was purchased from another farm	Yes	451	30.3
		No	196	69.7
		Total	647	

* Other breeds included Guernsey, Ayrshire, Swedish Red, Swiss Brown, Jersey, and several crossbreeds of Holstein with Jersey, Ayrshire, Angus, Blanco Orejinegro, Brahman, and Gir.

The herd-level characteristics of less than 50 hectares (66.2%), ≥ 30 and ≤ 60 cows in milk (45.8%), and a daily milk production between ≥ 500 and ≤ 1400 liters (46.2%) were the most common findings regarding farm size, herd size, and herd daily milk production, respectively (Table 3). The presence of other ruminants (i. e. goats, sheep, and/or buffalo), manure spreading on pastures as a method of fertilization, and cows staying with their calf after calving was reported in 17.9, 67.9, and 85.7% of the herds, respectively.

The percentage of herds certified in good farming practices (buenas prácticas ganaderas, BPG) and percentage of tuberculosis- and brucellosis-free herds was 25 and 75%, respectively (Table 3). The descriptive analysis of the quantitative variables is summarized in Table 4.

Table 3: Herd-level predictors in dairy herds of San Pedro de los Milagros, Antioquia, Colombia

Variable	Description	Unit/category	Observations	Distribution (%)
Farm size	Part of the herd dedicated to farming in hectares (Has)	< 50	19	66.2
		≥ 50–≤ 99	6	23.7
		≥ 100	3	10.1
		Total	28	
Herd size	Number of cows in milk	< 30	6	25
		≥ 30–≤ 60	11	45.8
		≥ 60	7	29.2
		Total	24	
Herd daily milk production	Total milk (in liters) obtained during a day in each herd considered in the screening, in average, to the moment of the testing	<500	7	26.9
		≥ 500–≤ 1400	12	46.2
		> 1400	7	26.9
		Total	26	
Presence of other ruminants	Co-existence with goats, sheep, and/or buffaloes in the same installations	Yes	5	17.9
		No	23	82.1
		Total	28	

Manure spreading	Use of cow manure as a fertilizer in the pastures	Yes	19	67.9
		No	9	32.1
		Total	27	
Cow stays with the dam after calving	After parturition the cow stays with the mother in direct contact	Yes	23	85.7
		No	5	14.3
		Total	28	
BPG[1] certification	Herd certified by the Instituto Colombiano Agropecuario (ICA) as a BPG practicant	Yes	8	25
		No	20	75
		Total	28	
Tuberculosis-free certification	Herd certified by Instituto Colombiano Agropecuario (ICA) as tuberculosis-free	Yes	20	75
		No	8	25
		Total	28	
Brucellosis-free certification	Herd certified by Instituto Colombiano Agropecuario (ICA) as brucellosis-free	Yes	21	75
		No	7	25
		Total	28	

[1] Buenas Prácticas Ganaderas (Good Farming Practices)

Table 4: Descriptive summary of quantitative variables in dairy herds of San Pedro de los Milagros, Antioquia, Colombia

Variable	Observations	Mean ± SD	Minimum	Maximum
Farm size (in Has)	28	50.87 ± 47.22	5	180
Herd size	24	63.66 ± 61.27	11	332
Herd daily milk production (L/d)[1]	26	1350 ± 1534	220	8132
Days in milk	532	199.67 ±	1	785
Parity	562	140.32	0	12
Individual milk production (L/d)[2]	529	3.06 ± 2.00	2	51
		20.42 ±7.39		

[1] Milk produced per herd/day.

[2] Milk produced per cow/day.

ELISA

Fourteen of 696 of the animals had a positive ELISA test, which resulted in an animal-level apparent prevalence of 2%. Eight of the seropositive animals were from one herd of the 28 included in the study. This herd was the only positive herd according to the case definition, resulting in a herd-level apparent prevalence of 3.6%.

Risk factors analysis

The two cow-level factors “days in milk” and “individual daily milk production” showed strong associations with the presence of ELISA positive results (Table 5). Biologically plausible interactions of predictor variables were assessed and found to be non-significant. The Hosmer-Lemeshow goodness of fit test suggested that the model fit the data ($p > 0.97$). The OR for seropositivity was increased with the number of days in milk and individual daily milk production ($p < 0.01$). The number of days in milk had a similar OR pattern for the 100 to 200 days interval (OR=4.42) as for > 200 days (OR=3.45).

Table 5: Final logistic regression model assessing the effect of selected herd and cow variables on the probability for animals to be serum-ELISA positive to MAP in San Pedro de los Milagros, Antioquia, Colombia (n=532 observations)

Variable	Odds ratio	SEM	p-value*	95% CI
Days in milk				
< 100	Referent			
≥ 100–≤ 200	4.42	0.86	0.00	2.89–6.76
> 200	3.45	0.92	0.00	1.93–6.17
Individual daily milk production				
< 20	Referent			
≥ 20–≤ 40	2.53	0.75	0.00	1.32–4.85
> 40	20.38	5.54	0.00	11.26–36.88

* Significant results ($p<0.05$).

Discussion

The present study was designed to identify the prevalence and explore the risk factors associated with seropositive results detected using an ELISA in one of the main dairy production areas of Colombia.

The current herd and animal-level prevalence is unknown in many countries. However, according to several authors, the prevalence of infection is increasing in some countries that do not have mandatory control programs (Salem *et al.,* 2013; Fernández-Silva *et al.,* 2014). Colombia lacks a mandatory program. However, no trend can be established with the currently available data. The animal- and herd-level prevalence estimated in the present study is lower than the prevalence found in cattle by other authors in European, Asian, North American, Latin American, and Caribbean countries (Clarke, 1997; Nielsen and Toft, 2009; Manning and Collins, 2010; Fernández-Silva *et al.* 2014). Nonetheless, Fernández-Silva *et al.* (2014) reported studies in Latin American and Caribbean countries with an overall prevalence of 16.9 (13.2–20.5) and 75.8% (50.1–101.5) in cattle, at the animal and herd levels, respectively, revealing the extreme limits that can be found in the PTB prevalence reports.

On a national scale, our results are similar to those obtained in a previous seroprevalence study in Normando cattle using an ELISA in the Colombian departments of Caldas and Tolima (animal-level 1.69%; 3/177; Patiño-Murillo and Estrada-Arbeláez, 1999). However, they contrast with MAP-detection results obtained in the department of Antioquia in which ELISA positive results were found for 10.1% (31/307) and 70% (10/14) at the animal and herd-level, respectively (Fernandez-Silva *et al.,* 2011a). It should be mentioned that in this previous study serum from asymptomatic cows was analyzed by an unabsorbed ELISA test, which could affect the specificity of the findings, leading to false-positive results.

In the other hand, in their study herds were selected attempting a representation of all productive districts of the municipality (not a random sampling), and, of these 14 herds, one herd had presented sporadic clinical cases compatible with paratuberculosis confirmed by PCR and histopathology (Zapata *et al.* 2010). These factors could have increased the prevalence reported. Our study attempts to, and, finally, reports a seroprevalence at the animal- and herd-level in a higher population of the department of Antioquia compared to previous studies carried out in the country and region. Those previous studies did not attempt to report prevalence in their study design, and used diagnostic tests with different characteristics.

Although the results obtained (2% and 3.6%, animal and herd-level, respectively) refer to the apparent MAP prevalence in the population being studied, no attempt to calculate the true prevalence was carried out due to a lack of information on the sensitivity and specificity of the test used, which should had been previously estimated in the same population for an accurate determination (Nielsen and Toft, 2009).

In any case, the low prevalence obtained could also been explained by the test´s characteristics that are mainly related to its sensitivity as a response to the silent and long-lasting behavior of the disease, than to failures of the test itself (Sweeney, 1996; Collins *et al.,* 2005; Mon *et al.,* 2012; Sorge *et al.,* 2012). According to Lavers *et al.* (2015), the sensitivity of serum and milk ELISA is approximately 25.6–45.3% and its specificity of 97.6–98.9%, which can lead to a misclassification of the cows and reporting infected cows as negative (Nielsen *et al.,* 2002). On the other hand, the low prevalence obtained could be related to sample handling. In the present study, the serum samples were frozen for 30 to 45 days at -20°C, which could have led to lower scores for the MAP ELISA, as previously reported by Alinovi *et al.* (2009).

The risk factors identified in this study (number of days in milk and individual daily milk production) are supported by the current data that parturition, stage of lactation, and metabolic stress, induced by milk production, can act as triggers and lead to

seroconversion or progression from stage II to stage III of the disease (Clarke, 1997; Nielsen *et al.,* 2002; Fecteau and Whitlock, 2010). Nielsen *et al.* (2002) reported that in serum ELISAs, the OR of being positive is highest at the end of lactation (> 203 days; OR=5.22), possibly indicating that cows with low antibody concentrations are infected but with a cell-mediated immune response, undetectable by ELISA. This statement is hypothetical and would have to be supported by a longitudinal study with repeated samplings on the same population to understand the serological patterns.

Our study reported similar results of odds over 3.45 for cows over 200 days in milk, indicating that the probability of being ELISA-positive is different across lactation progression and is higher in the middle of the lactation. From a diagnostic point of view, it is important to recognize the differences in ELISA-positive animals in different stages of lactation and different production levels, as these findings can help establish risk assessment-based control programs and guide owners to recognize the distinctive clinical signs of PTB at an early stage.

Some variables that we hypothesized to be important risks and were previously identified by other studies for seropositivity were not significant in the logistic regression analysis, including parity (p=0.160), physiological state (p=0.57), cow staying with the calf after calving (p=0.55), presence of other ruminants (p=0.62), and manure spreading as a fertilizer in the pastures (p=0.57; Goodger *et al.,* 1996; Cetinkaya *et al.,* 1997; Obasanjo *et al.,* 1997; Jakobsen *et al.,* 2000; Fredriksen *et al.,* 2004; Diéguez *et al.,* 2008; Nielsen and Toft., 2008b; Ansari-Lari *et al.,* 2009; Doré *et al.,* 2012; Nielsen and Toft, 2012).

Although previous studies have reported that the highest probability of a positive-ELISA is observed in older cows (parity ≥ 3; Sherman, 1985; Jakobsen *et al.,* 2000), a large herd (Braun *et al.,* 1990; Ott *et al.,* 1999; Jakobsen *et al.,* 2000; Muskens *et al.,* 2003; Hirst *et al*., 2004), and Jersey cows compared to larger breeds (including Holstein-Friesian; Jakobsen *et al.,* 2000; JØrgensen, 1972; McNab *et al.,* 1991; Cetinkaya *et al.,* 1997), no relationship between breed, parity, and herd size was found in our study.

However, the role of parity as a confounder was investigated by the fitting models considering MAP ELISA-positive results, with and without parity included. No confounding effect of parity was observed.

The practice of leaving a cow with her calf after birth was also representative of the herds of the study, and has been reported as a risk factor, increasing the within-herd transmission of PTB by Goodger *et al.* (1996), Obasanjo *et al.* (1997), and Ansari-Lari *et al*. (2009). Concerning the presence of other ruminants, Whittington et al. (2001) reported cases of bovine PTB due to S (sheep) strain that were confirmed in Australia, demonstrating the transmission opportunity between species. Manure spreading as a risk factor has been previously described (Goodger *et al.,* 1996; Obasanjo *et al.,* 1997), because of the potential exposure to younger and susceptible cattle.

BPG certification includes management practices which can be considered PTB-related, such as grazing strategies (i. e. rotational, rational, intelligent, stripped-rotational, altering, and extensive), fertilization strategies (i. e. organic and inorganic), other animal species in the farm (e. g. pigs, rabbits, goats, horses, buffaloes, and poultry), enteric disease cases in the last semester and their diagnosis, and tuberculosis and brucellosis sanitation status (ICA, 2007).

This study had several limitations. The design chosen for this study was not optimal for the evaluation of herd-level paratuberculosis risk factors. The study would have had much more power to evaluate herd-level effects if a cross-sectional study involving many more herds had been used. However, financial resources were limited to include more herds, but authors believe that herds included in this study were good examples of the specialized dairy herds in the region in an exploratory manner.

The Survey command in Stata version 12.0 (StataCorp, 2011) was used in the data analysis for several reasons. First, the variance linearization procedure used allows for the simultaneous evaluation of both cow-level and herd level risk factors, with appropriate standard error estimates. Second, it allows for the incorporation of sampling weights into all analyses to correctly account for the probability of a herd being sampled within a district.

Conclusion

In conclusion, we detected an apparent seroprevalence of 3.6% at the herd-level and 2% at the animal-level. The risk factors associated with MAP seropositivity were ≥ 100 days in milk and an individual daily milk production over 20 L/cow.

The information in this study indicates the importance of implementing protective management practices related to our results. Thus, it will be necessary to design risk-based programs in each country that are adapted to its specific conditions. Follow-up studies on herds with PTB over a long time period to investigate if the change of individual management practices leads to changes in PTB prevalence on these farms should be performed.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Chapter two

In order to accomplish the specific objective 3 (confirm ELISA positive results using fecal real-time PCR), an additional chapter was included in the present Master`s degree work. This part considers ELISA, fecal culture, an end-point IS900-specific nested PCR, and F57-real-time PCR results, from animals sampled in the study herd accomplishing case definition (under peer reviewing, submitted in 2016).

Fecal culture and two fecal-PCR methods for the diagnosis of Mycobacterium avium subsp*. paratuberculosis in a seropositive herd: a case report*

Cultivo y dos métodos de PCR en materia fecal para el diagnóstico de Mycobacterium avium subsp. paratuberculosis en un hato seropositivo: reporte de caso

Cultivo e dois PCR métodos fecal para o diagnóstico de Mycobacterium avium subsp. paratuberculosis em um rebanho soropositivo: relato de caso

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Nathalia M Correa Valencia[1]; Nicolás F Ramírez[1], Michael Bülte[2]; Jorge A Fernández Silva[1]

[1]*Grupo Centauro, Escuela de Medicina Veterinaria, Facultad de Ciencias Agrarias, Universidad de Antioquia, Medellín, Colombia.*

[2]*Institut für Tierärztliche Nahrungsmittelkunde, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Frankfurter Straße 92, Giessen 35392, Germany*.

Summary

Background: paratuberculosis is a slow-developing infectious disease, characterized by chronic granulomatous enterocolitis. This disease has a variable incubation period from 6 months to over 15 years, and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Its detection by direct and indirect diagnostic techniques has been of special interest. **Objective:** to report the diagnosis and detection of MAP using several diagnostic tests in a herd of the Northern region of Antioquia, Colombia. **Methods:** serum samples from the study herd were analyzed, using a commercial ELISA (enzyme-linked immunosorbent assay) kit. Fecal samples were cultured by duplicate using HEYM (Herrold´s egg yolk medium), and analyzed by an end-point IS*900*-specific nested PCR protocol, and a commercial *F57*-real-time PCR kit. **Results:** eight out of 27 serum samples in the study herd resulted ELISA-positive. None of fecal samples resulted positive to HEYM culture by duplicate and none were found to be positive by *F57*-real-time PCR. Seven of the 27 fecal samples were found to be positive by end-point IS*900*-specific nested PCR. Agreement was found between ELISA and end-point IS*900*-specific nested PCR in one of the animals. **Conclusion:** the present study gives information about agreement between direct and indirect MAP-detection techniques, using different matrixes from animals under the same husbandry conditions.

Keywords: *ELISA, Johne´s disease, culture medium, molecular diagnosis.*

Resumen

Antecedentes: la paratuberculosis es una enfermedad infecciosa de desarrollo lento, caracterizada por una enterocolitis granulomatosa crónica. Esta enfermedad tiene un periodo de incubación que varía entre los 6 meses hasta por más de 15 años, y es causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Su detección por técnicas diagnósticas directas e indirectas ha sido de interés especial. **Objetivo:** reportar el diagnóstico y detección de MAP utilizando varias técnicas diagnósticas en un hato de la región norte de Antioquia, Colombia. **Métodos:** se analizaron las muestras de suero del hato de estudio utilizando un kit comercial de ELISA (enzyme-linked immunosorbent assay). Las muestras de materia fecal fueron cultivadas por duplicado en HEYM (Herrold´s egg yolk medium), y analizadas mediante un protocolo de PCR anidado específico de IS*900* y un kit comercial de PCR en tiempo real para *F57*. **Resultados:** ocho de las 27 muestras de suero resultaron positivas a ELISA. Ninguna de las muestras de materia fecal resultó positiva al cultivo en HEYM por duplicado ni a PCR en tiempo real para *F57.* Siete de las 27 muestras de materia fecal resultaron positivas a PCR anidado específico de IS*900*. Se encontró concordancia entre el resultado de ELISA y de PCR anidado específico de IS*900* en uno de los animales. **Conclusión:** el presente estudio brinda información acerca de la concordancia entre técnicas directas e indirectas de detección de MAP, utilizando diferentes matrices a partir de animales bajo las mismas condiciones de manejo.
Palabras claves: *diagnóstico molecular, ELISA, enfermedad de Johne, medio de cultivo.*

Resumo

Antecedentes: paratuberculosis é uma doença infecciosa de evolução lenta, caracterizada por uma enterocolite granulomatosa crônica. Esta doença tem um período

de incubação que varia de 6 meses a 15 anos, e é causada pela *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Sua detecção por técnicas de diagnóstico diretos e indiretos foram de especial interesse. **Objetivo:** relatório de diagnóstico e detecção de MAP utilizando várias técnicas de diagnóstico em um rebanho na região norte de Antioquia, Colombia. **Métodos:** soro rebanho estudo amostras são analisadas utilizando um kit comercial de ELISA (enzyme-linked immunosorbent assay). As amostras de fezes foram cultivadas em duplicado em HEYM (Herrold´s egg yolk medium), e analisadas utilizando um protocolo de PCR aninhada IS*900* específico e um estojo de PCR para comercial *F57* tempo real. **Resultados:** oito das 27 amostras de soro eram positivas para ELISA. Nenhuma das amostras testadas cultura de fezes positiva HEYM duplicar ou PCR em tempo real *F57*. Sete das 27 amostras de fezes foram positivas para IS*900* específica de PCR aninhada. Foi encontrada concordância entre o resultado de ELISA e específico aninhado em um animal IS*900* PCR. **Conclusão:** este estudo fornece informações sobre a correlação entre técnicas de detecção direta e indireta MAP, utilizando diferentes matrizes de animais sob as mesmas condições de condução.
Palavras chave: *diagnóstico molecular, ELISA, doença de Johne, meio de cultura.*

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow-growing, mycobactin-dependent, acid fast bacterium that causes Johne's disease or paratuberculosis (PTB) in cattle and other susceptible species (Harris and Barletta, 2001). The disease produces a significant economic impact on the cattle industry, especially on milk and meat production (Sweeney, 1996; Chacon *et al.,* 2004; García and Shalloo, 2015; McAloon *et al.,* 2016), and the agent has also been associated to the chronic human enteritis known as Crohn's disease (Atreya *et al.,* 2014; Hanifian, 2014; Liverani *et al*., 2014; Waddell *et al.* 2015; 2016).

For the *ante-mortem* diagnosis of PTB in cattle, several types of test are available and proposed. These include tests to detect antibodies against MAP, detection of MAP genes, bacterial culture of fecal samples and test to detect MAP on tissue samples (Collins *et al.,* 2006; Nielsen and Toft, 2008; Stevenson, 2010a; 2010b). Sensitivity and specificity of tests for the *ante-mortem* diagnosis of PTB vary significantly depending on MAP infection stage and intrinsic characteristics of each test (Nielsen and Toft, 2008).

The antibody detection test known as enzyme-linked immunoassay (ELISA) is the most popular test to detect an immune response to infection by MAP. ELISA is also the most widely used to establish PTB status of herds, but it has shown limitations in some extend relating low sensitivity, primarily because of the slow progression of MAP infection. This does not ensure an adequate detection capacity of animals in an early stage of infection when fecal shedding is low (Kalis *et al.,* 2002; McKenna *et al.,* 2006; Nielsen, 2010). On the contrary, ELISA is highly specific, with a low presentation of false positive results (Harris and Barletta, 2001).

Cultivation of MAP from tissues and fecal samples (individual, in pool, and environmental) is the most reliable method of detecting infected animals (Nielsen and Toft, 2008; 2009; Fecteau and Whitlock, 2010). Usually, the specificity of fecal culture (FC) is considered to be almost 100% if the isolates obtained are confirmed to be MAP by molecular methods such as polymerase chain reaction (PCR; Nielsen and Toft, 2008; Schönenbrücher *et al.,* 2008; Whittington *et al.,* 2011). FC has been used as an acceptable standard technique for detecting the infection status of animals –related to elimination rate-, for estimating the sensitivity of other diagnostic tests (e.g. ELISA, PCR), and as an excellent confirmatory test for animals that tested positive with immunological tests (Motiwala *et al.,* 2005; Aly *et al*., 2012). Herrold´s egg yolk medium (HEYM) is the most frequently used for the primary cultivation of MAP from clinical samples (feces and tissue), and its sensitivity has been reported from 39 to 82%, compared to liquid media (Collins *et al.,* 1990; Eamens *et al.,* 2000; Stich *et al.,* 2004, Motiwala *et al.,* 2005; Cernicchiaro *et al.,* 2008; Whittington, 2009).

Special aspects of MAP and the disease dynamics can affect the FC accuracy, for example, MAP´s elimination through feces is intermittent and occurs in an advanced stage (stages III and IV) of the disease, mainly when the animals have clinical symptoms (Clarke, 1997; Whittington, 2010; Salem *et al.,* 2013). Although the FC has many limitations, such as a long incubation period (18 to 24 weeks), high costs, risk of contamination with other mycobacteria or fungi, and time required to report the results, it is still considered to be the "*gold standard*" for the detection of MAP (van Schaik *et al.,* 2007; Nielsen and Toft, 2008; Whittington, 2010).

The detection of MAP genes by PCR has shown advantages (rapidity, identification of agent, lack of contamination) and disadvantages (moderate sensitivity, high cost, special equipment and skilled personnel required; Collins, 1996). However, due to recent developments, PCR has been suggested for herd screening (Collins *et al.,* 2006; Anonymous, 2010), and it has been recently discussed as a possible new "*gold standard*" for PTB (Stevenson, 2010a; 2010b). The PCR technique is rapid and specific, and in contrast to a culture–based diagnostic, no additional tests are required to confirm the identity of the organism detected (Collins, 1996).

The most popular target gene for the detection of MAP is the multi-copy element IS*900* (Bolske and Herthnek, 2010; National Advisory Committee on Microbiological Criteria for Foods, 2010; Stevenson, 2010b; Gill *et al.,* 2011). However, mycobacteria other than MAP have been found to carry IS*900*-like elements with nucleotide sequences that are up to 94% identical to the nucleotide sequence of MAP IS*900* (Cousins *et al.,* 1999; Ellingson *et al.,* 2000; Englund *et al.,* 2002; Kim *et al.,* 2002; Taddei *et al.,* 2008). Some PCR systems that target IS*900* also can give false-positive results with DNA from mycobacteria other than MAP and with DNA from other types of organisms (Möbius *et al.,* 2008a; 2008b). Due to this, new protocols avoiding cross-reactions have been reported (Bull *et al.,* 2003; Herthnek and Bölske, 2006; Kawaji *et al.,* 2007). In response to the uncertainty about the specificity of PCR systems that target IS*900* for the identification of MAP, the use of several other target sequences for MAP identification systems have been

proposed: IS*Map02*, IS*Mav2*, hspX, locus 255, and *F57* (Stabel and Bannantine, 2005; Slana *et al.,* 2009; Kralik *et al.,* 2010; Sidoti *et al.,* 2011; Keller *et al.,* 2014).

PCR performs well as a confirmatory test on cultures, being its sensitivity close to 100% (Manning and Collins, 2001), but its application to clinical samples has been problematic, mainly due to the problems associated with DNA extraction from complex matrices such as milk, feces, and blood, and the presence of PCR inhibitors (Stevenson and Sharp, 1997; Grant *et al.,* 1998; Aly *et al.,* 2010; Stevenson, 2010b), decreasing its sensitivity. The limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, the matrix tested, and the PCR format (conventional gel-based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, or multiplex PCR; Möbius *et al.,* 2008a; Bolske and Herthnek, 2010; National Advisory Committee on Microbiological Criteria for Foods, 2010; Stevenson, 2010b). Ideally, sampling all adult cattle in every herd, environmental sampling, serial testing, and the use of two to three diagnostic tests would be the recommendation for herd screening, to increase the accuracy of MAP diagnosis (Collins *et al.,* 2006; Clark *et al.,* 2008; Stevenson, 2010b).

The aim of this study was to diagnose MAP using FC, *F57*-real-time PCR and end-point IS*900*-specific nested PCR in one herd previously screened positive for MAP antibodies by an indirect serum-ELISA.

Materials and methods

Ethical considerations

This research was approved by the Ethics Committee for Animal Experimentation of the Universidad of Antioquia, Colombia (Act number 88, from March 27, 2014; Annex 2).

Herd

The study herd was located in San Pedro de los Milagros, Antioquia (Colombia), one of the main dairy municipalities of the country, located in the Andean region of Colombia, with an area of 229 km^2, an altitude of 2,468 m. a. s. l, a mean annual temperature of 16 °C, and a cattle population of approximately 71,395 animals. The study herd was visited only once as part of a previous study in 2015, that aimed the determination of the seroprevalence of MAP and the exploration of the main risk factors associated with ELISA positive results in dairy cows of the municipality of interest (Correa-Valencia *et al.,* 2016). The study herd, reported a cattle population of 39 bovines, including 27 cows over 2 years of age at the moment of the sampling, the predominant breed was classified as “other” in the previous study (different from Holstein and Jersey), without history of farming other ruminants different from bovines (i.e. goats, sheep, buffaloes), spreading manure as a fertilizer in the pastures was a common practice in the herd, as well as, leaving the calves with their dams after parturition in direct contact, certified as free of tuberculosis and brucellosis, and never reported any compatible clinical case and/or followed any structured control program for prevention or control of PTB before the sampling in 2015.

Blood and fecal samples were taken from all animals over 2 years of age (n = 27). The sample collection was conducted according to standard methods to avoid unnecessary pain or stress to animals. Blood samples were taken from the coccygeal or jugular vein, collected in red-top plastic Vacutainer® tubes and transported refrigerated to the laboratory, where they were centrifuged at 1008 RCF for 5 minutes. Fecal samples were taken with a clean glove directly from the rectum of every adult animal, and then, transported refrigerated to the laboratory. The obtained serum and the fecal samples were stored at -20 °C until analysis.

ELISA

Serum ELISA was performed using the pre-absorbed ELISA kit Parachek®2 (Prionics AG, Schlieren, Switzerland) following the manufacturer's instructions. This test included a pre-absorption step with *Mycobacterium phlei* to reduce cross-reactions. An animal was considered ELISA-positive if serum sample was above or equal to the cut-off of 15 Percent Positivity (%P), as it is defined by the manufacturer of the diagnostic test used.

Fecal culture

Feces from all animals were thawed leaving the samples under 4 °C for 24 hours prior to decontamination procedure. Fecal culture was carried out according to the protocol reported previously by Fernández-Silva *et al.,* 2011. Briefly, 3 g of feces were added to a 50 ml sterile tube containing 30 ml of a 0.75% HPC (Hexadecyl Pyridinium Chloride) weight/volume (w/v) solution. This suspension was manually mixed by shaking, and let in a vertical position for 5 min at room temperature to allow precipitation and sedimentation of big particles. Approximately 20 ml of the upper portion of the supernatant was transfer to another 50 ml sterile tube, in which the whole suspension was agitated for 30 min at 200 U/min. Tubes were place in vertical position in the dark for 24 h at room temperature. Decontaminated pooled fecal samples were centrifuged at 900 x g during 30 min, supernatant was discarded. Duplicated Herrold's yolk agar medium (HEYM) slants, supplemented with mycobactin J and amphotericin B, nalidixic acid, and vancomycin mix (Becton Dickinson, Heidelberg, Germany) were inoculated with 300 µL of the decontaminated pellet (Fernández-Silva *et al.,* 2011). All culture media were incubated at 39 °C for 24 weeks and were checked weekly for mycobacterial growth or contamination with undesirable germs. MAP growth was visually monitored for typical slow growth rate and colony morphology according to previous descriptions (colonies developing after ≥3 weeks of incubation, initially round, smooth and white, then tending to heap up slightly and becoming dull light yellow with wrinkling of the surface; Whittington, 2010).

DNA isolation from individual fecal samples

Each fecal sample was homogenized for 5 min prior to DNA extraction procedure. DNA from individual fecal samples was extracted according to the following procedure reported previously by Leite *et al.* (2013) using a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, Irvine, CA, USA). Processing was done according to kit´s protocol for isolation of nucleic acids from bacteria and yeast. A mechanical cell disruptor step was carried out in an automated biological sample lyzer (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA) to achieve a more efficient cell lysis.

End-point IS900-specific nested PCR

DNA from individual fecal samples was tested for MAP by end-point IS*900*-specific nested PCR, using primers targeting IS*900* designated TJ1-4 [TJ1 (5´-GCT GAT CGC CTT GCT CAT-3´) and TJ2 (5´-CGG GAG TTT GGT AGC CAG TA-3´) in the first-round-PCR, and primer pair TJ3 (5´-CAG CGG CTG CTT TAT ATT CC-3´) and TJ4 (5´-GGC ACG GCT CTT GTT GTA GT-3´) in the second round-PCR] according to Bull *et al.* (2003), modified by Füllgrabe (2009) and Bulander (2009). The first and second-round PCR mixture comprised the same mix volumes in a final volume of 50 µl with 5 µl of TaqDNA polymerase buffer- $MgCl_2$, 1 µl of dNTP mix, 1 µl of each primer, and 0.4 µl of TaqDNA polymerase (AmpliTaq Gold® DNA Polymerase LD, recombinant; 5 U/µL; Applied Biosystems™, Foster City, CA, USA), and 5 µl of DNA from sample or from the first-round-PCR. Additionally to the samples, a positive (*Mycobacterium avium* subsp. *pararuberculosis*, strain K10 (ATCC® BAA-968™) and a negative control (DNA of a negative-known fecal sample from a seronegative cow), as well as, a blank control were included. Cycling conditions for both rounds were: 1 cycle of 95 °C for 10 min and then 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, followed by 1 cycle of 72 °C for 7 min. Amplicons of the expected size (355 and 294 bp, for the first and second round, respectively) were visualized with ethidium bromide on 1.5% agarose gels.

F57-real-time PCR

DNA from individual fecal samples was tested for MAP confirmation by *F57* using a commercial kit, including an internal amplification control (IAC) to avoid the misinterpretation of false negative results MAPsureEasy® (MSE) Real-Time PCR-Kit (TransMIT, Giessen, Germany). The positive control for MAP-DNA was *Mycobacterium avium* subsp. *pararuberculosis* strain K10 (ATCC® BAA-968™). Results were achieved looking for absolute quantification (presence/absence of MAP DNA). The double concentrated 2x Master Mix for real-time PCR included the Taq-polymerase, nucleotides, and buffer (MasterMix Plus SYBR Green® without UNG, Eurogentec, Ireland). The Oligonucleotid Mix included the primers and probes [*F57*-F 5‘– TAC GAG CAC GCA GGC ATT C – 3‘; *F57*-R 5‘– CGG TCC AGT TCG CTG TCA T – 3‘; *F57*po-TaqMan® Probe VIC-CCT GAC CAC CCT TC-MGB; and, IAK MSE TaqMan® Probe FAM-AGC AAT AAA CCA GCC AGC-MGB]. The PCR mixture was prepared according to the protocol, one sample in a final volume of 25 µl: 12.5 µl of real-time PCR Master Mix, 1 µl of MAP-Oligonucleotid Mix; 1 µl of the IAC-DNA, 5 µl of DNA probe, and 2.5 µl of DNA. Following this, the real-time PCR plate was sealed with adhesive film. After a brief centrifugation, the plate was introduced in the real-time PCR instrument. PCR was performed with the AbiPrism® 7000 Sequence Detection System (Applied Biosystems™, Foster City, CA, USA). Cycling conditions were: 1 cycle of 95 °C for 10 min, and then 45 cycles including two processes (95 °C for 15 sec and 60 °C for 1 min). A sample was considered positive for the detection of MAP-DNA when the detection system indicated a Ct value of <40. A sample was considered negative when the detection system indicated a Ct value ≥ 40.

Results

ELISA

Eight of the 27 (29.6%) animals were positive by serum-ELISA in the study herd (Table 1).

Fecal culture

None of the 27 fecal samples from animals of the study herd were positive by fecal culture based on growth rate and colony morphology (Table 1). Two duplicated cultures (four slants) presented contamination (7.4%).

End-point IS900-specific nested PCR and F57-real-time PCR

All the samples resulted negative by *F57*-real-time PCR, and seven (25.9%) resulted positive by end-point IS*900*-specific nested PCR (Table 1). Amplifications for end-point IS*900*-specific nested PCR in agarose gel results are shown in Figures 1 and 2. A compilation of individual information and tests results for animals tested (n = 27) of the study herd are shown in Table 1.

Table 1. Cow-level information and MAP-diagnostic tests results in a study herd in San Pedro de los Milagros, Antioquia, Colombia.

Cow ID	**Breed***	**Parity**	**Days in milk**	**Milk production per day (L)**	**Productive stage**	**Born in herd**	**Serum ELISA**	**Fecal culture**	**IS*900*-nested PCR**	***F57*-real-time PCR**
1	Other	2	192	23	Milking	Yes	−	−	+	−

2	Other	6	163	33	Milking	Yes	+	−	−	−
3	Other	2	372	n.d.	Dry	Yes	−	−	−	−
4	Other	5	72	34	Milking	No	−	−	−	−
5	Holstein	1	4	n.d.	Dry	Yes	−	−	−	−
6	Other	4	214	24	Milking	No	−	−	−	−
7	Other	6	182	21	Milking	No	+	−	−	−
8	Other	2	133	25	Milking	No	−	−	−	−
9	Other	2	235	14	Milking	No	−	−	−	−
10	Other	n.d.	n.d.	n.d.	Heifer	Yes	+	−	−	−
11	Other	1	37	27	Milking	Yes	−	−	−	−
12	Other	2	299	16	Milking	Yes	−	−	+	−
13	Holstein	2	88	31	Milking	Yes	+	−	−	−
14	Holstein	1	215	25	Milking	Yes	+	−	−	−
15	Other	1	52	21	Milking	Yes	−	−	+	−
16	Other	2	227	16	Milking	Yes	−	−	−	−
17	Holstein	6	324	n.d.	Dry	Yes	−	−	−	−
18	Holstein	2	197	19	Milking	Yes	+	−	−	−
19	Holstein	7	72	51	Milking	Yes	−	−	−	−
20	Other	5	18	25	Milking	Yes	−	−	−	−
21	Other	3	192	25	Milking	No	−	−	−	−

22	Holstein	n.d.	n.d.	n.d.	Heifer	Yes	−	−	+	−
23	Other	5	161	22	Milking	Yes	+	−	−	−
24	Holstein	5	89	37	Milking	Yes	−	−	+	−
25	Other	3	409	18	Milking	Yes	+	−	+	−
26	Other	3	184	24	Milking	Yes	−	−	−	−
27	Jersey	1	40	23	Milking	Yes	−	−	+	−

* Other breeds included Guernsey, Ayrshire, Swedish Red, Swiss Brown, Jersey, and several crossbreeds of Holstein with Jersey, Ayrshire, Angus, Blanco Orejinegro, Brahman, and Gir.

n.d.: no data available at the moment of sampling; +: positive result, −: negative result

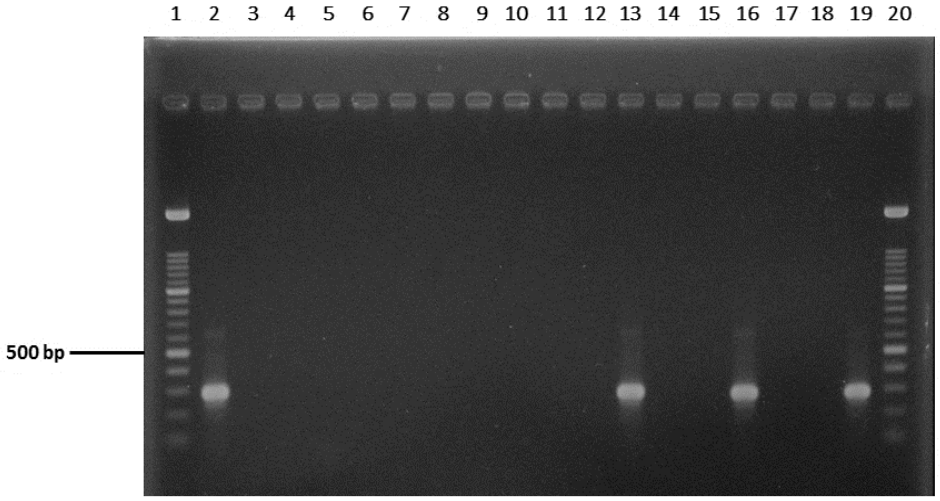

Figure 1. End-point IS*900*-specific nested PCR in agarose gel (final product of 294 bp), samples of cows 1-17. Molecular size marker (100 bp DNA ladder; Roche, Mannheim, Germany; lane 1 and 20), animal 1 (lane 2), animal 12 (lane 13), animal 15 (lane 16), positive control (*Mycobacterium avium subsp. pararuberculosis*, strain K10, ATCC® BAA-968™; lane 19), negative results (lanes 3-12, 14-15, and 17-18).

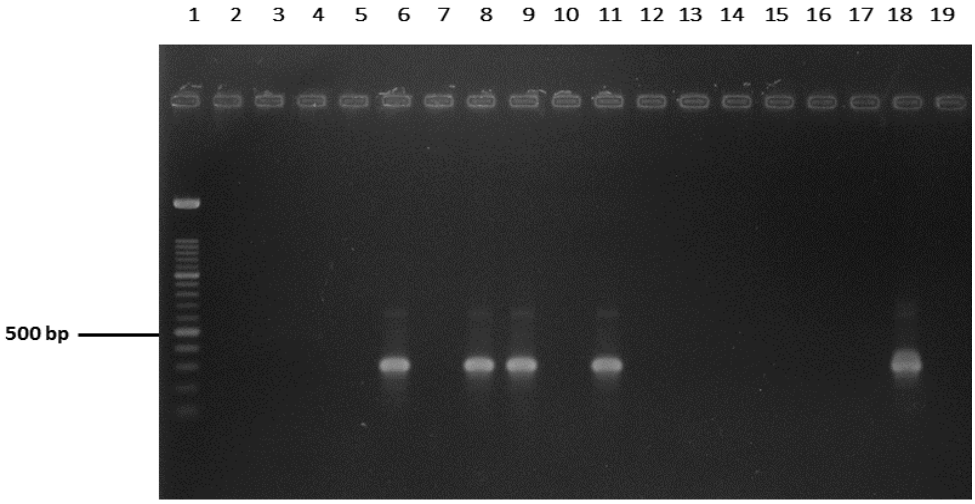

Figure 2. End-point IS*900*-specific nested PCR in agarose gel (final product of 294 bp), samples of cows 18-27. Molecular size marker (100 bp DNA ladder; Roche, Mannheim, Germany; lane 1 and 20), animal 22 (lane 6), animal 24 (lane 8), animal 25 (lane 9), animal 27 (lane 11), positive control (*Mycobacterium avium subsp. pararuberculosis*, strain K10, ATCC® BAA-968™; lane 18), blank control (master mixture blank; lane 19), negative results (lanes 2-5, 7, 10; empty lanes 12-17).

Discussion

The present study aimed to diagnose MAP using FC, *F57*-real-time PCR, and end-point IS*900*-specific nested PCR in one herd previously screened positive for MAP antibodies by an indirect serum-ELISA.

The confirmation of ELISA test results using FC and PCR was considered necessary to obtain a precise detection of PTB infected animals in an ELISA positive herd. Nevertheless, we expected to find a higher proportion of MAP-positive animals (by ELISA, as well as, by FC and PCR) in the study herd, considering inappropriate herd management practices present and known to be risk factors for the disease (e.g. presence of animals born at other dairies, exposure of calves 0-6 weeks to adults feces, feces spread on forage fed to any age group; Collins *et al.,* 1994; Goodger *et al.,* 1996; Jakobsen *et al.,* 2000;

Wells and Wagner, 2000; Diéguez *et al.,* 2008; Tiwari *et al.,* 2009; Sorge *et al.,* 2012; Künzler *et al.,* 2014; Fernández-Silva and Ramírez-Vásquez, 2015; Vilar *et al*., 2015). When a test combination is considered, it must be taken into account that some infected cows produce antibodies for several years prior to the fecal-shedding of detectable quantities of MAP. However, in other animals, antibodies may not be detectable during the early stages of infection when MAP fecal-shedding is minimal (Kalis *et al.,* 2002; McKenna *et al.,* 2006; Nielsen, 2010).

The ELISA results should be analyzed cautiously, mainly considering its sensitivity because of the silent and long-lasting behavior of the disease, more than as a failure of the test itself (Sweeney *et al.,* 1996; Collins *et al.,* 2005; Mon *et al.,* 2012; Sorge *et al*., 2012). According to Lavers *et al.* (2015), the sensitivity of serum ELISA is approximately 25.6-5.3% and its specificity of 97.6-98.9% in asymptomatic animals, which can lead to a misclassification of the cows and reporting infected cows as negative. On the other hand, the results could be related to sample handling. In the present study, the serum samples were frozen for 30 to 45 days at -20 °C, which could have led to lower scores for the MAP ELISA (Alinovi *et al.,* 2009).

Fecal culture did not report any positive result, which could be explained, among other aspects, by the storage conditions (4 °C for 12 h max, and then at -20 °C for 7 months). According to Khare *et al*. (2008) to store fecal samples at 4 °C for 48 h, and then at -20 °C for at least one week is limiting for the culture sensitivity, contrary to short-term storage at 4 °C and longer term storage at -70 °C, which appear to have no damaging effects on MAP viability in the fecal sample. On the other hand, there would be false-negative FC for samples that contain few organisms due to less of MAP during the culturing as a direct consequence of the process (Whittington, 2010). Dehydration and the possible reduction of viable microorganism by chemical decontamination are important data to interpret negative results, especially in low intensity fecal shedders (Reddacliff *et al.,* 2003).

Another point that should be considered to explain some of our results is the low-shedder status, considering that literature reports that about 75% of positive animals are either low or very low shedders (van Schaik *et al.,* 2003; USDA, APHIS, VS, CEAH, 2008). In view of the minimal amount of detectable MAP (100 CFU/g of feces; Merkal, 1970), only 15-25% of subclinical low and/or moderate fecal shedders can be detected by bacterial culture (Whitlock and Buergelt, 1996). The sensitivity of the FC in clinical stages can be 91% (Álvarez *et al.,* 2009), a value that can be reduced to 45-72% (Crossley *et al.,* 2005; Alinovi *et al.,* 2009) in subclinical stages, whereas the specificity is very good (100%) in all stages (Ayele *et al*., 2001). This information can explain some of our results, considerin the seroprevalenceresults for the whole municipality (3.6% and 2% at herd-level and animal-level, respectively; Correa-Valencia *et al.,* 2016), where no clinical animals were sampled.

The low agreement between tests results has been also reported before (Muskens *et al.,* 2003; Glanemann *et al*., 2004; Dreier *et al.,* 2006) and could be explained in the fact that ELISA negative or ELISA false-positive results have a low probability of delivering a positive culture result if just a single sampling is planned as normally done in a cross-sectional study, which was the case of the present study (Sweeney *et al*., 2006). Similar results on low agreement between ELISA and culture (Fernández-Silva *et al.* 2011b) and ELISA and PCR to MAP (Fernández-Silva *et al.* 2011a) were found in previous studies in asymptomatic animals from herds of the same dairy region.

The use of direct PCR to fecal DNA has several advantages as for example shorter times to diagnosis compared to culture (3 days vs. 14-22 weeks). In addition, the procedure for the extraction of fecal DNA in preparation for PCR has become easier and less expensive in the recent years (Stabel *et al.,* 2004). Considering an effective method to ensure a complete-DNA extraction, a mechanical disruption step (bead-beating) was included — which breaks up bacterial cell wall mechanically by vibrating bacteria at high speed— in addition to the commercial kit protocol (Odumero *et al.,* 2002; Zecconi *et al.,* 2002;

Herthnek, 2009) improving the sensitivity of the protocol applied, also reported by Leite *et al.* (2013) with the comparable performance results.

Special attention should be given to the inhibitory effects of certain components of the samples on Taq polymerase, which could cause false negative results, being a probable explanation for some of our negative outcomes (Tiwari *et al.,* 2006). Feces, especially ruminants´, are expected to include high levels of PCR inhibitors (Al-Soud and Radstrom, 1998; Inglis and Kalischuck, 2003; Thorton and Passen, 2004), and one of the main difficulties is to remove them to improved PCR sensitivity (Harris and Barletta, 2001). Although no clinical cows were found in our study herd, in some cases is highly probable that feces from cows with clinical PTB may contain heme (a complex of iron with protoporphyrin IX) and epithelial cells, being these components reported to be inhibitory to PCR (Inglis and Kalischuck, 2003).

The sensitivity and specificity of the end-point IS*900*-specific nested PCR used to test our samples are reported to be increased (Englund *et al.,* 2001; Ikonomopoulos *et al.,* 2004; Bölske and Herthnek, 2010). Any PCR inhibitors in the first run will be diluted when transferred as template to the second PCR (Bölske and Herthnek, 2010).

Our assays used two molecular elements found in different loci and ratios in MAP genome (IS*900* and *F57*), leading to non-comparable results related to their specificity and sensitivity. IS*900* is a repetitive DNA sequence present in 15-18 copies of MAP genome (Collins *et al.,* 1989; Green *et al.,* 1989). However, IS*900*-like elements have been described at low copy numbers in rarely encountered environmental mycobacteria (Cousins *et al*., 1999; Englund *et al.,* 2002; Tasara *et al.,* 2005), compromising its specificity. On the other hand, *F57,* a single copy-segment, has demonstrated high specificity for the detection of MAP (Coetsier *et al.,* 2000; Ellingson *et al.,* 2000; Harris and Barletta, 2001; Strommenger *et al.,* 2001; Vansnick *et al.,* 2004; Rajeev *et al*., 2005). The nested IS*900* assay can detect 0.01 pg of DNA (corresponding to 10 genomes) when extracted from a pure culture, while the *F57* assay can detect 0.1 pg of DNA

(corresponding to 100 genomes; Radomski *et al.,* 2013). Vansnicka *et al.* (2004), Tasara and Stephan (2005), and Schönenbrücher et al. (2008) recommend including the *F57*-PCR assay to confirm the presence of MAP after a positive IS*900*-PCR. According to this, our results (*F57*-PCR negative results and some positive results by IS*900*-PCR), can be considered MAP-unspecific by IS*900*-PCR, and confirmed as negative by the *F57* insertion detection.

Nevertheless, our results in the PCR protocols applied could be better explained by the already reported behavior of the disease than to PCR misclassification. According to Withlock *et al.* (2000), the disadvantages of some detection test are due mainly because of the intermittent shedding of microorganisms. This means that the sensitivity of direct tests to detect symptomatic animals is high, but low for detection of infected/subclinical animals (Nielsen and Toft, 2008; Schönenbrücher *et al.,* 2008; Whittington *et al.,* 2011).

On the other hand, the thawing of fecal samples stored at -20 °C was done in different times for fecal culturing process and for DNA extraction what could have affected the detection by PCR, leading to false negative results because of DNA damage during thawing-freezing re-processes, which can explain PCR results in this study (Bölske and Herthnek, 2010; Whittington, 2010).

Our results for all the tests used does not necessarily mean that the animals were not really infected, because the shedding phase has probably not yet started (infected animal in a noninfectious phase) or was absent at the moment of fecal sampling (intermittency). Another possibility is that in these animals MAP-antibodies were detected prior to the start of bacterial shedding, which could begin later and could be then detected by PCR or FC (Nielsen, 2008). Considering MAP-shedding characteristics as the major limitation in the detection of infected animals, it should be taken into account that the elimination of the bacteria through feces happens at all stages but at different levels and sporadically, which demands repeated testing to detect animals shedding very low number of MAP, which could anyway go undetected (Stevenson, 2010b). Nevertheless, we found a positive result

by serum-ELISA and fecal PCR in one of the cows in the study herd, revealing parallel detectable antibody levels and detectable MAP fecal-shedding, being this a biologically plausible result.

Alinovi *et al*. (2009) reported that test sensitivity for culture methods and real-time PCR, as well as, test accuracy, are comparable. This clearly demonstrates that in field applications, real-time PCR is as useful as solid or liquid culture methods while providing the producer with test results within hours, not weeks. Serum ELISA, although not as accurate as the other tests evaluated, continues to be a useful alternative because of its rapid turn-around. Now, with PCR, results that are more accurate can be available as fast as for ELISA.

Conclusion

Our results in a seropositive herd delivered one asymptomatic ELISA-positive cow with a negative FC, and a positive end-point IS*900*-specific PCR result. In addition, there were 26 asymptomatic ELISA-negative cows, producing negative results by FC, and negative results by two different PCR methods. We detected a low agreement between the diagnostic tests used (ELISA, FC, and PCR). These results evidence the perfect examples of MAP´s detection paradox and the most confounding component in PTB control: the detection of truly infected and uninfected animals. The information in this study indicates the importance of MAP detection and its direct impact in the implementation of strategic management practices to ensure the control of the disease and the dissemination of the agent. Thus, it will be necessary to design risk-based programs in each region in the country, adapted to its specific conditions, even considering production systems. Follow-up studies on herds with PTB over a long time to investigate whether the change of individual and herd-level management practices lead to changes in PTB control on this herd should be performed.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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General Conclusion

The specific objectives of this master´s degree work were oriented to stablish MAP-seroprevalence by ELISA, to confirm ELISA positive results using fecal real-time PCR, and to explore the main risk factors associated to MAP ELISA and/or real-time PCR positive results at animal and herd level. In addition, the hypotheses considered included an expected MAP sero-prevalence around 60% at herd level and 10% at animal level. And, that at least one individual animal feature, one herd characteristic and one herd management practice were potential risk factors for MAP ELISA positive results in the study herds.

During the investigative process, we found a low prevalence of PTB (at least, lower than expected) in the study population. In addition, there were no risk factors related to herd management practices or herd characteristics, as it was expressed in the hypothesis, and ELISA results could not be confirmed by real-time PCR, because of agreement conflicts exposed along this document and reported by literature.

Nevertheless, the results of this work confirm the presence of MAP in the dairy herds in the region of study, and the limitations of serum ELISA, FC, and fecal PCR for the detection of this microorganism in herds without history of PTB.

Further microbiological and epidemiological studies have to be carried out in Colombia, using higher populations and combining different testing and laboratory strategies and methodologies, in order to increase the knowledge about bovine PTB and the risk factors affecting its control in our specific conditions and agro-systems.

The main conclusion of this study is that tools for serological and fecal diagnosis of MAP (direct and indirect methods) were very useful to increase the knowledge of PTB in the

population of the study, but the results of each one must be considered strategically, as well as, their combination, to take decisions about the control measures for the disease.

Perspectives of Investigation

Future research opportunities should consider the determination of MAP herd and cow-level prevalences in a more representative population, including other municipalities, regions and departments of the country, as well as, other susceptible species, obtaining information about the epidemiological behavior of MAP at a molecular scale, leading to a better understanding of the disease´s dynamics. There is also a necessity of identifying the risk factors for MAP infection in dairy, beef, and double-purpose farms, with different characteristics and management practices.

Other opportunities include the identification of MAP in lake catchments, in river water abstracted for domestic use, and in effluent from domestic and farm sewage treatment works, obtaining information about the potential exposure of humans and other susceptible species to the bacterium.

Considering the availability of a PTB-endemic herd, it would be interesting to explore the differences on serum and milk-ELISA results across lactation in infected animals, assuming a change in antibody concentration throughout the lactation, improving disease detection strategies.

All this information (and other unexpected or not mentioned) will help to the establishment of a cost-effective basis for PTB control at a herd and local level, considering the recent concern about disease notification in Colombia.

Annexes

Annex 1: Authors guidelines

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Annex 2. Approval of Comité de Ética para la Experimentación Animal (CEEA), Universidad de Antioquia

UNIVERSIDAD DE ANTIOQUIA
1803

Comité de Ética para la Experimentación con Animales

Medellín, 11 de abril de 2014

Investigador
JORGE A. FERNANDEZ SILVA
Universidad de Antioquia

Solicitud: "Diagnóstico y factores de riesgo de *Mycobacterium avium subsp paratuberculosis* (MAP) en vacas de hatos lecheros de un municipio de la Región Norte de Antioquia, Colombia"

Cordial saludo.

Después de realizados los cambios sugeridos al protocolo en referencia, nos permitimos comunicarle que cumple con los lineamientos establecidos por el **Comité de Ética para la Experimentación con Animales** de la Universidad de Antioquia.

Con toda atención.

Juan Rpo.

JUAN GONZALO RESTREPO SALAZAR
Coordinador
Comité de Ética para la Experimentación con Animales
Universidad de Antioquia

Annex 3. Questionnaire for the determination of individual and herd risk factors for paratuberculosis

General information of herd	
Questionnaire number (consecutive herd number)	
Date	
Name of herd	
Name of owner	
Owner´s contact phone number	
Municipality	
Area of herd (in hectares)	
Herd daily average milk production (liters)	
Cattle population by groups Total cattle population	Calves ____ Heifers ____ Milking cows ____ Dry cows ____ Bulls ____ _____
Are you farming other kind of ruminants (goats, sheep or buffaloes) in your installations?	Yes No
Do you spread manure on pastures as a fertilizer?	Yes No
Are the calves staying with their dams after parturition?	Yes No
Certificated herd in BPG Tuberculosis-free Brucelosis-free	 _____ _____ _____
Individual cow information	
☐ Age group	1 (cows between 2-3 years) 2 (cows >3 years)
☐ Last parturition date	
☐ Sex	Male Female
☐ Breed	Holstein Jersey Other
☐ Parity	1 2 3 4 ≥5
☐ Individual daily average milk production (in liters)	
☐ Born in the herd	Yes No

Total of samples ☐