Epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in the Northern dairy region of Antioquia, Colombia

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"No pido milagros y visiones, Señor, pido la fuerza para la vida diaria. Enséñame el arte de los pequeños pasos. Hazme hábil y creativo, para notar a tiempo en la multiplicidad y variedad de lo cotidiano, los conocimientos y experiencias que me atañen personalmente. Ayúdame a distribuir correctamente mí tiempo: Dame la capacidad de distinguir lo esencial de lo secundario. Te pido fuerza, auto-control y equilibrio para no dejarme llevar por la vida y organizar sabiamente el curso del día. Ayúdame a hacer cada cosa de mi presente lo mejor posible, y a reconocer que esta hora es la más importante. Guárdame de la ingenua creencia de que en la vida todo debe salir bien. Otórgame la lucidez de reconocer que las dificultades, las derrotas y los fracasos son oportunidades en la vida para crecer y madurar. Envíame en el momento justo a alguien que tenga el valor de decirme la verdad con amor. Haz de mí un ser humano que se sienta unido a los que sufren. Permíteme entregarles en el momento preciso un instante de bondad, con o sin palabras. No me des lo que yo pido, sino lo que necesito. En tus manos me entrego. ¡Enséñame el arte de los pequeños pasos!"

Antoine de Saint-Exupéry

A mi familia, siempre.

Tabla de Contenido

List of Abbreviations and Acronyms

AGID	Agar Gel immunoDiffusion
CD	Crohn´s Disease
CI	Confidence Intervals
CIE	Counter Immuno-Electrophoresis
CF	Complement Fixation
Ct	Cycle Threshold
DISC	Data and Information Services Center
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunoabsorbent Assay
e.g.	exempli gratia (for example)
EROS	Earth Resources Observation and Science
et al.	Et alii (and others)
FC	Fecal Culture
FLA	Free-Living Amoebae
g	gram
GES	Goddard Earth Sciences
GIS	Geographical Information System
GFP	Good Farming Practices
HEYM	Herrold´s Egg Yolk Medium
HPC	Hexadecylpyridinium Chloride
IAC	Internal Amplification Control
ICA	Instituto Colombiano Agropecuario
i.e.	id est (that is)
IF	Indirect Immuno-Fluorescence
IGAC	Geográfico Agustín Codazzi
IJT	Intradermal Johnin Test

INF-γ	Interferon gamma
INPE	Intitudo de Pesquisas Espaciales
IS	Insertion Sequence
JD	Johne´s Disease
L	Liter
LAF	Laboratório de Sensoriamento Remoto Aplicado à Agricultura e Floresta
LPDAAC	Land Processes Distributed Active Archive Center
M.	*Mycobacterium*
MAA	*Mycobacterium avium* subsp. *avium*
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
min	Minute
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats
ml	Milliliter
MLSSR	Multi-Locus Short Sequence Repeats
MODIS	Moderate Resolution Imaging Spectroradiometer
NDVI	Normalized Vegetation Index
NDWI	Normalized Water Differential Index
OIE	World Organization for Animal Health
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivate
PTB	Paratuberculosis
qPCR	Quantitative (real-time PCR)
Se	Sensitivity
SNP	Single Nucleotide Polymorphism
Sp	Specificity
subsp.	subspecies
TRMM	Tropical Rainfall Measuring Mission

U	Unit
µl	Microliter
µM	Micromolar
USGS	U.S. Geological Survey
%	Percentage
ZN	Ziehl Neelsen

General Summary

Introduction: Paratuberculosis is an economically important, chronic, and incurable disease in ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Since the effects of the disease represent a major source of losses for the milk-producing sector worldwide, it is important to define its impact and epidemiological dynamics both at regional and country-level. **Objective:** This cross-sectional study aimed to determine MAP herd-level prevalence, the herd level risk factors associated, the circulating genotypes, and to describe the spatial distribution and the environmental variables related to MAP in dairy herds of the Northern region of Antioquia, Colombia. **Methods:** Study herds (n = 386) located in 62 different districts from six municipalities were randomly selected amongst 7,794 dairies registered in the foot-and-mouth disease vaccination records from 2015. The sampling strategy considered proportional allocation, both at municipality and district-level. Participant herds were visited once between June and October 2016 to collect composite environmental samples and to complete a risk assessment questionnaire. The study herds were clasified in two groups, according to their productive system, those with under mechanical milking parlor and pasture grazing-based systems, and those under with in-paddock milking facilities. Each composite environmental sample contained material from six different sites of concentration of adult cattle and/or high traffic areas (e.g. areas surrounding waterers and feeders, areas surrounding the current mobile milking-unit places) and, depending on the production system, a second composite was collected from the perimeter of the manure storage lagoon (containing manure from the milking parlor). Identification of MAP was achieved using a duplex IS*900*-qPCR (Bactotype MAP PCR Kit®, Qiagen). A herd was considered as MAP infected if the environmental sample was positive in the qPCR. Information about the general characteristics of the herd, management practices, and knowledge about the disease was collected using the risk-assessment questionnaire. The information on risk factors was analyzed using a multivariable logistic regression model. Environmental samples from the 25 MAP-qPCR positive dairy herds were cultured by duplicate in Herrold´s egg yolk medium with mycobactin J to obtain isolates. Suspicious colonies were confirmed by MAP-qPCR. Positive DNA was sub-typed using mycobacterial interspersed

repetitive units-variable number of tandem repeat (MIRU-VNTR) and multilocus short sequence repeats (MLSSR) techniques to analyze the genetic difference(s) between the isolates. To describe the spatial distribution and the environmental variables related to MAP, rainfall trends, day and nighttime surface temperature, and vegetation cover index were taken as environmental references of the physical background of the study area. **Results:** We found a herd-level prevalence of 4.1% (12/292; 95% CI: 1.8-6.4) and "having a history of mixed farming of cattle with other ruminants (i.e. sheep, goats) in the last 2 years" as a risk factor for MAP infection (OR = 3.9; 95% CI: 1.2-13.2) in 292 dairies under mechanical milking parlor and pasture grazing-based systems. On the other hand, we found a prevalence of 14.9% (14/94; 95% CI: 7.7-22.1) and "having other than Holstein breeds were predominant" (namely, Jersey, Jersey×Holstein crossbreeds, and Jersey×Swedish red crossbreeds) as a risk factor in 94 dairies with in-paddock milking facilities (OR = 3.7; 95% CI: 1.1-15.2). Sub-typing revealed two different genotypes by MIRU-VNTR (INMV 2 and INMV 36). MLSSR was carried out to increase the discriminatory power from what was obtained by MIRU-VNTR, but no differences were observed among the isolates recovered. According to the spatial distribution and the environmental analysis, an overall high rainfall regime was found in the study area. The daytime and nighttime surface temperature showed important variations during sampling months. No evidence of management of the vegetation cover was found. **Conclusions:** Our study reported the MAP prevalence in dairy herds from a representative dairy region in the Province of Antioquia (Colombia) and the possible relationship between MAP environmental positivity with the history of mixed farming of cattle with other susceptible ruminants and with the predominant breed of cattle in the herd. MAP genotypes INMV 2 and INMV 36 circulate in the study region. According to the spatial distribution and the environmental analysis, our study referred to an exploratory, non-experimental observational study carried out on an uncontrolled tropical and a real dynamic environment. Our purpose was to describe the general conditions of the environmental context where the detection of positive herds is most likely to happen, considering the same (or a very approximate) sample collection and handling, and molecular detection method. In general, our study represents an important approach to the knowledge on MAP epidemiological status in the Colombian dairy population of study.

Resumen General

Introducción: La paratuberculosis es una enfermedad económicamente importante, crónica e incurable en rumiantes, causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Dado que los efectos de la enfermedad representan una fuente importante de pérdidas para el sector productor de leche en todo el mundo, es importante definir su impacto y la dinámica epidemiológica tanto a nivel regional como nacional. **Objetivo:** El presente estudio transversal tuvo como objetivo determinar la prevalencia de MAP a nivel de hato, los factores de riesgo asociados, los genotipos circulantes y describir la distribución espacial y las variables ambientales relacionadas con MAP en hatos lecheros de la región norte de Antioquia. Colombia. **Métodos:** Los hatos de estudio (n = 386), ubicados en 62 distritos diferentes de seis municipios fueron seleccionados al azar entre 7,794 lecherías registradas en los registros de vacunación contra la fiebre aftosa en 2015. La estrategia de muestreo consideró la asignación proporcional, tanto a nivel de municipio como de distrito. Los hatos participantes fueron visitados una vez entre junio y octubre de 2016 para recolectar muestras ambientales en *pool* y completar un cuestionario de evaluación de factores de riesgo. Los hatos de estudio se clasificaron en dos grupos, de acuerdo con su sistema productivo, aquellos con sistema de ordeño mecánico y sistema de pastoreo en potrero, y aquellos bajo sistema de ordeño en sala Cada muestra ambiental en *pool* contenía material de seis sitios diferentes de concentración de ganado adulto y/o áreas de alto tráfico (e.g. áreas que rodean los bebederos y comederos, áreas que rodean los lugares de las unidades de ordeño móviles) y, dependiendo del sistema de producción, un segundo *pool* se recolectó en el perímetro del tanque estercolero (que contenía estiércol de la sala de ordeño). La identificación de MAP se logró utilizando un PCR en tiempo real dúplex (IS*900*-qPCR; Bactotype MAP PCR Kit®, Qiagen). Un hato se consideró como infectado por MAP si la muestra ambiental era positiva a qPCR. La información sobre las características generales del hato, las prácticas de manejo y el conocimiento sobre la enfermedad se recopilaron mediante el cuestionario de evaluación de factores de riesgo. La información sobre los factores de riesgo se analizó mediante un modelo de regresión logística multivariable. Las muestras ambientales de los 25 hatos positivos por MAP-qPCR se cultivaron por duplicado en medio de Herrold con yema de huevo con micobactina J para obtener aislamientos. Las colonias

sospechosas fueron confirmadas por MAP-qPCR. El ADN positivo se subtipó utilizando un número variable de unidades repetitivas intercaladas de micobacterias de repetición en tándem (MIRU-VNTR) y repeticiones de secuencia corta multilocus (MLSSR) para analizar las diferencias genéticas entre los aislamientos. Para describir la distribución espacial y las variables ambientales relacionadas con MAP, las tendencias de precipitación, la temperatura de la superficie diurna y nocturna y el índice de cobertura de vegetación se tomaron como referencias ambientales del fondo físico del área de estudio. **Resultados:** Encontramos una prevalencia a nivel de hato de 4,1% (12/292; IC 95%: 1,8-6,4) y "tener un historial de cría mixta de ganado con otros rumiantes (i.e. ovejas, cabras) en los últimos 2 años" como factor de riesgo para la infección por MAP (OR = 3,9; IC 95%: 1,2-13,2) en 292 hatos bajo sistemas de ordeño mecánico y sistemas basados en pastoreo. Por otro lado, encontramos una prevalencia de 14,9% (14/94; IC 95%: 7,7-22,1) y "raza predominante diferente a Holstein en el hato" (i.e. Jersey, cruces de Jersey×Holstein y cruces de Jersey×Rojo sueco) como factor de riesgo en 94 hatos con instalaciones de ordeño en sala (OR = 3,7; IC del 95%: 1,1-15,2). El subtipado reveló dos genotipos diferentes por MIRU-VNTR (INMV 2 e INMV 36). MLSSR se llevó a cabo para aumentar el poder discriminatorio de lo que se obtuvo por MIRU-VNTR, pero no se observaron diferencias entre los aislamientos recuperados. De acuerdo con la distribución espacial y el análisis ambiental, se encontró un régimen general de alta precipitación en el área de estudio. La temperatura de la superficie diurna y nocturna mostró importantes variaciones durante los meses de muestreo. No se encontró evidencia de manejo de la cubierta vegetal. **Conclusiones:** Nuestro estudio reportó la prevalencia de MAP en hatos de una región lechera representativa en la Provincia de Antioquia (Colombia) y la posible relación entre la positividad ambiental a MAP con la historia de la cría de ganado bovino con otros rumiantes susceptibles y con la raza predominante del ganado en el hato. Los genotipos de MAP INMV 2 e INMV 36 circulan en la región de estudio. De acuerdo con la distribución espacial y el análisis ambiental, nuestro estudio se refirió a un estudio observacional no experimental, exploratorio, realizado en un entorno tropical no controlado y en un entorno real dinámico. Nuestro propósito fue describir las condiciones generales del contexto ambiental donde es más probable que ocurra la detección de hatos positivos, considerando la misma recolección y manejo de la muestra (o una muy aproximada) y método de detección molecular. En general, nuestro estudio representa un

enfoque importante del conocimiento sobre el estado epidemiológico del MAP en la población colombiana lechera de estudio.

General Introduction

Paratuberculosis (PTB) is a severe enteritis that affects cattle and other domestic and wild ruminants worldwide (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 (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 (Kaevska *et al.,* 2014; Salgado *et al.,* 2015).

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).

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-level, the risk factors assessment, the molecular diversity, and MAP-related spatial and environmental features are 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 also of major interest of the proposing line of research, because it investigates phenomena that relate animal and human health, using molecular and culture-based diagnostic tests, and epidemiology-related approaches as basis to achieve its goals on health improvement.

The present *Doctoral Thesis* includes a narrative Literature Review, exploring the foundations of the investigation of the disease and its causative agent in Colombia. Secondly, an Original Article (chapter 1) is included, aiming to determine the prevalence of MAP in dairy herds using

environmental sampling and real-time-PCR, and the herd-level risk factors associated in dairy herds with in-paddock milking facilities in the study population, and later, an Original Article (chapter 2) accomplishing to determine the prevalence the herd-level risk factors associated under a similar methodology, in dairy herds under mechanical milking parlor and pasture grazing-based systems in the study population is incorporated. In addition, an Original Article on the isolation of MAP by means of fecal culture from real-time PCR-positive samples (previously defined) and the genotypes isolated from them was included. And, finally, an Original Article describing the spatial distribution and the environmental variables related to MAP in the dairy herds of study was also incorporated.

References

Góngora OA, Villamil JC. La paratuberculosis bovina desde la óptica de la salud pública. Holstein Colomb 1999; 147:44-48.

Harris NB, Barletta RG. *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. Clin Microbiol Rev 2001; 14:489-512.

Kaevska M, Lvoncik S, Lamka J, Pavlik I, Slana I. Spread of *Mycobacterium avium* subsp. *paratuberculosis* through soil and grass on a mouflon (*Ovis aries*) pasture. Curr Microbiol 2014; 69(4):495-500.

Nielsen SS, Toft N. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev Vet Med 2009; 88:1-14.

Plata Guerrero R. La paratuberculosis bovina en Cundinamarca. Rev Med Vet 1931 (cited by Vega-Morales A, 1947).

Salgado M, Alfaro M, Salazar F, Badilla X, Troncoso E, Zambrano A, González M, Mitchell RM, Collins MT. Application of cattle slurry containing *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to grassland soil and its effect on the relationship between MAP and free-living amoeba. Vet Microbiol 2015; 175(1):26-34.

Sweeney RW. Transmission of paratuberculosis. Vet Clin North Am Food Anim Pract 1996; 12:305-312.

Vega Morales A. Relación entre el diagnóstico de la paratuberculosis bovina por el examen coprológico y de la prueba alérgica de termorreacción con la tuberculina aviaria por vía subcutánea [Thesis]. Bogotá, Colombia. UNAL; 1947.

Objectives

General Objective

To determine MAP herd-level prevalence, the herd level risk factors associated, the circulating genotypes, and to describe the spatial distribution and the environmental variables related to MAP in dairy herds of the Northern region of Antioquia, Colombia.

Specific Objectives

1. Determine the presence of MAP in dairy herds using environmental sampling and real-time-PCR.

2. Isolate MAP by means of fecal culture of real-time PCR-positive samples.

3. Genotype MAP isolates by means of PCR-based methods (MIRU-VNTR, MLSSR).

4. Determine herd-level risk factors for MAP real-time-PCR positivity using multivariate analysis.

5. Describe the spatial distribution and the environmental variables related to MAP using geostatistical analysis.

Literature Review

It was back in 1895 when Johne and Frottingham described the disease for the first time. By 1902 and 1908 the disease was reported almost worldwide. Exploring the basis of the investigation of the disease and its causative agent, we aimed to search for the history of paratuberculosis in Colombia, because it deserves the intensive investigation its importance demands, being this review the first approach (published in the Revista Colombiana de Ciencias Pecuarias, —RCCP, in September 2018; doi: 10.17533/udea.rccp.v31n3a01).

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Mycobacterium avium subsp. paratuberculosis in Colombia (1924-2016): A review

Mycobacterium avium subsp. paratuberculosis en Colombia (1924-2016): Revisión de literatura

Mycobacterium avium subsp. paratuberculosis na Colômbia (1924-2016): Revisão de literatura

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Abstract

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 potentially zoonotic agent due to its relation to Crohn's disease (CD) in humans is still under debate and investigation. The aim of the present systematic review is to summarize original studies on MAP carried out in Colombia since 1924, as well as to establish strengths, weaknesses, and future research opportunities in the country with emphasis on diagnosis and epidemiology. The initial search for existing publications reporting original studies on MAP, PTB, and the relationship between MAP and CD was carried out in the available databases and national libraries. After compilation of the available studies (n = 20), the relevant data was extracted (year, province of report, species studied, diagnostic tests used, study design, summary of results, and authors). Recommendations for future research opportunities on MAP in Colombia are made.

Keywords: *Buffalo, cattle, epidemiology, goats, Johne's disease, sheep.*

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, así como establecer 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 (n = 20), los datos relevantes fueron extraídos (año, provincia de reporte, especie estudiada, prueba diagnóstica usada, diseño del estudio, resumen de resultados y autores). Se hacen recomendaciones para futuras investigaciónes de MAP en Colombia.

Palabras clave: *búfalos, cabras, enfermedad de Johne, epidemiología, ganado bovino, ovejas.*

Resumo

Mycobacterium avium subsp. *paratuberculosis* (MAP) é um ácido forte, bacilo gram-positivo. O MAP é o agente causador da paratuberculosis (PTB) ou doença de Johne, uma doença infecciosa que afecta os ruminantes domésticos e algumas espécies selvagens. Sua importância como um agente zoonótico por causa de sua relação com a doença de Crohn (CD) em humanos ainda está em discussão e sob investigação. O objetivo desta revisão é apresentar a estudos mapa original realizado na Colômbia desde 1924, e estabelecer pontos fortes, pontos fracos e oportunidades para futuras pesquisas no país com ênfase no diagnóstico e epidemiológico pontos de vista. A busca inicial da literatura sobre estudos originais sobre MAP, PTB e a relação MAP e CD foi feita nas bases de dados disponíveis e bibliotecas nacionais. Após a compilação dos estudos disponíveis (n = 20), os dados relevantes foram extraídos (ano, relatório província, espécies estudadas, teste de diagnóstico utilizado, desenho do estudo, os resultados resumo e autores). Foram feitas recomendações sobre futuras oportunidades de pesquisa em MAP em Colômbia.

Palavras-chave: *búfalo, cabras, doença de Johne, epidemiologia, gado, ovelhas.*

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an obligate intracellular, gram-positive, acid-fast bacterium, causing a persistent infection of host macrophages leading to a strong immune response (Harris and Barletta, 2001). This bacterium has a remarkable tropism for the intestine, a characteristic not seen in any other mycobacterial species (Clarke, 1997), and can impact a wide variety of domestic and wild species (Stief *et al.,* 2012; Sweeney *et al,* 2012; Carta *et al.,* 2013; Kukanich *et al.,* 2013) and humans (Rani *et al.,* 2010; Rosenfeld and Bressler, 2010; Cossu *et al.,* 2011; Chiodini *et al.,* 2012). 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; Alexander *et al.,* 2009). 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 (Stevenson, 2010a).

Paratuberculosis (PTB) or Johne's disease (JD), a slow-developing and incurable infectious animal disease (caused by MAP) is characterized by chronic granulomatous enterocolitis. This disease has a variable incubation period from 6 months to over 15 years (Clarke, 1997). The PTB is transmitted between animals by a fecal-oral route, but intra-uterine and trans-mammary pathways occur (Sweeney, 1996; Lambeth *et al.,* 2004; Whittington and Windsor, 2009). Animals from 0 to 6 months of age are thought to be most susceptible (Windsor and Whittington, 2010). Experimental infection studies have demonstrated that goats are naturally less resistant to PTB compared to sheep and cattle (Stewart *et al.,* 2007). Chronic, progressive weight loss and chronic or intermittent diarrhea are the primary clinical signs of bovine PTB (Clarke, 1997). Symptoms are vague and unspecific in goats and sheep and, like many other diseases, are only characterized by weight loss (Djønne, 2010). Diarrhea is a common clinical sign in cattle but not in small ruminants (Clarke, 1997; Begg and Whittington, 2010; Robbe-Austerman, 2011). The clinical disease is most frequent among cattle 2-5 years old, although younger and older cattle (0-13 years old) can be affected (Nielsen and Toft, 2008). In other domesticated and wild ruminants, the course of infection and clinical disease are poorly described (Djønne, 2010).

In sheep, clinical signs are limited to weight loss, which can occur from 2 years of age and animals succumb to MAP infection from 3-5 years of age (Lugton, 2004; Begg and Whittington, 2010). In goats, the clinical development of the disease is similar to that in sheep (Djønne, 2010; Manning and Collins, 2010; Robbe-Austerman, 2011). Parturition, lactation, or other stresses may provoke clinical manifestations (Clarke, 1997; Fecteau and Whitlock, 2010).

Several tests are used to diagnose MAP infection in cattle, sheep, goats, and humans. The most common are histopathology (on intestinal tissue and regional lymph nodes using Ziehl Neelsen (ZN) stain, enzyme-linked immunoassay (ELISA; in serum and milk), microbiological (in tissues, feces, and environmental samples), and the detection of MAP DNA by polymerase chain reaction (PCR; in feces, milk, tissue, and blood). Less common tests include interferon gamma (IFN-γ) assay and intradermal Johnin test (IJT).

In all affected species, the necropsy findings are commonly restricted to the ileum and the mesenteric and ileocecal lymph nodes. In most cases, congestive and “wrinkled” surfaces of the ileum, cecum, and colon are observed (Manning and Collins, 2001; Olsen *et al.,* 2002).

The ELISA is the most widely used tests for detecting an antibody response to MAP infection. Several commercial ELISA kits for PTB diagnosis are currently available and multiple studies have compared their performance (Sonawane and Tripathi, 2013; Donat *et al.,* 2014; Nielsen and Toft, 2014; Lavers *et al.,* 2014; 2015). The main advantages of ELISAs are that they are inexpensive, rapid and easy to perform (1-2 hours) and provide quantitative results (Constanzo *et al.,* 2012). A major disadvantage of ELISA is its low sensitivity (Se) in subclinical animals (7-15%; Gilardoni *et al.,* 2012). It must be also considered that ELISA results should be interpreted given the different objectives for screening (*e.g.,* identification of infected animals, identification of the most likely to shed mycobacteria; OIE, 2011; Nielsen *et al.,* 2002; Nielsen and Toft, 2012).

Cultivation of MAP from feces and tissues is the most reliable method of detecting infected animals (Nielsen and Toft, 2009; Fecteau and Whitlock, 2010). Usually, its Se is 30-70% and its specificity (Sp) 98% if the isolates obtained are confirmed to be MAP by molecular methods such

as PCR (Whittington *et al.,* 2011; Gilardoni *et al.,* 2012). Although fecal culture (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 the most commonly used reference test for the detection of MAP (Whittington, 2010).

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, 2010b), and it has been recently discussed as a possible new reference test for PTB (Stevenson, 2010b). The PCR´s Se is 70-97% and its Sp 95% (Gilardoni *et al.,* 2012) and, in contrast to a culture-based diagnostic, it is rapid and no additional tests are required to confirm the identity of the organism detected (Collins, 1996).

The PPD antigens used in the *in vivo* IFN-γ test and in the IJT are crudely steam-sterilized mycobacterial culture extracts containing many cross-reacting antigens with other related bacteria (Stabel and Whitlock, 2001). Unapparent infected 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 antigens and/or increased antibody response to MAP (Nielsen, 2010; Gilardoni *et al.,* 2012). The IFN-γ (*in vivo*/*in vitro*) Se is around 41% and its Sp 10% (Gilardoni *et al.,* 2012).

The lack of a perfect *ante-mortem* reference test is a significant obstacle for PTB diagnostic test evaluation (McKenna *et al.,* 2006). Complicating diagnostic test evaluations is the high degree of variability among animals in their response to MAP infections; some may produce antibodies (ELISA positive) years before consistently shedding MAP in feces (culture or PCR-positive), while others will be fecal shedders of MAP long before becoming ELISA-positive (Kalis *et al.,* 2002; McKenna *et al.,* 2006; Nielsen, 2010). Current diagnostic tests cannot, with a single application, discriminate between MAP-infected and uninfected animals at any age with 100% accuracy, highlighting the need for improved tests (Nielsen and Toft, 2009).

Dalziel (1913) described the clinical and pathologic similarities between PTB in cattle and Crohn´s disease (CD) in humans, which are 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 (Uzoigwe *et al.,* 2007; Sechi and Dow, 2015). In agreement with this, MAP has been detected in the tissues of CD patients (Di Sabatino *et al.,* 2011; Tuci *et al.,* 2011; Wagner *et al.,* 2013; Dalton *et al.,* 2014). The source, the route of infection, the persistence mechanisms, and the consequences of MAP infection in humans are unknown (Uzoigwe *et al.,* 2007; Lowe *et al.,* 2008). Therefore, an association between CD and PTB has been shown, but a causal relationship remains to be demonstrated (Liverani *et al.,* 2014; Sechi and Dow, 2015).

The MAP-infected animals, whether clinically normal or showing signs of disease, can shed live bacteria in both feces and milk. If these animals are farmed for food production, the safety of foods derived from them becomes important because of its impact on public health (Sweeney *et al.,* 2012; Atreya *et al.,* 2014; Liverani *et al.,* 2014).

Both MAP infections and clinical cases of JD have been reported from all continents that have ruminant populations in any degree of husbandry (Barkema *et al.,* 2010), and countries acquire the infection by animal importation. Multiple studies on the determination of the within-herd and between herd prevalence of MAP infections around the world have been carried out (Nielsen and Toft, 2009; Salem *et al.,* 2013; Fernández-Silva *et al.,* 2014). 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 are reported as risk factors worldwide (Lepper *et al.,* 1989; Johnson-Ifearulundu and Kaneene, 1998; Wells and Wagner, 2000; Dieguez *et al.,* 2008; Ansari-Lari *et al.,* 2009; Tiwari *et al.,* 2009; Pithua *et al.,* 2013; Benavides *et al.,* 2016; Correa-Valencia *et al.,* 2016).

The apparent prevalence among cattle appears to be at least 0 and 24% in several European countries (Nielsen and Toft, 2009). Between-herd prevalence estimates appear to be >50% (Clarke, 1997; Nielsen and Toft, 2009). Wells and Wagner (2000) reported an apparent 3.4 and 21.6% at the animal and herdlevel, respectively, using ELISA to test US dairy cattle herds in

1996. According to Manning and Collins (2010) and Nielsen and Toft (2009), over 60% of dairy cattle herds in Europe and >50% in North America are infected, respectively. According to Fernández-Silva *et al.* (2014), 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 (Attili *et al.,* 2011), 46.7% in sheep in Portugal (Coelho *et al.,* 2007), and 52% in sheep and 50% in goats in Cyprus (Liapi *et al.,* 2011). The prevalence of infection tends to increase in countries that do not have control programs (Salem *et al.,* 2013; Fernández-Silva *et al.,* 2014).

The JD causes important economic losses in infected flocks and herds (Nielsen and Toft, 2009) and produces a 6-19% decrease in the production of meat, milk, or both (Djønne, 2010; Kostoulas *et al.,* 2006; Marce *et al.,* 2009). Ovine and caprine PTB causes losses related to death, early culling, and reduced milk production (Arsenault, 2001). Control of PTB in farm ruminants by testing, culling, and herd/flock management helps limiting the economic impact of the disease and are used in control programs in the USA, Australia, and Europe (Bakker, 2010; Kennedy and Citter, 2010; Whitlock, 2010; Khol and Baumgartner, 2012). The lack of a fully functional immune system renders neonatal ruminants more susceptible to MAP infection than adult animals. Thus, control programs are primarily focused on limiting opportunities for MAP contamination of colostrum, milk, water and feed by hygienic programs (Tiwari *et al.,* 2009; Whitlock, 2010; Khol and Baumgartner, 2012).

The aim of the present review was to summarize original studies and abstracts on MAP carried out in Colombia since 1924, as well as to highlight the strengths, weaknesses, and future research opportunities for PTB research in the country with emphasis on diagnostic and epidemiology.

Materials and methods

The review of MAP/PTB original 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. Searching was done using electronic databases (*i.e.,* Scielo, Medline/Pubmed, and Virtual Health Library), national libraries, institutional repositories, and the Internet. Because the aim of the review was to summarize only original studies, publications not considered original by the three authors of this report were excluded through consensus and not further analyzed. The main characteristics (year of publication, province of report, species, diagnostic test used, study design, and results) of selected MAP original studies were extracted and analyzed.

Results

The review process produced 20 original studies and abstracts on MAP carried out in Colombia (Table 1). These studies refer to PTB and MAP detection. No studies in Colombia attempted detection of MAP in food or humans. One publication by Albornoz (1949) comparing bovine PTB with human leprosy was not available. Its significance as an original study could not be evaluated, therefore it was not considered in this review.

Thirteen publications not considered original studies were not further analyzed or discussed in this review, but they are of great value for the national knowledge base about MAP. These reviews, case reports, case series reports, and editorials demonstrate the national academic concern about MAP and its impacts in Colombia (García, 1957; Góngora and Villamil, 1999; Calderón and Góngora, 2008; Zapata *et al.,* 2008; Villalobos *et al.,* 2008; Anonymous, 2010a; de Waard, 2010; Peña *et al.,* 2011; Ramírez *et al,* 2011; Ramírez and Maldonado, 2013a; 2013b; Fernández *et al.,* 2014; Correa *et al.,* 2015).

The existence of MAP in Colombia was first documented in 1924 by the Cuban veterinarian Ildefonso Pérez Vigueras in cattle with PTB (reported by Plata, 1931 according to Vega, 1947).

This documentation was the first confirmation of PTB in the country and occurred in the municipality (primary political division of provinces in Colombia) of Usme (Cundinamarca) in imported cattle. Most studies on MAP or PTB (60%, 12/20) were carried out during the present decade (2010-2020). No more than two studies on MAP or PTB in Colombia were published in previous decades. Most studies were carried out in the provinces of Antioquia (60%; 12/2) and Cundinamarca (30%; 6/20), Caldas and Tolima (5%; 1/20), as well as in Nariño (5%; 1/20). The original studies concerning MAP in Colombia reported the results from cattle, sheep, goats, and buffaloes. Studies on cattle were the most common (80%; 16/20) compared to sheep and goats (15%; 3/20), and buffaloes (5%; 1/20). Other relevant species in the country (wild mammals or humans) were not found or cited in any original study reviewed.

The most common diagnostic test used to investigate MAP in Colombia is ELISA (36.1%; 13/36), followed by microscopy on ZN-stained samples (on feces, rectal mucosa scrapings, or tissues; 19.4%; 7/36), PCR (13.9%; 5/36), IJT (with bovine and/or avian-PPD; 11.1%; 5/36), culture (from feces or tissues, and individual or pooled; 8.3%; 2/36), CF (complement fixation; 5.6%; 2/36), IF (indirect immuno-fluorescence; 2.8%; 1/36), and CIE (counter immuno-electrophoresis; 2.8%; 1/36).

The studies reviewed included cross-sectional, diagnostic test comparisons, risk factor analyses, and clinical trials (on treatments). Thus far, no cohort or case and control studies have been published in Colombia.

Discussion

This review summarizes for the first time the original studies on MAP carried out in Colombia since 1924. In recent years, MAP presence and distribution in the country, especially in farmed animals and humans, have been reviewed (Góngora and Villamil, 1999; Calderón and Góngora, 2008; Zapata *et al.,* 2008; Fernández *et al.,* 2014). However, no review of the original studies has been undertaken. According to several anecdotal reports, opinions about the national -not

even regional- distribution of MAP or 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 number of publications reporting original studies on MAP, especially PTB, in recent years is relatively low compared to other countries in Latin America (Fernández *et al.,* 2014), 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) from academic and productive perspectives.

Although PTB is a notifiable disease in Colombia (ICA, 2015), it is not of major concern to animal health authorities and its control is a responsibility of the farmer (Anonymous, 2010a; Fedegán, 2010; Fernández *et al.,* 2014). 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 countrywide PTB review has been published (Yamasaki *et al.,* 2013). According to this review, 35 studies have been carried out in Brazil since its first report in 1915. These studies were carried out in cattle, sheep, goats, and buffaloes and using the same diagnostic tests that have been used in Colombia according to the present report.

The locations of most studies do not follow a clear trend, but could be related to the high concentrations of cattle in some of the provinces (*i.e.,* Antioquia and Cundinamarca; ICA, 2016), or to the interests of academics, scientists, or cattle producers. Since the first report in 1924, Cundinamarca has been a province with common reports of PTB (Vega, 1947; Huber, 1954; Isaza, 1978; Mogollón et al., 1983; Góngora and Perea, 1984; Mancipe et al., 2009). This could be explained by the long tradition of the Facultad de Medicina Veterinaria of the Universidad Nacional de Colombia in Bogotá, the oldest veterinary school in the country, where the first studies in the early 20th century were carried out, most of them being degree works. More recently, Antioquia province has been publishing the majority of original studies, all of them from academics at Universidad de Antioquia and Universidad CES. As expected, studies on cattle

were the most common, most likely due to the size of the population in the country and to the production systems related to milk and meat. In contrast, studies on sheep are less common in the country probably due to its smaller population (ICA, 2016).

The common use of ELISA, ZN-staining, IJT using bovine and/or avian-PPD is not surprising given their relatively low cost and availability of materials, qualified personnel, and infrastructure for these tests (Collins, 1996). However, the use of culture and PCR is becoming more common and could be related to the recent development of diagnostic capacities in universities compared to national laboratories, 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 common in animal health research in Colombia. These high-profile observational studies, as well as experimental approaches, are more complex, laborious, demanding and expensive, given the microbiological and pathophysiological characteristics of MAP. 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.

Definitively, Colombia needs to cover some knowledge gaps to get to a true understanding of the disease. It is necessary to define the exact status of the disease through well-designed prevalence/incidence studies, considering that no whole national data is available. In this regard, just some local estimates are currently available (Patiño and Estrada, 1999; Ramírez *et al.,* 2001; Fernández *et al.,* 2011a; 2011b; Benavides *et al.,* 2016; Correa *et al.,* 2016). Harmonization of diagnostic methods, considering the epidemiologic and biological behavior of MAP under local agro-ecological, productive, and cultural conditions are also needed. In addition, 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 (Calderón and Góngora, 2008), improving their testing capacity and the access to diagnostic reagents.

Table 1. Summary of published original studies on *Mycobacterium avium* subsp. *paratuberculosis* in Colombia, 1924-2016.

Year of publication	Province of report	Species	Diagnostic test	Study design	Summary of results	Reference
1947	Cundinamarca	Bovine	IJT- avian PPD; ZN	30 animals with different ZN-fecal staining results (6 negative, 8 suspicious, and 16 positive) were inoculated with PPD. Body temperature was taken three times before PPD inoculation. Next day results were determined and temperature was measured every 2 h	6 animals negative to the ZN were also negative by IJT; 12.5% (1/8) of the animals were suspicious and 37.5% (6/16) were positive by ZN and by IJT, respectively	Vega
1954	Cundinamarca	Bovine	IJT - avian PPD; ZN	9 medical cases were reviewed looking for PTB. Administration of isonicotimilhidrazina (orally) and cortisone (intramuscular) in the treatment of AFB-related diseases was performed	AFB were confirmed in all the animals which were also negative to IJT; body temperature and weight after treatment improved in 40% (4/9) of the cases	Huber
1978	Cundinamarca	Bovine	ZN; CF; IF	2 groups of adult cattle (>2 years of age) were sampled for serum and feces. Sixty-seven Holstein and Normando clinically normal animals, and 65 animals clinically compatible with PTB were tested twice with 6 months of difference	3.51% (7/199) were positive to ZN; 2.02% (4/199) serums were positive to CF; 5.52% (11/199) serums reacted positively to IF	Isaza
1983	Cundinamarca	Ovine	CF; ZN	Blood and fecal samples were taken from 480 adult sheep	11.25% (54/480) of the serums were positive to CF; 5.62 % (27/480) of the fecal samples were positive to ZN. A necropsy was performed and PTB was confirmed	Mogollón *et al.*
1984	Cundinamarca	Bovine	ZN and HE-staining; CIE	94 older than 3 years Holstein, Normando, and cross-breed cows and bulls were sampled. The groups were designated according to presence of diarrhea compatible with 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) of the animals were positive to ZN and HE tissue staining; 6.38% (6/94) of the animals were positive to ZN in rectal mucosa scrapings; 9.57% (9/94) were positive to CIE in serum samples	Góngora and Perea
1999	Caldas and Tolima	Bovine	ELISA	177 Normando animals from 3 farms were serum sampled	Seroprevalence for each farm was 3.4% (2/59), 1.7% (1/59), and, 0% (0/59)	Patiño and Estrada
2001	Antioquia	Bovine	IJT - avian and bovine PPD	3 farms from 3 municipalities were sampled: San Pedro de los Milagros (n = 77), Gómez Plata (n = 76), and Barbosa (n = 78). The study population was 176 animals over 1 year of age	11% (19/176) of animals were positive to bovine PPD, all of them from San Pedro de los Milagros, while 27.8% (49/176) were suspicious; 2% (1/49) of suspicious was positive to avian PPD, and 2% (1/49) was suspicious; four clinical animals were confirmed by necropsy	Ramírez *et al.*

Year of publication	Province of report	Species	Diagnostic test	Study design	Summary of results	Reference
2009	Cundinamarca	Ovine	ZN; IJT- bovine PPD; ELISA	250 female sheep (Black face, Cheviot, Corriedale, Hampshire, Merino rambouillet, Romney marsh, Mora, creole, and cross-breeds) aged between 1-9 years old were sampled	4% (10/250) of fecal samples were positive to ZN; animals between 2-6 years old presented AFB in fecal samples, whereas animals older than 8 years were all suspicious; 4.9% (16/250) were positive to the IJT, and 1.1% (3/250) were suspicious; 0.8% (2/250) were positive to ELISA. Animals that resulted suspicious and positive to intradermal test were confirmed by ZN in fecal samples: 62.5% (10/16) were positive to both (ZN and IJT), 18.8% (3/16) were negative to both, and other 18.8% (3/16) were positive to the IJT only	Mancipe *et al.*
2010	Antioquia	Bovine	ZN; FC; IS*900* q-PCR	15 Holstein and BON x Holstein cows in a herd enzootic for PTB were sampled. Average age of sampled cows was 6.7 years. Fecal samples were individually taken from clinical healthy cows and cows with diarrhea	56% (9/15) were positive to ZN to FC, whereas 20% (3/15) that were positive by PCR applied to positive FC	Zapata *et al.*
2011a	Antioquia	Bovine	Non-absorbed indirect ELISA (A); Pre-absorbed indirect ELISA (B); IS*900* nested-PCR; F5/ISMav2 q-PCR; FC	14 dairy herds of 9 districts were fecal and serum sampled. Only 1 herd had presented sporadic clinical cases compatible with PTB confirmed by PCR and histopathology	10% (31/315), 87% (268/315), and 2.6% (8/315) of samples were positive, negative, and doubtful, respectively, to ELISA A; 70% (10/14) of 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% (6/31) positive animals with ELISA A were positive to nested-PCR. One positive animal to q-PCR were also positive to nested-PCR; 19 and 6.5% of the ELISA A-positive animals were positive to nested PCR and q-PCR, respectively. The FC was negative in all samples	Fernández *et al.*
2011b	Antioquia	Bovine	Pre-absorbed indirect ELISA (C); FC; F57/ ISMav2 PCR; IS*900* nested-PCR	5 herds previously tested by the authors, referring to those that resulted ELISA and PCR positive but FC negative for MAP, and one additional herd not previously tested were included in the study. The herds participated with 384 cows (>2 years of age). 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	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; ELISA C results were confirmed by FC in only one symptomatic animal of one herd; eight MAP isolates were recovered	Fernández *et al.*
2013	Antioquia	Bovine	IS*900* q-PCR	48 cows with compatible signs of PTB were euthanized. Lymph nodes were cultured and DNA from macrophages was extracted	Macrophages from four cows were infected by MAP; 8.51% of the cultures were positive and confirmed by q-PCR	Del Río *et al.*

Year of publication	Province of report	Species	Diagnostic test	Study design	Summary of results	Reference
2015	Antioquia	Ovine and caprine	Pre-absorbed indirect ELISA	Blood samples from all animals in a farm in Barbosa (>2 years of age) (n = 53 goats and 6 sheep) were obtained	ELISA results were all negative	Hernández *et al.*
2015	Antioquia	Bovine	Pre-absorbed indirect ELISA	Blood samples from all animals in a farm at San Pedro de los Milagros, (>2 years of age) (n = 83) were obtained	17% (14/83) of animals were ELISA positive; statistical association between ELISA results and breed was found	Jaramillo *et al.*
2015a	Antioquia	Bovine	Pre-absorbed indirect ELISA	Blood samples from all animals in a farm in Gómez Plata, (>2 years of age) (n = 50) were obtained	4% (2/50) of the animals were ELISA positive	Tuberquia *et al.*
2015b	Antioquia	Buffalo	Pre-absorbed indirect ELISA	Blood samples from all animals in a farm in Gómez Plata, (>2 years of age) (n = 21) were obtained	ELISA results were all negative	Tuberquia *et al.*
2016	Nariño	Bovine	Pre-absorbed indirect ELISA	Blood samples were obtained from 958 cows (>2 years of age) in 16 dairy farms	94% (15/16) of the farms with at least one positive animal and 8% (77/958) of the cows were ELISA positive; statistical association between ELISA results and BCS was found	Benavides *et al.*
2016	Antioquia	Bovine	Pre-absorbed indirect ELISA	Risk factors assessment, related to seropositive results from screening 696 randomly selected bovines in 28 dairy herds located in 12 districts of San Pedro de los Milagros was done	3.6% (1/28) and 2% (14/696) of the herds and animals were ELISA positive, respectively. 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, were associated with MAP seropositivity with OR of 4.42, 3.45, 2.53, and 20.38, respectively	Correa *et al.*
2016	Antioquia	Bovine	Pre-absorbed indirect ELISA	Blood samples from all animals in a farm in the municipality of Caucasia (>2 years of age) (n = 151) were obtained	33.8% (51/151) of the animals were ELISA positive	Vélez *et al.*
2016	Antioquia	Bovine	Pre-absorbed indirect ELISA	Risk factors assessment was performed. Blood samples were obtained from 19-25 cows, (>2 years of age) randomly selected in 14 dairy herds located in 9 districts of Belmira and San Pedro de los Milagros	10.09% (31/307) of the animals and 70% (10/14) of the herds were ELISA positive; OR for PTB seropositivity increased 20% in cows with >1 parity; OR was 0.74 times lower in herds feeding calves with pooled colostrum from several cows, compared to herds feeding calves with colostrum from their own dams	Fernández *et al.**

IJT: Intradermal Johnin test; PPD: Purified protein derivate; ZN: Ziehl-Neelsen; PTB: Paratuberculosis; AFB: Acid fast bacteria; CF: Complement fixation; IF: Indirect immuno-fluorescence; HE: Hematoxylin and eosin; CIE: Counter immuno-electrophoresis; ELISA: Enzyme-linked immunosorbent assay; FC: Fecal culture; BON: Blanco orejinegro; PCR: Polymerase chain reaction; q-PCR: Quantitative, real-time polymerase chain reaction; ELISA (A): Svanovir Para-TB Ab ELISA Kit (Svanova Biotech AB, Uppsala, Sweden); ELISA (B): ELISA paratuberculosis antibody verification (Institut Pourquier, Montpellier, France); ELISA (C): ID Screen Paratuberculosis Indirect (IDVET, Montpellier, France); MAP: *Mycobacterium avium* subsp. *paratuberculosis*; BCS: Body condition score; OR: Odds ratios. *Refers to an analysis of the information from the study done in 2011a by Fernandez *et al.*

It is also necessary to improve farmer-training highlighting the importance of disease control, not only for PTB, but also other diseases that cause economic losses and are considered of sanitary risk. Only one previous study reported molecular characterization of strains isolated in Colombia (Fernández *et al.,* 2011b), being this insufficient to consider the definition of “indigenous strains” and the ulterior design of vaccines. It would be necessary to conduct studies on wider regions, considering infectionassessment on cattle and other-than-cattle susceptible populations (even local wildlife) to generate prophylactic strategies according to Colombian MAP molecular and epidemiological diversity.

The relationship between MAP and CD has been not discussed in the country, except for some sporadic reviews (Góngora and Villamil, 1999; Calderón and Góngora, 2008; de Waard, 2010). 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 of MAP zoonotic potential has not been proven, but should not be ignored (Patel and Shah, 2011). The CD has been known in Colombia since the 1950s and the incidence and prevalence rates are increasing (estimated point prevalence of 77,000 CD cases), but no national consolidated information about the disease is available (Calderón and Góngora, 2008). According to some researchers, efforts should be made to correlate these two diseases in areas with high prevalence or incidence of both.

In general, progress has been made on MAP diagnosis and epidemiology as reported in the studies covered in this review. However, unanswered questions remain, offering many research opportunities.

Conclusion

In Colombia 20 original studies about MAP have been carried out so far in four different animal species, mainly using ELISA, and predominantly in Antioquia and Cundinamarca provinces. 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, limits the evidence about the magnitude of MAP circulation in animals, humans, the environment, and food in Colombia.

It is imperative to improve the laboratory diagnostic capabilities 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. Determination of at least regional prevalence 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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Conflicts of interest

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

References

Alexander DC, Turenne CY, Behr MA. Insertion and deletion events that define the pathogen *Mycobacterium avium* subsp.*paratuberculosis*. J Bacteriol 2009; 191(3):1018-1025.

Anonymous. Situación en Colombia de enfermedades bovinas no sujetas al control oficial. Fedegan. Primera edición. Bogotá, Colombia: Sanmartín Obregón & Cia; 2010a. 126 p.

Anonymous. Uniform program standards for the voluntary bovine Johne´s disease control program. In: United States Department of Agriculture-USDA, Animal and Plant Health Inspection Service-APHIS; 2010b. 40 p.

Ansari-Lari M, Haghkhah M, Bahramy A, Novin Baheran AM. Risk factors for *Mycobacterium avium* subspecies *paratuberculosis* in Fars province (Southern Iran) dairy herds. Trop Anim Health Prod 2009; 41(4):553-557.

Arsenault J. Prévalence et impact du maedi–visna, de la lymphadénite caséeuse et de la paratuberculose chez les ovins du Québec. [Thesis]. Québec, Canada. Université de Montréal; 2001.

Atreya R, Bülte M, Gerlach GF, Goethe R, Hornef MW, Köhler H, Meens J, Möbius P, Roeb E, Weiss S. Facts, myths, and hypotheses on the zoonotic nature of *Mycobacterium avium* subspecies *paratuberculosis*. Int J Med Microbiol 2014; 304(7):858-867.

Attili AR, Ngu-Ngwa V, Preziuso S, Pacifici L, Domesi A, Cuteri V. Ovine paratuberculosis: A seroprevalence study in dairy flocks reared in the Marche Region, Italy. Vet Med Int 2011:782875.

Bakker D. Paratuberculosis control measures in Europe. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 306-315.

Barkema HW, Hesselink JW, McKenna SL, Benedictus G, Groenendaal H. Global prevalence and economics of infection with *Mycobacterium avium* subsp. *paratuberculosis* in ruminants. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 10-7.

Begg D, Whittington R. Paratuberculosis in sheep. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 157-164.

Benavides B, Arteaga A, Montezuma CA. Estudio epidemiológico de paratuberculosis bovina en hatos lecheros del sur de Nariño, Colombia. Rev Med Vet 2016; 31:57-66.

Calderón J, Góngora A. Similaridades clinopatológicas entre paratuberculosis y enfermedad de Crohn ¿posible vínculo zoonótico? Rev MVZ Córdoba 2008; 13(1):1226-1239.

Carta T, Álvarez J, Pérez de la Lastra JM, Gortázar C. Wildlife and paratuberculosis: A review. Res Vet Sci 2013; 94(2):191-197.

Chiodini RJ, Chamberlin WM, Sarosiek J, McCallum RW. Crohn's disease and the mycobacterioses: A quarter century later. Causation or simple association? Crit Rev Microbiol 2012; 38(1):52-93.

Clarke CJ. The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Path 1997; 116(3):217-261.

Coelho AC, Pinto ML, Silva S, Coelho AM, Rodrigues J, Juste RA. Seroprevalence of ovine paratuberculosis infection in the Northeast of Portugal. Small Rumin Res 2007; 71(1-3):298-303.

Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. J Am Vet Med Assoc 2006; 229(12):1912-1929.

Collins MT. Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 1996; 12(2):357-371.

Correa NM, Ramírez NF, Olivera M, Fernández JA. 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. Trop Anim Health Prod 2016; 48(6):1191-1200.

Correa NM, Ramírez NF, Fernández JA. Diagnóstico de la paratuberculosis bovina: Revisión. Rev ACOVEZ 2015; 44(1):12-16.

Cossu A, Rosu V, Paccagnini D, Cossu D, Pacifico A, Sechi LA. MAP3738c and MptD are specific tags of *Mycobacterium avium* subsp. *paratuberculosis* infection in type I diabetes mellitus. Clin Immunol 2011; 141(1):49-57.

Costanzo G, Pinedo FA, Mon ML, Viale M, Gil A, Illia MC, Gioffré A, Arese A, Travería G, Romano MI. Accuracy assessment and screening of a dairy herd with paratuberculosis by three different ELISAs. Vet Microbiol 2012; 156(1-2):183-188.

Dalton JP, Desmond A, Shanahan F, Hill C. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in patients with Crohn's disease is unrelated to the presence of single nucleotide polymorphisms rs2241880 (ATG16L1) and rs10045431 (IL12B). Med Microbiol Immunol 2014; 203(3):195-205.

Dalziel TK. Chronic interstitial enteritis. British Med J 1913; 2(2756):1068-1070.

de Waard JH. ¿Ordeñando micobacterias del ganado? Impacto económico y en salud de tuberculosis bovina y paratuberculosis en Colombia. Rev MVZ Córdoba 2010; 15(2):2037-2040.

Del Río D, Jaramillo L, Ramírez R, Maldonado JG. Amplificación del genoma de *Mycobacterium avium* subespecie *paratuberculosis* mediante qPCR a partir de tejido linfoide de bovinos con cuadros clínicos compatibles con enfermedad de Johne. Rev Colomb Cienc Pecu 2013; 26(Sup):408.

Di Sabatino A, Paccagnini D, Vidali F, Rosu V, Biancheri P, Cossu A, Zanetti S, Corazza GR, Sechi LA. Detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-specific IS900 DNA and antibodies against MAP peptides and lysate in the blood of Crohn's disease patients. Inflamm Bowel Dis 2011; 17(5):1254-1255.

Dieguez FJ, Arnaiz I, Sanjuán ML, Vilar MJ, Yus E. Management practices associated with *Mycobacterium avium* subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. Vet Rec 2008; 162(19):614-617.

Djønne B. Paratuberculosis in goats. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 169-178.

Donat K, Schlotter K, Erhardt G, Brandt HR. Prevalence of paratuberculosis in cattle and control measures within the herd influence the performance of ELISA tests. Vet Rec 2014; 174(5):119.

Fecteau ME, Whitlock RH. Paratuberculosis in cattle. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 144-153.

Federación colombiana de ganaderos (Fedegán). Situación en Colombia de enfermedades bovinas no sujetas al control oficial. Primera edición. Bogotá, Colombia: 2010. 118 p.

Fernández JA, Abdulmawjood A, Akineden O, Bulte M. Serological and molecular detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle of dairy herds in Colombia. Trop Anim Health Prod 2011a; 43(8):1501-1507.

Fernández JA, Abdulmawjood A, Bulte M. Diagnosis and molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* from dairy cows in Colombia. Vet Med Int 2011b; 352561.

Fernández JA, Correa NM, Ramírez N. Systematic review of the prevalence of paratuberculosis in cattle, sheep, and goats in Latin America and the Caribbean. Trop Anim Health Prod 2014; 46(8):1321-1340.

Fernández JA, Ramírez N, Correa NM. Factors associated with *Mycobacterium avium* subsp. *paratuberculosis* in dairy cows from Northern Antioquia, Colombia. Rev Colomb Cienc Pecu 2017; 30(1):48-59.

García A. Comprobaciones de la trichomoniasis bovina y contribución al estudio de la paratuberculosis en el departamento de Nariño. [Tesis]. Bogotá, Colombia. UNAL; 1957.

Gilardoni LR, Paolicchi FA, Mundo SL. Bovine paratuberculosis: A review of the advantages and disadvantages of different diagnostic tests. Rev Argent Microbiol 2012; 44(3):201-215.

Góngora OA, Perea J. Evaluación de tres métodos diagnósticos en paratuberculosis bovina. [Tesis]. Bogotá, Colombia. UNAL; 1984.

Góngora OA, Villamil JC. La paratuberculosis bovina desde la óptica de la salud pública. Holstein Colomb 1999; 147:44-48.

Harris NB, Barletta RG. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin Microbiol Rev 2001; 14:489-512.

Hernández JM, García YM, Fernández J. Seroprevalencia de *Mycobacterium avium* subsp. *paratuberculosis* (MAP) em caprinos y ovinos de un aprisco de bosque húmedo premontano del departamento de Antioquia. Rev Colomb Cienc Pecu 2015; 28(Sup):103.

Huber G. La administración de la Isonicotimilhidrazina de cortisona en la paratuberculosis bovina (enfermedad de Johne). UNAL. 1954.

Instituto Colombiano Agropecuario (ICA). Consolidado nacional bovinos 2016-Poblacion y Predios. In: Censo Pecuario Nacional, 2016. [Access date: August 20th, 2016]. URL: http://www.ica.gov.co/getdoc/8232c0e5-be97-42bd-b07b-9cdbfb07fcac/Censos-2008.aspx

Instituto Colombiano Agropecuario (ICA). Resolución 3714 de 2015. [Access date: December 20th, 2016]. URL: http://www.ica.gov.co/getattachment/3188abb6-2297-44e2-89e6-3a5dbd4db210/2015R3714.aspx

Isaza PF. Diagnóstico de paratuberculosis en bovinos por los métodos de baciloscopia, fijación de complemento e inmunofluorescencia. UNAL; 1978.

Jaramillo S, Uribe JS, Montoya MA, Ramírez NA, Fernández J. Seroprevalencia de *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) y exploración de factores asociados al estatus serológico en un hato de lechería especializada en el altiplano norte de Antioquia, Colombia. Rev Colomb Cienc Pecu 2015; 28(Sup):104.

Johnson-Ifearulundu YJ, Kaneene JB. Management-related risk factors for *M. paratuberculosis* infection in Michigan, USA, dairy herds. Prev Vet Med 1998; 37(1-4):41-54.

Kalis CHJ, Barkema HW, Hesselink JW, van Maanen C, Collins MT. Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding *Mycobacterium avium* subspecies *paratuberculosis*. J Vet Diagn Invest 2002; 14(3):219-224.

Kennedy D, Citter L. Paratuberculosis control measures in Australia. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 330-341.

Khol JL, Baumgartner W. Examples and suggestions for the control of paratuberculosis in European cattle. Jpn J Vet Res 2012; 60(Sup):1-7.

Kostoulas P, Leontides L, Billinis C. The association of subclinical paratuberculosis with the fertility of Greek dairy ewes and goats varies whit parity. Prev Vet Med 2006; 74:226-238.

Kukanich KS, Vinasco J, Scott HM. Detection of *Mycobacterium avium* subspecies *paratuberculosis* from intestinal and nodal tissue of dogs and cats. ISRN Vet Sci 2013:1-4.

Lambeth C, Reddacliff LA, Windsor P, Abbott KA, McGregor H, Whittington RJ. Intrauterine and transmammary transmission of *Mycobacterium avium* subsp *paratuberculosis* in sheep. Aust Vet J 2004; 82(8):504-508.

Lavers CJ, Barkema HW, Dohoo IR, McKenna SL, Keefe GP. Evaluation of milk ELISA for detection of *Mycobacterium avium* subspecies *paratuberculosis* in dairy herds and association with within-herd prevalence. J Dairy Sci 2014; 97(1):299-309.

Lavers CJ, Dohoo IR, McKenna SL, Keefe GP. Sensitivity and specificity of repeated test results from a commercial milk enzyme-linked immunosorbent assay for detection of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. J Am Vet Med Assoc 2015; 246(2):236-244.

Lepper AW, Wilks CR, Kotiw M, Whitehead JT, Swart KS. Sequential bacteriological observations in relation to cellmediated and humoral antibody responses of cattle infected with *Mycobacterium paratuberculosis* and maintained on normal or high iron intake. Aust Vet J 1989; 66(2):50-55.

Liapi M, Leontides L, Kostoulas P, Botsarisc G, Iacovoua Y, Reesc C, Georgioua K, Smithd GC, Nasebye DC. Bayesian estimation of the true prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in Cypriot dairy sheep and goat flocks. Small Rumin Res 2011; 95(2-3):174-178.

Liverani E, Scaioli E, Cardamone C, Dal Monte P, Belluzzi A. *Mycobacterium avium* subspecies *paratuberculosis* in the etiology of Crohn's disease, cause or epiphenomenon? World J Gastroenterol 2014; 20(36):13060-13070.

Lowe AM, Yansouni CP, Behr MA. Causality and gastrointestinal infections: Koch Hill, and Crohn's. Lancet Infect Dis 2008; 8(11):720-726.

Lugton IW. Cross-sectional study of risk factors for the clinical expression of ovine Johne's disease on New South Wales farms. Aust Vet J 2004; 82(6):355-365.

Mancipe LF, Sánchez L, Rodríguez G. Estudio de la paratuberculosis en un rebaño de ovinos de la Sabana de Bogotá mediante la utilización de tres técnicas diagnósticas. Rev Med Vet 2009; 18:33-51.

Manning EJ, Collins MT. Epidemiology of paratuberculosis. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 22-26.

Manning EJ, Collins MT. *Mycobacterium avium* subsp. *paratuberculosis*: Pathogen, pathogenesis, and diagnosis. Rev Sci Tech. 2001; 20(1):133-150.

Marce C, Beaudeau F, Bareille N, Seegers H, Fourichon C. Higher non-return rate associated with *Mycobacterium avium* subspecies *paratuberculosis* infection at early stage in Holstein dairy cows. Theriogenology 2009; 71(5):807-816.

McKenna SLB, Barkema HW, Keefe GP, Sockett DC. Agreement between three ELISA´s for *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. Vet Microbiol 2006; 114(3-4):285-291.

Mogollón JD, Hernández AL, Tovar AL, Murillo BN, Peña NE, Mossos NA. Prevalencia de paratuberculosis ovina en el altiplano cundi-boyacense. Revista ICA (Colombia) 1983; 18:479-484.

Nielsen SS, Toft N. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev Vet Med 2009; 88:1-14.

Nielsen SS, Enevoldsen C, Gröhn YT. The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev Vet Med 2002; 54:1-10.

Nielsen SS, Toft N. *Ante-mortem* diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-gama assay, and faecal culture techniques. Vet Microbiol 2008; 129(3-4):217-235.

Nielsen SS. Immune-based diagnosis of paratuberculosis. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 284-91.

Nielsen SS, Toft N. Effects of days in milk and milk yield on testing positive in milk antibody ELISA to *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. Vet Immunol Immunopathol 2012; 149(1-2):6-10.

Nielsen SS, Toft N. Bulk tank milk ELISA for detection of antibodies to *Mycobacterium avium* subsp. *paratuberculosis*: Correlation between repeated tests and within-herd antibodyprevalence. Prev Vet Med 2014; 113(1):96-102.

Office International des Epizooties (OIE). Manual of diagnostic tests and vaccines for terrestrial animals 2011. Paris, France.

Olsen I, Sigurðardóttir ÓG, Djønne B. Paratuberculosis with special reference to cattle. A review. Vet Q 2002; 24:12-28. Patel A, Shah N. *Mycobacterium avium* subsp *paratuberculosis*—incidences in milk and milk products, their isolation, enumeration, characterization, and role in human health. J Microbiol Immunol
Infect 2011; 44(6):473-479.

Patiño DA, Estrada M. Determinación de la prevalencia de paratuberculosis en tres hatos del Páramo de Letras. [Thesis]. Caldas, Colombia. Universidad de Caldas; 1999.

Peña MA, Góngora A, Jiménez C. Infectious agents affecting fertility of bulls, and transmission risk through semen. Retrospective analysis of their sanitary status in Colombia. Rev Colomb Cienc Pecu 2011; 24(4):634-646.

Pithua P, Espejo LA, Godden SM, Wells SJ. Is an individual calving pen better than a group calving pen for preventing transmission of *Mycobacterium avium* subsp *paratuberculosis* in calves? Results from a field trial. Res Vet Sci 2013; 95(2):398-404.

Plata R. La paratuberculosis bovina en Cundinamarca. Rev Med Vet. 1931 (cited by Vega-Morales A, 1947).

Ramírez R, Maldonado JG. Detection of macrophages infected with *Mycobacterium avium* subspecies *paratuberculosis* in a cow with clinical stage IV of the disease. A case report. Rev Colomb Cienc Pecu 2013a; 26(3):219-225.

Ramírez R, Maldonado JG. Evasión molecular de la activación del macrófago bovino por *Mycobacterium avium* subespecie *paratuberculosis*. Rev MVZ Córdoba 2013b; 18(3):3897-3907.

Ramírez N, Gaviria G, Restrepo LF, Gómez C. Diagnóstico epidemiológico referente a varias patologías de bovinos en tres haciendas de la Universidad de Antioquia. (Unpublished document), 2001.

Ramírez N, Rodríguez B, Fernández JA. Diagnóstico clínico e histopatológico de paratuberculosis bovina en un hato lechero en Colombia. Rev MVZ Córdoba 2011; 16(3):2742-2753.

Rani PS, Sechi LA, Ahmed N. *Mycobacterium avium* subsp. *paratuberculosis* as a trigger of type-1 diabetes: Destination Sardinia, or beyond? Gut Pathogens 2010; 2:1.

Robbe-Austerman S. Control of paratuberculosis in small ruminants. Vet Clin North Am Food Anim Pract 2011; 27(3):609-620.

Rosenfeld G, Bressler B. *Mycobacterium avium paratuberculosis* and the etiology of Crohn's disease: A review of the controversy from the clinician's perspective. Can J Gastroenterol 2010; 24(10):619-624.

Salem M, Heydel C, El-Sayed A, Ahmed SA, Zschöck M, Baljer G. *Mycobacterium avium* subspecies *paratuberculosis*: Na insidious problem for the ruminant industry. Trop Anim Health Prod 2013; 45(2):351-366.

Sechi LA, Dow CT. *Mycobacterium avium* ss. *Paratuberculosis* Zoonosis - The hundred-year war - Beyond Crohn's disease. Front Immunol 2015; 6:96.

Sonawane GG, Tripathi BN. Comparison of a quantitative realtime polymerase chain reaction (qPCR) with conventional PCR, bacterial culture, and ELISA for detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep showing pathology of Johne's disease. Springerplus 2013; 2(1):45.

Stabel JR, Whitlock RH. An evaluation of a modified interferongamma assay for the detection of paratuberculosis in dairy herds. Vet Immunol Immunopath 2001; 79:69-81.

Stevenson K. Comparative differences between strains of *Mycobacterium avium* subsp. *paratuberculosis*. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010a. p. 126-132.

Stevenson K. Diagnosis of Johne´s disease: Current limitations and prospects. Cattle Practice 2010b; 18:104-109.

Stewart D, Vaughan J, Stiles P. A long-term bacteriological and immunological study in Holstein- Friesian cattle experimentally infected with *Mycobacterium avium* subsp. *Paratuberculosis* and necropsy culture results for Holstein- Friesian cattle, merino sheep, and angora goats. Vet Microbiol 2007; 122(1-2):83-96.

Stief B, Möbius P, Türk H, Hörügel U, Arnold C, Pöhle D. Paratuberculosis in a miniature donkey (*Equus asinus* f. asinus). Berl Munch Tierarztl Wochenschr 2012; 125(1-2):38-44.

Sweeney RW, Collins MT, Koets AP, McGuirk SM, Roussel AJ. Paratuberculosis (Johne's disease) in cattle and other susceptible species. J Vet Intern Med 2012; 26(6):1239-1250.

Sweeney RW. Transmission of paratuberculosis. Vet Clin North Am Food Anim Pract 1996; 12(2):305-312.

Tiwari A, Van Leeuwen JA, Dohoo IR, Keefe GP, Haddad JP, Scott HM, Whiting T. Risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* seropositivity in Canadian dairy cows and herds. Prev Vet Med 2009; 88:32-41.

Tuberquia BC, Uribe F, Medrano MX, Ramírez NA, Fernández J. Seroprevalencia de *Mycobacterium avium* subsp. *Paratuberculosis* y exploración de factores asociados en una población bovina del municipio de Gómez Plata, Antioquia. Rev Colomb Cienc Pecu 2015a; 28(Sup):104.

Tuberquia BC, Uribe F, Medrano MX, Ramírez NA, Fernández J. Seroprevalencia de *Mycobacterium avium* subsp. *Paratuberculosis* y exploración de factores asociados en una población de búfalos del municipio de Gómez Plata, Antioquia. Rev Colomb Cienc Pecu 2015b; 28(Sup):105.

Tuci A, Tonon F, Castellani L, Sartini A, Roda G, Marocchi M, Caponi A, Munarini A, Rosati G, Ugolini G, Fuccio L, Scagliarini M, Bazzoli F, Belluzzi A. Fecal detection of *Mycobacterium avium paratuberculosis* using the IS*900* DNA sequence in Crohn's disease and ulcerative colitis patients and healthy subjects. Dig
Dis Sci 2011; 56(10):2957-2962.

Uzoigwe JC, Khaitsa ML, Gibbs PS. Epidemiological evidence for *Mycobacterium avium* subspecies *paratuberculosis* as a cause of Crohn's disease. Epidemiol Infect 2007; 135(7):1057-1068.

Vega A. Relación entre el diagnóstico de la paratuberculosis bovina por el examen coprológico y de la prueba alérgica de termorreacción con la tuberculina aviaria por vía subcutánea [Thesis]. Bogotá, Colombia. UNAL; 1947.

Vélez M, Rendón Y, Valencia A, Ramírez N, Fernández J. Seroprevalencia de *Mycobacterium avium* Subsp. *Paratuberculosis* (MAP) en una granja de ganado de carne de bosque húmedo tropical en Caucasia, Antioquia, Colombia. Rev Colombiana Cienc Anim 2016; 8(2):167-176.

Villalobos R, Hernández I, Tibata V, Rueda E. Diagnosis of mycobacteria important for veterinary medicine in Colombia. Instituto colombiano agropecuario –ICA, laboratorio nacional de diagnóstico veterinario (LNDV). First international congress on mycobacteria: A challenge for the 21st century. Third meeting of the SLAMTB; 2008; Bogotá, Colombia.

Wagner J, Skinner NA, Catto-Smith AG, Cameron DJ, Michalski WP, Visvanathan K, Kirkwood CD. TLR4, IL10RA, and NOD2 mutation in paediatric Crohn's disease patients: An association with *Mycobacterium avium* subspecies *paratuberculosis* and TLR4 and IL10RA expression. Med Microbiol Immunol 2013; 202(4):267-276.

Wells SJ, Wagner BA. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. JAVMA 2000; 216(9):1450-1457.

Whitlock RH. Paratuberculosis control measures in the USA. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 319-326.

Whittington RJ, Marsh IB, Saunders V, Grant IR, Juste R, Sevilla IA, Manning EJ, Whitlock RH. Culture phenotypes of genomically and geographically diverse *Mycobacterium avium* subsp. *paratuberculosis* isolates from different hosts. J Clin Microbiol 2011; 49(5):1822-1830.

Whittington RJ, Windsor PA. *In utero* infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: A critical review and meta-analysis. Vet J 2009; 179(1):60-69.

Whittington RJ. Cultivation of *Mycobacterium avium* subsp. *paratuberculosis*. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 244-260.

Windsor PA, Whittington RJ. Evidence for age susceptibility of cattle to Johne's disease. Vet J 2010; 184(1):37-44.

Yamasaki EM, Brito MF, Mota RA, McIntoshe D, Tokarnia CH. Paratuberculose em ruminantes no Brasil. Pesq Vet Bras 2013; 33(2):127-140.

Zapata MM, Arroyave O, Ramírez R, Piedrahita C, Rodas JD, Maldonado JG. Identification of *Mycobacterium avium* subspecies *paratuberculosis* by PCR techniques and establishment of control programs for bovine paratuberculosis in dairy herds. Rev Colomb Cienc Pecu 2010; 23(1):17-27.

Zapata MM, Rodas JD, Maldonado JG. Paratuberculosis bovina: ¿conocemos la situación real de la enfermedad en la ganadería colombiana? Rev Colomb Cienc Pecu 2008; 21(3):420-435.

Chapter one

The present article was constructed to accomplish the specific objectives 1 (determine the presence of MAP in dairy herds using environmental sampling and real-time-PCR) and 4 (determine herd-level risk factors for MAP real-time-PCR positivity using multivariate analysis) in dairy herds with in-paddock milking facilities of the Northern region of the Province of Antioquia (Colombia). This material was presented as a poster in the 14th International Colloquium on Paratuberculosis in Cancun (Mexico), June 4th to 8th, 2018) as "Co-existence of cattle with other ruminants is associated with Mycobacterium avium subsp. paratuberculosis presence in environmental samples from dairy herds in Northern Antioquia, Colombia" (2.66; URL: http://www.paratuberculosis.net/proceedings/proc14.pdf). The manuscript is under review by Preventive Veterinary Medicine journal (submitted in December 2018).

URL for the Author Guidelines (Preventive Veterinary Medicine):
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Prevalence of Mycobacterium avium subsp. paratuberculosis in dairy herds in Northern Antioquia (Colombia) and associated risk factors using environmental sampling

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Abstract

This cross-sectional study aimed to determine *Mycobacterium avium* subsp. *paratuberculosis* (MAP) herd-level prevalence using a quantitative real-time PCR method (qPCR), performed on environmental samples. Secondly, the study aimed to explore herd-level risk factors associated with the presence of MAP in dairy herds with in-paddock milking facilities of the Northern region of the Province of Antioquia (Colombia). Study herds (*n* = 292) located in 61 different districts from six municipalities were randomly selected amongst 7,794 dairies registered in the foot-and-mouth disease vaccination records from 2015. The sampling strategy considered a proportional allocation, both at municipality and district level. Participant herds were visited once between June and October 2016 to collect one composite environmental sample and to complete a risk assessment questionnaire. Each composite environmental sample contained material from six different sites of concentration of adult cattle and/or high traffic areas (e.g. areas surrounding waterers and feeders, areas surrounding the current mobile milking-unit places). Identification of MAP was achieved using a duplex qPCR (Bactotype MAP PCR Kit®, Qiagen). A herd was considered as MAP infected if the environmental sample was positive in the qPCR. Information about the general characteristics of the herd, management practices, and knowledge about the disease was collected using the risk-assessment questionnaire. The information on risk factors was analyzed using a multivariable logistic regression model. The apparent herd-level prevalence was 4.1% (12/292; 95% CI: 1.8-6.4). Herds with a history of mixed farming of cattle with other ruminants had higher odds of being MAP infected than herds without (OR = 3.9; 95% CI: 1.2-13.2). Our study demonstrates the MAP prevalence in dairy herds from Antioquia, Colombia and the possible relationship between MAP environmental positivity with the history of mixed farming of cattle with other susceptible ruminants.

Keywords: environmental sampling, IS*900*-qPCR, Johne's disease, risk factor.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a Gram-positive acid-fast bacillus and the causal agent of paratuberculosis (PTB), also known as Johne's disease (Sweeney, 1996). MAP is resistant to both environmental and chemical adverse conditions and can persist in the environment, including soil, stream water, and manure slurry storage, for up to one year (Kaevska et al., 2014; Salgado et al., 2015).

Several diagnostic tests can be used to detect MAP (Nielsen and Toft, 2008). Fecal culture (FC) is considered the *ante-mortem* reference test for PTB (Whittington, 2010). However, polymerase chain reaction (PCR) on environmental samples has been suggested for herd screening (Collins et al., 2006; USDA, 2010) with comparable results to culture (Douarre et al., 2010). Quantitative real-time-PCR method (qPCR) has been found to be a found to be a relatively sensitive and specific procedure, 60 and 97%, respectively (Aly and Mangold, 2010; Logar et al., 2012), and enables both detection and accurate quantification of a specific target sequence of MAP directly in milk and fecal samples (Tiwari et al., 2006; Alinovi et al., 2009; Hanifian et al., 2013; Kruze et al., 2013; Donat et al., 2015). In addition, analysis of environmental samples by PCR is a cost-saving and easy-to-use approach to diagnose PTB at the herd-level, as it does not require sample collection from individual animals. It is considered the most cost-effective diagnostic strategy to classify herds as infected or non-infected (Donat et al., 2015; Wolf et al., 2015).

Herd-level prevalence among cattle herds in Europe appeared to be >50% (Nielsen and Toft, 2009). In the United States, results from ELISA-based testing revealed that 21.6% to 70.4% of dairy herds were infected with MAP (Wells and Wagner, 2000; Lombard et al., 2013). Herd-level prevalence of PTB in cattle from South America and the Caribbean range from 18.7 to 100%, using different diagnostic tests available (i.e. ELISA, individual and pooled-fecal culture —FC, skin test, culture of environmental samples, agar gel immunodiffusion —AGID, fecal PCR, individual milk culture, and bulk tank milk PCR; Fernández-Silva et al., 2014). In Colombia, PTB was first reported in cattle in 1924 (Vega, 1947). According to previous estimations in dairy cattle from different provinces of the country, apparent herd-level seroprevalence appeared to be

>50% based on both unabsorbed and absorbed ELISA tests (Fernández-Silva et al., 2011; Benavides et al., 2016). According to Correa-Valencia *et al.* (2016), the apparent seroprevalence at herd-level was 3.6% in a municipality of the dairy Northern region in the Province of Antioquia, using an absorbed ELISA test.

Practices acting as triggers for MAP entrance and persistence may vary between countries or agro-ecological zones, or also between regions or herds (Doré et al., 2012; Rangel et al., 2015). This situation leads to the need for a local determination of specific factors associated with the PTB in a specific region (Fernández-Silva et al., 2014). Different herd characteristics have been reported to influence MAP status in dairy cattle worldwide [e.g. herd size (Tavornpanich et al., 2008; Wolf et al., 2016; Corbett et al., 2018), predominant breed in the herd (Jakobsen et al., 2000), purchasing herd practices (Wells and Wagner, 2000; Sorge et al., 2012; Wolf et al., 2016), pasture fertilization (Goodger et al., 1996; Daniels et al., 2002; Wolf et al., 2016)]. In addition, some herd management practices have been reported to influence MAP status in dairy cattle worldwide [e.g. contact of calves with adult cattle (Dieguez et al., 2008; Tiwari et al., 2009), practices regarding colostrum and milk feeding to calves (Nielsen et al., 2008).

In general, data regarding individual animal or herd risk factors associated with PTB in Colombian dairy herds, as well as a broader population-based prevalence estimation are still limited and only explored in the last three years (Benavides et al., 2016; Correa-Valencia et al., 2016; Fernández-Silva et al., 2017).

The aim of this cross-sectional study was to determine the herd-level prevalence and to explore herd-level risk factors associated with the presence of MAP in dairy herds with in-paddock milking facilities in the Northern region of the Province of Antioquia (Colombia), using environmental sampling and MAP detection by IS*900*-qPCR.

Materials and methods

Study design and herd selection

This research was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act number 71, June 15th, 2011). A cross-sectional study was carried out from July to October 2016 in the Northern region of the Province of Antioquia (Colombia). Study herds were located in districts of six municipalities (San José de la Montaña, Belmira, Santa Rosa de Osos, Entrerríos, San Pedro de Los Milagros, and Donmatías) which produce 70% of the milk in the Province of Antioquia (Corantioquia, 2016). The study area is located between 1,090 and 2,979 meters above sea level, and the temperature ranges from 12 to 16°C during the year. According to the Caldas-Lang climate classification, the study areas are classified cold-humid (Santa Rosa de Osos, San Pedro de Los Milagros, Entrerríos, Donmatías, San José de La Montaña) and cold-very humid (Belmira; Gobernación de Antioquia, 2016). The herd was considered as the unit of analysis.

Proportional allocation at municipality-level was considered in the study design, according to the adult cattle population (> 2 years of age; Fedegán, 2015). Similarly, the districts to be sampled into each municipality were established according to the specific weight of each district inside its corresponding municipality, only considering the largest districts until the sum of their census accounted for the 70% of the adult cattle population in each municipality. A probabilistic design using a simple random sampling strategy with restitution and without replacement was performed to define the herds to be included in the study. Restitution means that when a farmer did not agree on participating or the contact phone number was wrong or not registered or out of service, another herd with the same characteristics and location was considered from the computer-generated random numbers. Without replacement means that once a herd was chosen, it was not put back again into the sample population base-pack. The sampling frame was 7,794 herds registered on the foot-and-mouth disease vaccination records of the six municipalities of interest and under in-paddock milking facilities. From these registered herds,

292 herds in 61 districts were randomly selected, according to sampling strategy and inclusion criteria.

The sample size was defined according to the formula for prevalence estimation from a finite population (Dohoo et al., 2014). The formula included an unknown PTB prevalence for the study region (50%), a 95% confidence level, and a maximum acceptable error rate of 6%.

In all cases, herds had to fulfill the following inclusion criteria to be finally enrolled in the study: Having adult cattle, in-paddock milking facilities —mobile units, geographic accessibility, not previously defined as MAP-infected (by any method), and willingness of the owner to participate (i.e. allowing the sampling of all necessary areas in the herd and giving information regarding herd characteristics and management practices).

Sample collection

Environmental sampling as reported by the literature (Raizman et al., 2004; Collins et al., 2006; Pillars et al., 2009a; 2009b; USDA, 2010; Kruze et al., 2013; Donat et al., 2015; Wolf et al., 2015) was modified due to differences in management systems and facilities in the region of study and budget restrictions. Each participating herd was visited once during the study to collect one composite environmental sample containing material from at least six different sites (subsamples) of the concentration of adult cattle and/or high traffic areas in grazing paddocks (e.g. areas surrounding waterers and feeders, areas surrounding the current mobile milking-unit place). Approximately 20 g of manure was placed into a plastic container labeled with the herd number and date of collection to constitute each composite environmental sample. Each subsample was collected taking into account that the feces were not previously exposed to direct sunlight. Subsamples were placed into a bigger container, then pooled and manually mixed at the farm, and then conserved refrigerated at 4 °C during transport back to the laboratory. At the laboratory, samples were homogenized for 5 min each one and then frozen at -20˚C until DNA extraction (from a minimum of 56 to a maximum of 248 days).

Information collection

A one-page questionnaire was administered to the herd owner or herdsman at the time of sample collection in a face-to-face interview. Information on herd characteristics, management practices, and knowledge about the disease was collected using the questionnaire (available from the authors upon request). Three different research assistants, previously trained for the task, administered the questionnaire. All questionnaires included an introductory paragraph explaining the rationale and importance of the questions, how data was going to be used, and a confidentiality agreement. The questions were divided into three sections: 1) General information of herd, 2) herd management practices, and 3) knowledge about the disease. All questions were two-choice/multiple-choice, considering a semi-open alternative in some of the questions. Possible answers by each question were exclusive and jointly exhaustive, and no abbreviations or complex technical terminologies were considered in the design as previously recommended (Dohoo et al., 2014).

All information collection procedures were pre-tested at small scale to evaluate their effectiveness. In relation to the questionnaire, two expert colleagues in the field evaluated the structure to ensure that all important issues were identified and covered. The pre-test of the questionnaire at a small scale to some farmers (n = 5) was oriented to identify problems such as excessive length, poorly worded, confusing questions (using technical manners), or allowance of subjective responses (Dohoo et al., 2014).

Laboratory analysis

DNA extraction. DNA isolation from environmental fecal samples for IS*900*-qPCR was carried out using a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, CA, USA). The protocol included a *prior* bead-beating (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA) of 150 mg of sample in lysis solution for 20 min at maximum speed. The following procedures were carried out following the instructions of the manufacturer. A NanoDrop 2000® spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the

purity and yield of nucleic acids at two wavelengths (A_{260} and A_{280} nm). DNA integrity was confirmed using an only-agarose gel on a representative sub-sample of each extraction batch (10%). DNA extraction efficiency was confirmed by PCR using bacterial constitutive genes to the same sub-samples mentioned above (Weisburg et al., 1991; Inokuma et al., 2001). The extracted DNA was conserved at -20°C until qPCR analysis.

IS900-qPCR. DNA from environmental samples was tested for MAP using a duplex IS*900*-qPCR (Bactotype MAP PCR Kit®, Qiagen, Leipzig, Germany), including an internal amplification control and a MAP-positive and negative controls. The total reaction volume was 25.2 µl (17 µl of master mix, 8 µl of sample, and 0.2 µl of the IAC, diluted at 1:5 in ultrapure DNase- and RNase-free distilled water). The reaction began with 1 cycle at 95°C for 15 min and consisted of 45 cycles of denaturation at 95°C for 15 sec and annealing-extension at 60°C for 30 secs, and a final extension at 72°C for 35 sec. The sample was considered positive when it emitted a signal on the FAM and MAX channels or strongly positive if only emits a signal on the FAM channel with a Ct ≤ 40 and a sigmoid-pattern curve result (according to MIQE guidelines; Bustin et al., 2009). Procedures were carried out following the instructions of the manufacturer.

Statistical analysis

The outcome variable was herd status (positive/negative) as determined by IS*900*-qPCR on environmental samples. All the information generated during the study was entered into Excel worksheets (Microsoft Corp., Redmond, WA, USA) and then exported to Stata 15.0 (StataCorp, 2017, College Station, Texas, USA) for statistical analysis. Descriptive statistics were computed for all the variables of interest. Variables were checked for more than 30% missing values, a case in which they were not considered for further analysis. Categorical variables with multiple answers were dichotomized according to risk and distribution. A complex design analysis was conducted according to a cluster effect by district and the stratified nature of the study using the *Survey* command. Univariable analysis was performed to assess unconditional associations between the outcome (MAP-herd status) and each independent predictor using simple logistic regression. Associations with a $P \leq 0.20$ were considered for inclusion in the multivariable logistic

regression model. Conditional associations were explored. Evaluation of potential confounders was then performed by assessing the change in the β-coefficient of the variables of the adjusted model compared to the non-adjusted model. Confounders were only retained if a change greater than 15% was observed, regardless of the significance of the coefficient of the confounding variable in the model. The variables to be explored as confounders (i.e. herd size, predominant breed) were considered according to literature. Biologically plausible interactions were studied between significant variables from the multivariable models, as well as the 2-way interactions between significant predictors with a significant unconditional association with the dependent variable. Selection of the independent variables included in the final model was performed based on statistical considerations using a backward stepwise procedure with *P*-values of entry and removal of 0.2 and 0.25, respectively. The results from the final model are presented as odds ratios (OR) with 95% CIs. The model fit was assessed using the Hosmer-Lemeshow goodness-of-fit test (Dohoo et al., 2014).

Results

Environmental samples were collected from 292 dairy herds located in 61 districts of six municipalities of the Province of Antioquia (Colombia). Eight percent (*n* = 24) of the herds primarily selected as potentially eligible did not agree to participate when contacted by phone and 3% of the contact phone numbers were out of service or not registered. The non-participating population was composed by small dairy herds (<30 milking cows) according to foot-and-mouth disease vaccination records (Fedegán, 2015) and were mainly located in the municipalities of Donmatías and Entrerríos. Those who refused to participate in the study supported their decision to a reason related with the detection of bovine tuberculosis in their herds, even after the differences between diseases and causal agents was explained. All herds that agreed to participate by phone also allowed the sampling and the interview (*n* = 292).

Herd characteristics

Herd characteristics explored and then considered as predictors for the risk factor assessment are presented in Table 1. The study herds were all pasture-based dairies. The study population was mainly composed of small herds with access to veterinary assistance, Holstein being the predominant breed. Most of the herds were classified as closed according to cattle purchasing and co-grazing practices in the last 10 years. The presence of other ruminants (e.g. goats, sheep) in the herd during the last 2 years was a less frequent practice. Approximately half of the herds were neither tuberculosis-free and a small proportion were certified in good farming practices (GFP) the time of sampling. The GFP certification explored in this study included management practices which can be considered PTB-related, such as fertilization strategies (i.e. organic and inorganic), other animal species in the farm (e.g. goats, sheep), and tuberculosis sanitation status (ICA, 2007). Most of the interviewed owners/managers reported not having heard about the disease before and have not seen PTB-symptomatic animals in their herds in the last 2 years.

Herd management practices

Herd management practices explored and then considered as predictors for the risk factor assessment are presented in Table 2. Manure spreading on pastures as a method of fertilization was found to be a less frequent practice by most of the herds. Allowing calves to spend extended time with the dams (≥ 2 days after calving) was found in most of the cases. Nose-to-nose contact between adult cattle and ≤ 6 months-old calves was not allowed in most of the herds. Own-dam's colostrum and milk without antibiotics represented the main feeding sources used to feed pre-weaned calves.

Herd-level prevalence

Twelve herds out of 292 were positive to the IS*900*-qPCR (4.1%; 95% CI: 1.8-6.4; Fig. 1), ranging from 0.8 to 11.8%. MAP-apparent prevalence by municipality is shown in Table 3.

Risk factors assessment

Among the 17 risk factors explored in the univariable analysis (12 herd characteristics and five management practices), three were associated with the IS*900*-qPCR MAP-herd status ($P \leq 0.20$; Tables 1 and 2). These variables were selected for the multivariable analysis.

The variables “own animals grazing in non-proper pastures”, “producer´s knowledge of the disease”, and “colostrum fed to calves” were excluded from all logistic regressions. There was no variation among herds with a MAP positive status and the maximum likelihood estimation procedure in the logistic regression analysis would not converge if the variable was included. The variables “herd size” and “cattle purchasing practices” were considered as potential confounders, but none of them was found as such. A final model was built (Table 4). In the best fit of the model (Hosmer-Lemeshow´s $P = 0.97$), having a history of mixed farming of cattle with other ruminants in the last 2 years was significantly associated with a positive MAP-herd status as determined by qPCR performed on environmental samples (OR = 3.89; 95% CI: 1.2-13.2).

Discussion

This study was undertaken to better understand the presence and distribution of MAP in Colombian typical specialized in-paddock milking facilities. To the authors´ knowledge, this is the first approach using a regional-scale environmental sampling and qPCR to determine herd-level infection status in the country so far.

The apparent MAP herd-level prevalence of 4.1% (0.8-11.8%) estimated in the present study at municipality-level seems lower than the prevalence found in cattle by other authors in European, Asian, North American, and Latin American and Caribbean countries (Nielsen and Toft, 2009; Fernández-Silva et al., 2014).

Table 1.

Herd-level characteristics considered to be risk factors for *Mycobacterium avium* subsp. *paratuberculosis* presence in the environment in the Province of Antioquia, Colombia (2016).

Herd characteristic (description, when needed)	Categories	N positive herds	N negative herds	N	Distribution (%)	OR (95% CI)	*P*-value
Herd size	≤ 30	5	151	156	53.4		
(number of lactating cows)	>30	7	129	136	46.6	1.6 (0.5-5.3)	0.409
Predominant breed	Holstein	11	239	250	85.6		
(the most common cattle breed in the herd)	Other[a]	1	41	42	14.4	0.5 (0.7-4.2)	0.548
Veterinary assistance	Yes	10	246	256	87.7		
(availability of veterinarian assistance in the farm)	No	2	34	36	12.3	0.7 (0.1-3.3)	0.642
Purchasing practices	Yes	6	142	148	50.7		
(use of external replacement cattle in the last 10 years)	No	6	138	144	49.3	1.00 (0.3-3.1)	0.970
Outsider animals grazing in own pastures	Yes	1	7	8	2.7		
	No	11	273	284	97.3	3.5 (0.4-31.4)	0.255
Own animals grazing in non-proper pastures	Yes	0	28	28	9.6		
	No	12	252	264	90.4	0	Inestimable
Mixed farming in the last 2 years	Yes	5	50	55	18.8		
(cattle co-existence with goats and/or sheep in the last 2 years)	No	7	230	237	81.2	3.3 (1.0-10.8)	0.050*

Type of ruminants co-existing with the cattle in the last 2 years	Goats	4	23	27	9.2	5.7 (1.5-20.9)	0.099
	Sheep	1	24	25	8.6	1.4 (0.2-11.6)	0.773
	Sheep and goats	0	3	3	1.0	0	Inestimable
	Not applicable	7	230	237	81.2		
Good farming practices-status [herd certified by the Colombian Agricultural Institute (ICA; by its name in Spanish, Instituto Colombiano Agropecuario) as a GFP certified herd]	Yes	3	45	48	16.4		
	No	9	235	244	83.6	1.7 (0.5-6.7)	0.419
Bovine tuberculosis status (herd certified by the ICA as free from bovine tuberculosis)	Yes	7	138	145	49.7		
	No	5	142	147	50.3	1.4 (0.5-4.7)	0.541
Producer´s knowledge about the disease	Some[b]	0	30	30	10.3		
	Never heard about it before	12	250	262	89.7	0	Inestimable
PTB-compatible signs´ history (report of animals with compatible PTB symptoms in the herd —diarrhea and progressive weight loss, refractory to treatment)	Yes[c]	2	84	86	29.5		
	Never	10	196	206	70.5	1.0 (0.2-4.8)	0.991

OR: Odds Ratio. 95% CI: 95% confidence interval. [a] Includes: Jersey, Guernsey, Ayrshire, Swedish Red, Swiss Brown, and crossbreeds. [b] Includes: recognized name only, knew some basics, and fairly knowledgeable. [c] Includes: at present and in the last 2 years. * Variables used for the multivariable analysis ($P \leq 0.20$).

Table 2.

Herd management practices considered to be risk factors for *Mycobacterium avium* subsp. *paratuberculosis* presence in the environment in the Province of Antioquia, Colombia (2016).

Herd management practice (description, when needed)	Categories	N positive herds	N negative herds	N	Distribution (%)	OR (95% CI)	*P*-value
Manure spreading (use of cow manure as a fertilizer)	Yes	8	113	121	41.4		
	No	4	167	171	58.6	3.0 (0.9-10.1)	0.083*
Typical time of separation (separation of newborn calf from their dam after birth, in days)	≤ 1	2	27	29	9.9	0.5 (0.1-2.6)	0.433
	≥ 2	10	253	263	90.1		
Calves ≤ 6 months old sharing spaces with adult cows (in nose-to-nose contact)	Yes	2	53	55	18.8		
	No	10	227	237	81.2	0.9 (0.2-4.0)	0.845
Colostrum fed to calves (source)	From multiple cows	0	27	27	9.3	0	Inestimable
	From its own dam	12	253	265	90.7		
Milk fed to unweaned calves (source)	Unsalable milk	1	95	96	32.9		
	Other sources[a]	11	185	196	67.1	5.64 (0.7-44.4)	0.110*

OR: Odds Ratio; 95% CI: 95% confidence interval. [a] Includes: milk without antibiotic (salable milk) and milk replacer. * Variables used for the multivariable analysis ($P \leq 0.20$).

Table 3.

Mycobacterium avium subsp. *paratuberculosis* apparent prevalence by municipality in the Province of Antioquia, Colombia (2016) using qPCR performed on environmental samples.

Municipality	Sample weight* (%)	Herds of study (n)	N positive herds (%)
San José de La Montaña	5.8	17	2 (11.8)
Belmira	10.6	31	2 (6.5)
Santa Rosa de Osos	45.2	132	1 (0.8)
Entrerríos	11.3	33	1 (3.0)
San Pedro de Los Milagros	17.8	52	3 (5.7)
Donmatías	9.3	27	3 (11.1)
Total	100	292	12 (4.1)

*****According to foot-and-mouth disease vaccination records (Fedegán, 2015).

Table 4.

Final multivariable logistic regression model to identify herd characteristics and management practices associated to a positive *Mycobacterium avium* subsp. *paratuberculosis* status determined by IS*900*-qPCR on environmental samples in 292 herds located in six municipalities of the Province of Antioquia, Colombia (2016).

Risk factor	OR	Standard error	*P*-value	OR 95% CI
Mixed farming in the last 2 years				
No	Reference			
Yes	3.9	2.4	0.029	1.2-13.2
Manure spreading				
No	Reference			
Yes	2.5	1.5	0.146	0.7-8.9
Milk fed to unweaned calves				
Unsalable milk	Reference			
Other sources	5.1	5.5	0.126	0.6-41.8

OR: Odds Ratio. 95% CI: 95% confidence interval.

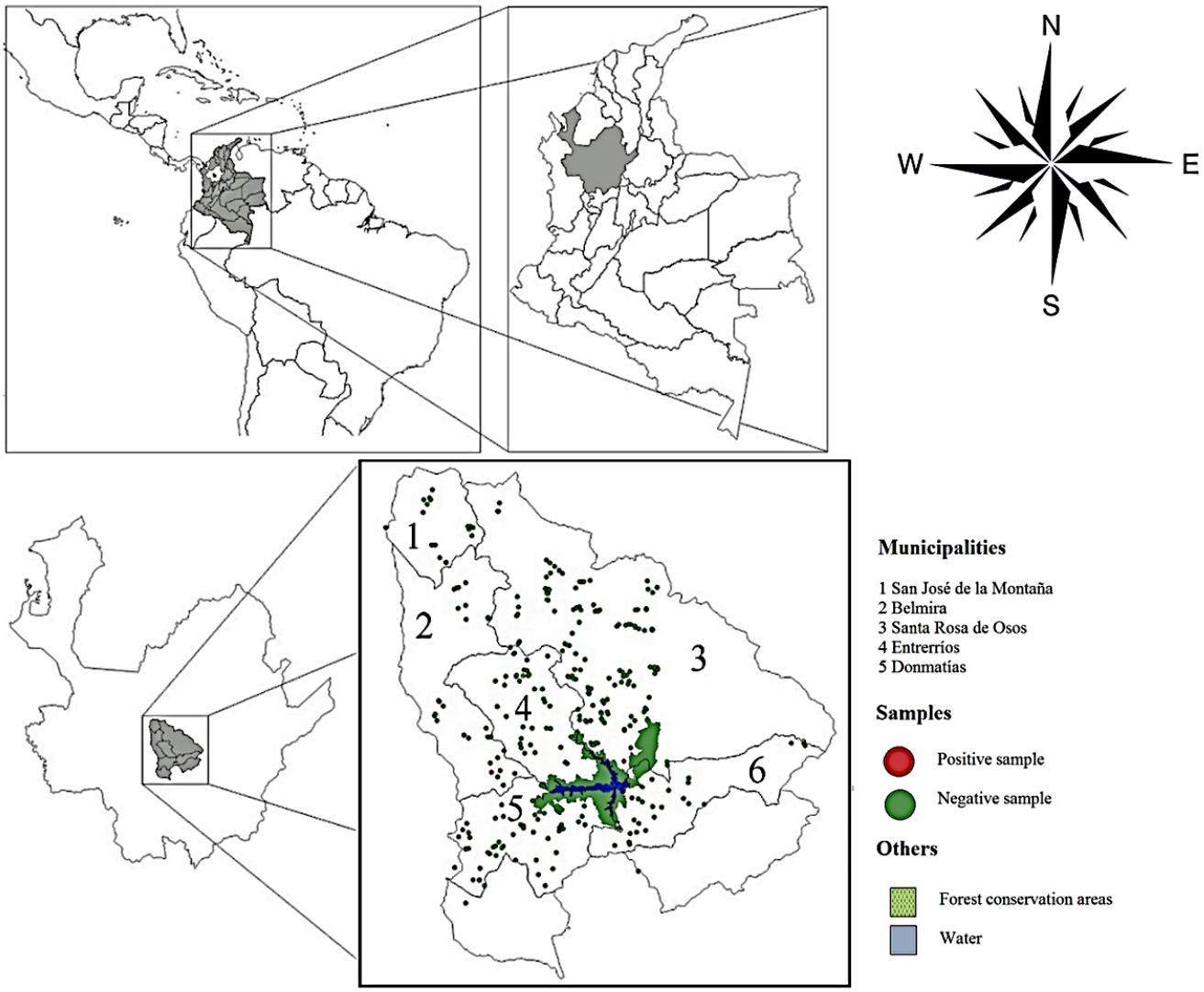

Fig. 1. Geographic location of the six municipalities and the 292 dairy herds sampled in the Northern region, Province of Antioquia, Colombia (2016). Green dots refer to the herds found to be negative while red dots refer to those found positive to *Mycobacterium avium* subsp. *paratuberculosis* by IS*900*-qPCR on environmental samples.

Fernández-Silva et al. (2014) reported findings from a systematic review of studies done in Latin American and Caribbean countries with a non-adjusted overall herd-level prevalence of 75.8% (50.1-100%) in cattle, revealing the extreme limits that can be found in the PTB apparent

prevalence reports.According to the authors of the systematic review, the high heterogeneity detected in overall prevalence estimations could be easily attributed to the high diversity in study design, the variable quality of measures, or to the test used. Scientific literature hypothesizes that up-to-date MAP-herd-level prevalence is increasing in some countries that do not have mandatory control programs (Salem et al., 2012), such as Colombia, but not enough data can support that statement.

At a national scale, PTB in cattle was first reported in Colombia more than 90 years ago and thereafter it has been also reported in sheep and goats (Correa-Valencia et al., 2018). However, precise and updated estimations of herd-prevalence in cattle at national or regional-level are not available. It should be mentioned that prevalence records of MAP infection in Colombia are limited because regular testing for the disease is not performed among dairies, or it is done only on selected cows suspicious to be clinically affected. Only three studies have been previously published concerning herd-prevalence of MAP infection in Colombian dairy herds (Fernández-Silva et al., 2011; Benavides et al., 2016; Correa-Valencia et al., 2016) and, although these studies suggest important information of infected herds, their estimations cannot be considered as representative of a national situation given the low number of herds sampled and features of their designs which could have overestimated the prevalence reported, both at animal and at herd-level.

On the other hand, one concern about using environmental sampling for the determination of herd-level infection status could be the possibility of MAP being detected in the environment without being present in the cattle. Nevertheless, an active shedder-cow culled from the herd before the sampling visit will be missed by the individual sampling but could be detected by environmental sampling since its feces could still remain on the farm. In such cases, environmental samples represent an advantage compared to single animal-testing. In addition, MAP is classified as an obligate pathogen and is not believed capable of replicating outside a suitable host (Manning, 2001). Therefore we considered all herds found positive to qPCR as infected, based on a potential (previous or during the time of sampling) MAP-elimination source leading to environmental fecal contamination with the bacteria, and therefore to the risk of

ingestion by susceptible cattle (Elliott et al., 2015). In addition, shedding of MAP from infected cows is not a continuous event, especially in the early stages of infection (Mitchell et al., 2012), explaining the possibility of false negative results in our study. Recognition of shedding can occur more or less throughout the entire infection period, but only a few animals test positive in the early phases of infection, and at early ages following natural infection (Weber et al., 2010). Another phenomenon that could explain negative results in the present report is that the environmental sampling method used in this study was a form of pooling. Samples collected from operations with low levels of MAP in the environment may not be detected as positive with this method because of bacterial numbers falling below the threshold of the detection method. According to Pillars et al. (2009b), because of the relatively low sensitivity threshold of the qPCR method, it is certainly possible that some infected herds were misclassified as ‘‘negative’’ using the sampling protocol described, but misclassification would only occur in herds with prevalences below 7% of fecal shedding.

Regarding the sampling methodology used, we considered a single pool of at least six different points of adult concentration areas. This strategy was applied due to budget restrictions and the need to cover a larger geographical area that would give broader information about MAP-infection status. A well-defined place known as suitable to yield a herd-level MAP-positive result is the wastewater storage lagoon (Lombard et al., 2006; Pillars et al., 2009a; 2009b). This location was not included in our screening because it is not a representative feature of local dairy herds. Only one in four of the dairies visited during this sampling had this location available to be sampled. Other alternative places to collect samples according to literature (Collins et al., 2006) are the maternity-related areas —in our case paddocks, because of the concentration of adult cattle in peripartum. No sampling was considered from these areas because just a small fraction of the sampled herds has these facilities (one in six). The nursing zone that some publications refer to (Berghaus et al., 2006; Collins et al., 2006), highly differs in our dairy systems, as it is seen on maternity facilities, so, those were not considered either.

The herd-level risk factor identified in this study was having a history of mixed farming of cattle with sheep and goats in the last 2 years. Barrett et al. (2011) were the first to report this risk

factor for MAP infection, by individual FC, using a case-control design. Concerning the presence of other ruminants, Whittington et al. (2001) reported cases of bovine PTB due to S (sheep) strain that was confirmed in Australia and Iceland under pasture-based systems, demonstrating the transmission opportunity between species. Wolf et al. (2014) proposed that an environmental sample could be contaminated with MAP bacteria by a source other than cattle. As MAP is an intracellular pathogen that does not reproduce in the environment, possible sources for contamination would be other domestic ruminants such as goats or sheep. Considering the literature support of cross-contamination between susceptible species we consider that this practice can increase the risk to find MAP in the environment. Further analysis is needed to establish the MAP-strain which is circulating in the study herds.

Several studies have explored herd size as a predictor linked to MAP-infection status or detection worldwide (Wells and Wagner, 2000; Hirst et al., 2004; Ridge et al., 2010; Kruze et al., 2013), reporting an association for herds ≥ 300, ≥ 600, ≥ 345, and ≥ 200 lactating cows, respectively. According to Fedegán (2015), most of the herds in the Province of Antioquia are small (approximately 30 lactating cows; ranging from 1 to 821 milking cows) and most of the herds include cattle on pasture and mobile milking facilities. Vilar et al. (2015) found that herd size ≥ 12 lactating cows aged over 24 months was associated to MAP-seroprevalence in the State of Paraíba, Northeastern Brazil, being the closest cultural and technical approach to our conditions. The question included in the questionnaire related to herd size was found (and expected) to be “reluctant” because producers are discrete about this information.

The purchase of infected cattle is considered the primary way of JD transmission between herds (Sweeney, 1996). Therefore, several studies have reported an association between purchase policies and MAP-herd-level status (Wells and Wagner, 2000; Hirst et al., 2004; Pillars et al., 2009a; 2009b; Ridge et al., 2010; Vilar et al., 2015; Puerto-Parada et al., 2018). Frequent cattle purchasing from other herds without knowledge of their disease status increased the risk for MAP culture-positive environmental samples (Wolf et al., 2016). This practice was explored from three points of view in the present study (outsider animals grazing in own pastures, own animals grazing in non-proper pastures, and cattle purchasing practices) and was grouped as part of the

classification of the herd as *open* or *closed,* being the first group the most representative one. The main reasons to introduce/allow to grass/purchase animals for Colombian dairy farmers are to expand herd size because most herds cannot do this by producing their own heifers.

In our study, two producers that had not heard of PTB had MAP-qPCR positive environmental samples, suggesting that clinical disease was not occurring, or at least was not recognized in their herds. Moreover, only two of the 12 positive herds did not have access to veterinary assistance, and then, may not have information available when compatible cases of the disease were presented in the herd.

Milk and colostrum can be contaminated with MAP, either through fecal contamination of teats or shedding from the udder (Nielsen et al*.,* 2008). In our case, milk replacers and salable milk (without antibiotics) were the main source used to fed unweaned calves. Nevertheless, according to the authors´ experience, it is still a common practice to use discarded milk to feed the calves. Feeding practices in dairies are closely related to post-partum husbandry practices, such as the practice of leaving a cow with her calf after birth, which was also representative of the herds of our study and has been reported as a risk factor by Goodger et al. (1996), Obasanjo et al. (1997), Ansari-Lari et al. (2009), increasing the within-herd transmission of PTB.

Misclassification biases of farms with low within-herd prevalence as negative is likely to occur because it was expected that the characteristics of the diagnostic test (Se and Sp) would deliver false negatives. On the other hand, the misclassification of herds due to laboratory features is not likely to occur because the laboratory procedures were done by the same trained person (as well as DNA extraction). Selective entry bias may be presented since only registered herds, according to foot-and-mouth disease vaccination records (Fedegán, 2015), were considered as the source for eligible herds. Nevertheless, considering that this vaccination is mandatory in the country, it is unlikely that any herd remains outside the registry.

Selection biases were not likely to occur because of randomization and representativeness of the target population in the source and study populations; non-response biases, by the

replacement sampling selection; detection biases, by inclusion and exclusion criteria established; and, missing data biases, by the fact we applied a face-to-face interview at the moment of sampling, assuring the complete filling out of the questionnaire. Inter-observer variability could induce bias. However, the impact of this bias was considered low, as all interviewers received standardized training and sample collection followed a strict protocol outlined in an instruction sheet that accompanied every sampling kit. While the effect of the Sp may be important, the effect of the Se may be as important since it may lead to the low apparent prevalence found. No duplicated run or confirmations to PCR were available because of a limited budget. One of the limitations of the current study with respect to the identification of risk factors was that MAP was only identified on a relatively small number of farms (*n* = 12). Consequently, a better approach aiming to define prevalence in a certain region should include a comparative test applied to a different matrix (i.e. serum, feces, milk) and also at a different level (i.e. pooled samples, animal-level), so misclassification biases due to the environmental sampling strategy could be controlled. Accordingly, there was low power to identify statistically significant associations with herd-level MAP-infection status.

Conclusion

Our results provide evidence that the prevalence found in the herds of study, representative of the area and of the dairy production system in the country, was 4.1% (95% CI: 1.8-6.4). In addition, having a history of mixed farming of cattle with other ruminants (i.e. sheep, goats) in the last 2 years is a risk factor for MAP infection at herd-level, and could be considered for PTB control, particularly in typical dairies in Colombia.

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Conflict of interest

The authors are not aware of any financial or personal relationships with other people or organizations that could inappropriately influence the work reported in this paper. The study sponsors (CODI-Universidad de Antioquia) had no direct role in developing the study design, data collection analysis or interpretation.

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References

Alinovi, C.A., Ward, M.P., Lin, T.L., Moore, G.E., Wu, C.C., 2009. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp *paratuberculosis*. Vet. Microbiol. 136(1-2), https://doi.org/10.1016/j.vetmic.2008.10.012

Aly, S., Mangold, B., 2010. Correlation between Herrold egg yolk medium culture and real-time quantitative polymerase chain reaction results for *Mycobacterium avium* subspecies. J. Vet. 683, 677–683. https://doi.org/10.1177/104063871002200501

Ansari-Lari, M., Haghkhah, M., Bahramy, A., Novin Baheran, A.M., 2009. Risk factors for *Mycobacterium avium* subspecies *paratuberculosis* in Fars province (Southern Iran) dairy herds. Trop. Anim. Health Prod. 41, 553–557. https://doi.org/10.1007/s11250-008-9221-7

Barrett, D.J., Mee, J.F., Mullowney, P., Good, M., McGrath, G., Clegg, T., More, S.J., 2011. Risk factors associated with Johne's disease test status in dairy herds in Ireland. Vet. Rec. 168, 0–2. https://doi.org/10.1136/vr.c6866

Benavides, B., Ángela, B., Arteaga, V., Carlos, C., Montezuma, A., 2016. Estudio epidemiológico de paratuberculosis bovina en hatos lecheros del sur de Nariño, Colombia. Rev. Med. Vet. (Bogotá). 31, 57–66. http://www.scielo.org.co/pdf/rmv/n31/n31a06.pdf

Berghaus, R.D., Farver, T.B., Anderson, R.J., Jaravata, C.C., Gardner, I.A., 2006. Environmental Sampling for Detection of *Mycobacterium avium* ssp. *paratuberculosis* on Large California Dairies. J. Dairy Sci. 89, 963–970. https://doi.org/10.3168/jds.S0022-0302(06)72161-0

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: Minimum Information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797

Collins, M.T., Gardner, I.A., Garry, F.B., Roussel, A.J., Wells, S.J., 2006. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. J. Am. Vet. Med. Assoc. 229, 1912–1919. https://doi.org/10.2460/javma.229.12.1912

Corantioquia. 2016. Manual de Producción y Consumo Sostenible. Fincas Lecheras. http://www.corantioquia.gov.co/SiteAssets/PDF/Gesti%C3%B3n%20ambiental/Producci%C3%B3n%20y%20Consumo%20Sostenible/Manuales_GIRH/Fincas%20Lecheras.pdf

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W., 2018. Environmental sample characteristics and herd size associated with decreased herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis*. J. Dairy Sci. 101(9), 8092–8099. https://doi.org/10.3168/jds.2018-14661

Correa-Valencia, N.M., Ramírez, N.F., Olivera, M., Fernández-Silva, J.A., 2016. 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. Trop. Anim. Health Prod. 48, 1191–1200. https://doi.org/10.1007/s11250-016-1074-x

Correa-Valencia, N.M., García-Tamayo, Y.M., Fernández-Silva, J.A., 2018. *Mycobacterium avium* subsp. *paratuberculosis* in Colombia, 1924-2016. Rev. Colom. Cienc. Pecu. 31(3), 165–179. https://doi.org/10.17533/udea.rccp.v31n3a01

Daniels, M.J., Hutchings, M.R., Allcroft, D.J., McKendrick, J., Greig, A., 2002. Risk factors for Johne's disease in Scotland--the results of a survey of farmers. Vet. Rec. 150, 135–139.

Dieguez, F.J., Arnaiz, I., Sanjuan, M.L., Vilar, M.J., Yus, E., 2008. Management practices associated with *Mycobacterium avium* subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. Vet. Rec. 162, 614–617. https://doi.org/10.1136/vr.162.19.614

Dohoo, I., Martin, W., Stryhn, H., 2014. Veterinary Epidemiologic Research. 8 Berkeley Way, Charlottetown, Prince Edward Island, Canada, VER Inc.

Donat, K., Kube, J., Dressel, J., Einax, E., Pfeffer, M., Failing, K., 2015. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiol. Infect. 143, 975–985. https://doi.org/10.1017/S0950268814002465

Doré, E., Paré, J., Côté, G., Buczinski, S., Labrecque, O., Roy, J.P., Fecteau, G., 2012. Risk factors associated with transmission of *Mycobacterium avium* subsp. *paratuberculosis* to calves within dairy herd: A systematic review. J. Vet. Intern. Med. 26, 32–45. https://doi.org/10.1111/j.1939-1676.2011.00854.x

Douarre, P.E., Cashman, W., Buckley, J., Coffey, A., O'Mahony, J.M., 2010. Isolation and detection of *Mycobacterium avium* subsp *paratuberculosis* (MAP) from cattle in Ireland using both traditional culture and molecular based methods. Gut Pathog. 2(1), 11. https://doi.org/10.1186/1757-4749-2-11

Elliott, G.N., Hough, R.L., Avery, L.M., Maltin, C.A., Campbell, C.D., 2015. Environmental risk factors in the incidence of Johnes disease. Crit. Rev. Microbiol. 41, 488–507. https://doi.org/10.3109/1040841X.2013.867830

Fedegán. Registro de vacunación, primer ciclo, 2015 (official restricted access material).

Fernández-Silva, J.A., Abdulmawjood, A., Bülte, M., 2011. Diagnosis and molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* from dairy cows in Colombia. Vet. Med. Int. 2011, 1–12. https://doi.org/10.4061/2011/352561

Fernández-Silva, J.A., Correa-Valencia, N.M., Ramírez, N.F., 2014. Systematic review of the prevalence of paratuberculosis in cattle, sheep, and goats in Latin America and the Caribbean. Trop. Anim. Health Prod. 46, 1321–1340. https://doi.org/10.1007/s11250-014-0656-8

Fernàndez, J.A., Ramìrez, N.F., Correa, N.M., 2017. Factors associated with *Mycobacterium avium* subsp. *paratuberculosis* in dairy cows from Northern Antioquia, Colombia. Rev. Colomb. Cienc. Pecu. 30, 48–59. file:///C:/Users/Natalia/Downloads/325782-117575-5-PB.pdf

Gobernación de Antioquia, 2016. Fichas municipales de Antioquia 2015-2016. Colombia. http://www.antioquia.gov.co/planeacion/fichas_municipales_web/index.html Accessed September 8 2018.

Goodger, W.J., Collins, M.T., Nordlund, K., Eisele, C., Pelletier, J., Thomas, C.B., Sockett, D.C., 1996. Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle. J. 208, 1877–1881.

Hanifian, S., Khani, S., Barzegari, A., Shayegh, J., 2013. Quantitative real-time PCR and culture examination of *Mycobacterium avium* subsp *paratuberculosis* at farm level. Vet. Microbiol. 162(1), 160–165. https://doi.org/10.1016/j.vetmic.2012.08.026

Hirst, H.L., Garry, F.B., Morley, P.S., Salman, M.D., Dinsmore, R.P., Wagner, B. a, McSweeney, K.D., Goodell, G.M., 2004. Seroprevalence of *Mycobacterium avium* subsp *paratuberculosis* infection among dairy cows in Colorado and herd-level risk factors for seropositivity. J. Am. Vet. Med. Assoc. 225, 97–101. https://doi.org/10.2460/javma.2004.225.97

ICA (Instituto Colombiano Agropecuario), 2007. Resolución 0002341 de 2007. URL: http://www.ica.gov.co/getattachment/0b5de556-cb4a-43a8-a27a-cd9a2064b1ab/2341.aspx. Accessed April 30 2018.

Inokuma, H., Parola, P., Raoult, D., Brouqui, P., 2001. Molecular survey of *Ehrlichia* infection in ticks from animals in Yamaguchi Prefecture, Japan. Vet. Parasitol. 99, 335–339. https://doi.org/10.1016/S0304-4017(01)00470-8

Jakobsen, M.B., Alban, L., Nielsen, S.S., 2000. A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. Prev. Vet. Med. 46, 15–27. https://doi.org/10.1016/S0167-5877(00)00138-0

Kaevska, M., Lvoncik, S., Lamka, J., Pavlik, I., Slana, I., 2014. Spread of *Mycobacterium avium* subsp. *paratuberculosis* through soil and grass on a mouflon (*Ovis aries*) pasture. Curr. Microbiol. 69, 495–500. https://doi.org/10.1007/s00284-014-0618-4

Kruze, J., Monti, G., Schulze, F., Mella, A., Leiva, S., 2013. Herd-level prevalence of Map infection in dairy herds of southern Chile determined by culture of environmental fecal samples and bulk-tank milk qPCR. Prev. Vet. Med. 111, 319–324. https://doi.org/10.1016/j.prevetmed.2013.05.011

Logar, K., Kopinč, R., Bandelj, P., Starič, J., Lapanje, A., Ocepek, M., 2012. Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in subclinically infected dairy cattle: Comparison with faecal culture, milk real-time PCR and milk ELISA. BMC Vet. Res. 8, 1–10. https://doi.org/10.1186/1746-6148-8-49

Lombard, J.E., Wagner, B.A., Smith, R.L., McCluskey, B.J., Harris, B.N., Payeur, J.B., Garry, F.B., Salman, M.D., 2006. Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. J. Dairy Sci. 89, 4163–4171. https://doi.org/10.3168/jds.S0022-0302(06)72461-4

Lombard, J.E., Gardner, I.A., Jafarzadeh, S.R., Fossler, C.P., Harris, B., Capsel, R.T., Wagner, B.A., Johnson, W.O., 2013. Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in United States dairy herds in 2007. Prev. Vet. Med. 108(2-3), 234–238. https://doi.org/10.1016/j.prevetmed.2012.08.006

Manning, E.J.B., 2001. *Mycobacterium avium* subspecies *paratuberculosis*. J. Zoo Wildl. Med. 32, 293–304.

Mitchell, R.M., Medley, G.F., Collins, M.T., Schukken, Y.H., 2012. A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiol. Infect. 140, 231–246. https://doi.org/10.1017/S0950268811000689

Nielsen, S.S., Bjerre, H., Toft, N., 2008. Colostrum and milk as risk factors for infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. J. Dairy Sci. 91, 4610–4615. https://doi.org/10.3168/jds.2008-1272

Nielsen, S.S., Toft, N., 2009. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev. Vet. Med. 88, 1–14. https://doi.org/10.1016/j.prevetmed.2008.07.003

Nielsen, S.S., Toft, N., 2008. Ante-mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet. Microbiol. 129(3-4), 217–235. https://doi.org/10.1016/j.vetmic.2007.12.011

Obasanjo, I.O., Gröhn, Y.T., Mohammed, H.O., Grohn, Y.T., Mohammed, H.O., 1997. Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in dairy herds on the New York State Paratuberculosis Control Program. Prev. Vet. Med. 32, 243–251. https://doi.org/10.1016/S0167-5877(97)00027-5

Pillars, R.B., Grooms, D.L., Kaneene, J.B., 2009a. Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project. Can. Vet. J. 50(10), 1039–1046.

Pillars, R.B., Grooms, D.L., Woltanski, J.A., Blair, E., 2009b. Prevalence of Michigan dairy herds infected with *Mycobacterium avium* subspecies *paratuberculosis* as determined by environmental sampling. Prev. Vet. Med. 89, 191–196. https://doi.org/10.1016/j.prevetmed.2009.02.022

Puerto-Parada, M., Arango-Sabogal, J.C., Paré, J., Doré, E., Côté, G., Wellemans, V., Buczinski, S., Roy, J.P., Labrecque, O., Fecteau, G., 2018. Risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* herd status in Québec dairy herds. Prev. Vet. Med. 152, 74–80. https://doi.org/10.1016/j.prevetmed.2018.02.010

Raizman, E.A., Wells, S.., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C., Olsen, K.E., 2004. The Distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the Environment Surrounding Minnesota Dairy Farms. J. Dairy Sci. 87, 2959–2966. https://doi.org/10.3168/jds.S0022-0302(04)73427-x

Rangel, S.J., Paré, J., Doré, E., Arango, J.C., Côté, G., Buczinski, S., Labrecque, O., Fairbrother, J.H., Roy, J.P., Wellemans, V., Fecteau, G., 2015. A systematic review of risk factors associated with the introduction of *Mycobacterium avium* spp. *paratuberculosis* (MAP) into dairy herds. Can. Vet. J. 56, 169–177. https://doi.org/10.1016/S0167-5877(97)00027-5

Ridge, S.E., Heuer, C., Cogger, N., Heck, A., Moor, S., Baker, I.M., Vaughan, S., 2010. Herd management practices and the transmission of Johne's disease within infected dairy herds in Victoria, Australia. Prev. Vet. Med. 95, 186–197. https://doi.org/10.1016/j.prevetmed.2010.05.001

Salem, M., Heydel, C., El-Sayed, A., Ahmed, S.A., Zschöck, M., Baljer, G., 2012. *Mycobacterium avium* subspecies *paratuberculosis*: An insidious problem for the ruminant industry. Trop. Anim. Health Prod. 45, 351–366. https://doi.org/10.1007/s11250-012-0274-2

Salgado, M., Aleuy, O.A., Sevilla, I.A., Troncoso, E., Salgado, M., Aleuy, O.A., Sevilla, I.A., Troncoso, E., 2015. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in a cattle/pudu interface. Arq. Bras. Med. Veterinária e Zootec. 67, 1205–1209. https://doi.org/10.1590/1678-4162-7530

Sorge, U.S., Lissemore, K., Godkin, A., Jansen, J., Hendrick, S., Wells, S., Kelton, D.F., 2012. Risk factors for herds to test positive for *Mycobacterium avium* ssp. *paratuberculosis*-ani\bod\es with a commercial milk enzyme-linked immunosorbent assay (ELISA) in Ontario and Western Canada. Can. Vet. J. 53, 963–970. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3418782/pdf/cvj_09_963.pdf

StataCorp, 2017. Stata Statistical Software: Release 15. 2017.

Sweeney, R.W., 1996. Transmission of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12(2), 305–312. https://doi.org/10.1016/S0749-0720(15)30408-4

Tavornpanich, S., Johnson, W.O., Anderson, R.J., Gardner, I.A., 2008. Associated with seroprevalence infection in dairy herds. Am. J. Vet. Res. 2008 69(7), 904–911. http://doi.org/10.2460/ajvr.69.7.904

Tiwari, A., VanLeeuwen, J.A., Dohoo, I.R., Keefe, G.P., Haddad, J.P., Scott, H.M., Whiting, T., 2009. Risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* seropositivity in Canadian dairy cows and herds. Prev. Vet. Med. 88, 32–41. https://doi.org/10.1016/j.prevetmed.2008.06.019

Tiwari, A., VanLeeuwen, J.A., McKenna, S.L.B., Keefe, G.P., Barkema, H.W., 2006. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can. Vet. journal. La Rev. vétérinaire Can. 47, 874–82. https://doi.org/10.1515/semi.1969.1.3.339

USDA (2010). Uniform Program Standards for the Voluntary Bovine Johne´s Disease Control Program. Washington D.C. United States, Department of Agriculture-USDA, Animal and Plant Health Inspection Service-APHIS. https://johnes.org/handouts/files/USDA_Program_Standards_Sept-2010.pdf

Vega, A., 1947. Relación entre el diagnóstico de la paratuberculosis bovina por el examen coprológico y de la prueba alérgica de termorreación aviar por vía subcutánea, Universidad Nacional de Colombia.

Vilar, A.L.T., Santos, C.S.A.B., Pimenta, C.L.R.M., Freitas, T.D., Brasil, A.W.L., Clementino, I.J., Alves, C.J., Bezerra, C.S., Riet-Correa, F., Oliveira, T.S., Azevedo, S.S., 2015. Herd-level prevalence and associated risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in cattle in the State of Paraíba, Northeastern Brazil. Prev. Vet. Med. 121, 49–55. https://doi.org/10.1016/j.prevetmed.2015.06.003

Weber, M.F., Kogut, J., de Bree, J., van Schaik, G., Nielen, M., 2010. Age at which dairy cattle become *Mycobacterium avium* subsp. *paratuberculosis* faecal culture positive. Prev. Vet. Med. 97, 29–36. https://doi.org/10.1016/j.prevetmed.2010.07.004

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Wells, S.J., Wagner, B.A., 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am.Vet. Med. Assoc. 216, 1450–1457. https://doi.org/doi:10.2460/javma.2000.216.1450

Whittington, R.J., Taragel, C.A., Ottaway, S., Marsh, I., Seaman, J., Fridriksdottir, V., 2001. Molecular epidemiological confirmation and circumstances of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. Vet. Microbiol. 79, 311–322. https://doi.org/10.1016/S0378-1135(00)00364-3

Whittington, R., 2010. Cultivation of *Mycobacterium avium* subsp. *paratuberculosis*. In: Paratuberculosis: Organism, Disease, Control. CAB International,, Oxfordshire, England, pp. 244-266.

Wolf, R., Barkema, H.W., De Buck, J., Slomp, M., Flaig, J., Haupstein, D., Pickel, C., Orsel, K., 2014. High herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Western Canadian dairy farms, based on environmental sampling. J. Dairy Sci. 97, 6250–6259. https://doi.org/10.3168/jds.2014-8101

Wolf, R., Barkema, H.W., De Buck, J., Orsel, K., 2015. Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results. J. Dairy Sci. 98, 275–287. https://doi.org/10.3168/jds.2014-8676

Wolf, R., Barkema, H.W., De Buck, J., Orsel, K., 2016. Dairy farms testing positive for *Mycobacterium avium* ssp. *paratuberculosis* have poorer hygiene practices and are less cautious when purchasing cattle than test-negative herds. J. Dairy Sci. 99, 4526–4536. https://doi.org/10.3168/jds.2015-10476

Chapter two

The present article was constructed to accomplish the specific objectives 1 (determine the presence of MAP in dairy herds using environmental sampling and real-time-PCR) and 4 (determine herd-level risk factors for MAP real-time-PCR positivity using multivariate analysis) in dairy herds under mechanical milking parlor and pasture grazing-based systems of the Northern region of the Province of Antioquia (Colombia). The manuscript is under review by the Veterinary Research Communications (submitted in June 2019).

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Breed and its association with Mycobacterium avium subsp. paratuberculosis infection on dairy herds under mechanical milking parlor-systems in the Northern Antioquia, Colombia

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Abstract

Paratuberculosis or Johne's disease (JD) is a chronic enteritis of ruminants caused by *Mycobacterium avium* subsp *paratuberculosis* (MAP). This study aimed to determine MAP herd-level prevalence according to environmental samples and to explore the herd-level risk factors

associated to MAP infection in dairy herds under mechanical milking parlor and pasture grazing-based systems in the Northern Antioquia, Colombia. The study herds (n = 94) were located in 60 different districts from five municipalities. Participant herds were visited once between June and October 2016 to collect two composite environmental samples and to complete a risk assessment questionnaire. Identification of MAP was achieved using a duplex quantitative real-time PCR method (IS*900*-qPCR; Bactotype MAP PCR Kit®, Qiagen). A herd was considered as MAP-infected if one or both of the environmental samples were found positive by the molecular technique. The information on risk factors was analyzed using a multivariable logistic regression model. The apparent herd-level prevalence found was 14.9% (14/94; 95% CI: 7.7-22.1), ranging from 0 to 33.3% at municipality-level. Herds where other than Holstein breeds were predominant (namely, Jersey, Jersey×Holstein crossbreeds, and Jersey×Swedish red crossbreeds) were more likely to be MAP-qPCR positively infected using environmental sampling than those on which Holstein was predominant (OR = 3.7; 95% CI: 1.1-15.2). Our study reports MAP prevalence in dairy herds under mechanical milking parlor and pasture grazing-based systems in the Province of Antioquia (Colombia), and the possible association between MAP environmental positivity in such herds with the predominant breed of cattle in the herd.

Keywords: environmental sampling, Holstein, Johne's disease.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an obligate intracellular pathogen of several mammals and the causal agent of Johne´s disease (JD) (Sweeney 1996). MAP is resistant to both environmental and chemical changes and can persist in the environment, including soil, stream water, and manure slurry storage, for up to one year (Whittington et al. 2005; Elliott et al. 2014). The JD refers to a chronic granulomatous enterocolitis and regional lymphangitis and lymphadenitis, characterized by a slow-developing course (Clarke 1997). The JD has a worldwide distribution and causes production losses (Ott et al. 1999; Kudahl et al. 2007; McAloon et al. 2016).

Several diagnostic tests can be used to detect MAP, and each one presents advantages and disadvantages, depending on the matrix and the different stages of the infection and subsequent illness (Nielsen and Toft 2008; Stevenson 2010). Polymerase chain reaction (PCR) on environmental samples has been suggested for herd screening (Collins et al. 2006; Stevenson 2010; USDA 2010), with comparable results to culture (Douarre et al. 2010). The sensitivity (Se) of PCR can vary due to the irregular fecal shedding organisms, whereas its specificity (Sp) is close to 100% in all stages of the disease (Eamens et al. 2000; Sweeney et al. 2012). PCR is also a rapid method (2-3 days) and highly specific (close to 99%; analytical and diagnostic-related), and no additional tests are required to confirm the identity of the organism detected (Collins et al. 2006). Quantitative real-time-PCR method (qPCR) has been found to be a sensitive (≈ 60%) and specific (≈ 97%) procedure (Aly and Mangold 2010; Logar et al. 2012), which enables both detection and accurate quantification of a specific target sequence of MAP directly in milk and fecal samples (Alinovi et al. 2009; Donat et al. 2015; Soumya et al. 2009). The analysis of environmental samples using PCR is considered nowadays as a cost-saving and easy-to-use approach to diagnose JD at herd-level and to classify the herd as infected or not, since it does not require sample collection from individual animals, reducing the inherent stress of the sampling process (Donat et al. 2015; Wolf et al. 2015).

The JD herd-level prevalence worldwide seems to be >18%, with reports much higher than 50% (Wells and Wagner 2000; Nielsen and Toft 2009; Fernández-Silva et al. 2014). In Colombia, JD was first reported in cattle in 1924 (Vega 1947). According to previous estimations in dairy cattle from different provinces of the country, apparent herd-level seroprevalence appeared to be >50% based on both unabsorbed and absorbed ELISA tests (Fernández-Silva et al. 2011; Benavides et al. 2016). Nevertheless, other studies have reported lower prevalences [3.6 and 4.1% by Correa-Valencia et al. (2016) and Correa-Valencia et al. (2019; unpublished results), respectively]. It seems that the range of possible results about prevalence estimation in the country is wide, and depends largely on the characteristics of the diagnostic test used and the tested population.

It is important to considerer that some herd-level management practices may vary between countries or agro-ecological zones, or also between regions or herds (Doré et al. 2012; Rangel et al. 2015), leading to the need of a local definition of the specific factors associated to the disease. Different herd characteristics have been reported worldwide, known to influence MAP or JD-status in dairy cattle, including herd size (Muskens et al. 2003; Hirst et al. 2004; Tavornpanich et al. 2008; Bolton et al. 2011; Wolf et al. 2016; Corbett et al. 2018a, b), predominant breed in the herd (Çetinkaya et al. 1997; Jakobsen et al. 2000), purchasing herd practices (Pillars et al. 2009; Tiwari et al. 2009; Wells and Wagner 2000; Sorge et al. 2012; Wolf et al. 2016), and pasture fertilization (Goodger et al. 1996; Daniels et al. 2002; Wolf et al. 2016). Also, some herd management practices have been reported acting as triggers for MAP infection, including contact of young with adult cattle (Goodger et al. 1996; Obasanjo et al. 1997; Dieguez et al. 2008; Tiwari et al. 2009), and colostrum and milk feed to calves (Nielsen et al. 2008). In Colombia, data regarding animal or herd-level risk factors associated with JD in dairy herds are still limited and only explored in the last three years.

Therefore, the aim of this cross-sectional study was to determine MAP herd-level prevalence according to IS*900*-qPCR results on environmental samples and to explore herd-level risk factors associated to MAP infection in dairy herds under mechanical milking parlor-systems of the Northern region of the Province of Antioquia (Colombia).

Materials and methods

Study design and herd selection

A cross-sectional study was carried out from July to October 2016 in the Northern dairy region of the Province of Antioquia (Colombia). Study herds were located in districts of five municipalities (San Pedro de Los Milagros, Entrerríos, Santa Rosa de Osos, Donmatías, and Belmira), known for their considerable volumes of dairy production. The study area is located between 1,090 and 2,979 meters above sea level, and the temperature ranges from 12 to 16°C

during the year. According to the Caldas-Lang climate classification, Santa Rosa de Osos, San Pedro de Los Milagros, Entrerríos, and Donmatías municipalities are classified as cold-humid, and Belmira municipality as cold-very humid (Gobernación de Antioquia 2016). The herd was considered as the unit of analysis.

A probabilistic design using a simple random sampling strategy with restitution and without replacement was performed. The sample size was defined according to the formula for prevalence estimation from a finite population (Dohoo et al. 2014). The formula included an *a priori* JD-prevalence proportion estimation of 0.118 (11.8%) from a previous report in the study region (Correa-Valencia et al. 2019; unpublished results) —achieved under similar methodological conditions and population and a maximum acceptable error rate of 7%. The sampling frame was 7,794 herds registered on the foot-and-mouth disease vaccination records of the six municipalities of interest (Fedegán 2015). From the registered herds, 94 herds in 60 districts were randomly selected, according to sampling strategy and inclusion criteria.

Districts to be sampled into each municipality were established according to the specific weight of each district inside its corresponding municipality, only considering the largest districts until the sum of their census accounted for the 70% of the adult cattle population in each municipality. In all cases, herds had to fulfill the following criteria to be finally enrolled in the study: Having adult cattle, mechanical milking in parlor facilities and pasture grazing-based systems, geographic accessibility, no previous history or report of JD or MAP detection by any method, and willingness of the owner to participate (*i.e.* allowing the sampling of all necessary areas in the herd and giving information regarding herd characteristics and management practices).

Sample collection

Environmental sampling was carried out as reported by the literature (Raizman et al. 2004; Collins et al. 2006; Pillars et al. 2009; Kruze et al. 2013; Donat et al. 2015; Wolf et al. 2015; Corbett et al. 2018), with some modifications due to differences in management systems and facilities in the study region (*e.g.* maternity, quarantine and/or nursing area not always existing) and due to budget restrictions.

Each participating herd was visited once during the study period to collect two composite environmental samples. The first one contained material from at least six different sites (subsamples) of concentration of adult cattle and/or high traffic areas (*e.g.* paddocks, areas surrounding waterers and feeders, alleyways, gutters, milking parlor holding areas). Each subsample was collected taking into account that the feces were not previously exposed to direct sunlight. The second one contained manure from the milking parlor collected in the manure storage lagoon, after mixing its content for at least 5 min. The six subsamples from the second place were obtained from different places of the perimeter of the lagoon by submerging the sampling container up to 10 cm beneath the surface. Each environmental sample was collected using a clean latex glove. Subsamples of each of the two collection places were pooled and manually mixed at the farm. Then, approximately 20 g of each of the two pooled samples (separately) was placed into a plastic container labeled with the herd number and collection date and place (first place: Concentration of adult cattle and/or high traffic areas or second place: Manure storage lagoon) to constitute the definitive material for each of the two composite environmental sample per herd. Definitive samples were conserved refrigerated at 4 °C during transport to the laboratory, where they were homogenized for 5 min each one and then frozen at -20˚C until DNA extraction, from a minimum of 41 to a maximum of 245 days.

Questionnaire and strategy of information collection

The questionnaire used for the collection of information in the study reported by Correa-Valencia et al. (2019; unpublished results) was used herein. Briefly, a one-page questionnaire (available upon request) was administered to the herd owner or herdsman present at the time of the samples collection in a face-to-face interview. Information on herd characteristics, management practices, and knowledge about the disease was collected, considering the same subjects as sections of the questionnaire. Three different people, previously trained for the task, administered the questionnaire. All information collection procedures were pre-tested at a small scale to evaluate their effectiveness as has been previously recommended by the literature (Dohoo et al. 2014).

Laboratory analysis

The DNA extraction and IS*900*-qPCR were carried out as previously reported by Correa-Valencia et al. (2019; unpublished results). Briefly, DNA isolation was carried out using a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, CA, USA). The protocol included a bead-beating *prior* step (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA). A NanoDrop 2000® spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the purity and yield of nucleic acids. DNA integrity was confirmed using an only-agarose gel on a representative sub-sample of each extraction batch (10%). DNA extraction efficiency was confirmed by PCR using bacterial constitutive genes to the same sub-samples mentioned above (Weisburg et al.1991; Inokuma et al. 2001). The extracted DNA was conserved at -20 °C until qPCR analysis using a duplex IS*900*-qPCR. The sample was considered positive when it emitted a signal on the FAM and MAX channels or strongly positive if only emits a signal on the FAM channel with a Ct ≤ 40 and a sigmoid-pattern curve result (according to MIQE guidelines; Bustin et al. 2009).

Statistical analysis

The outcome variable was herd status (positive/negative) as determined by IS900-qPCR on environmental samples. All the information generated during the study was entered into Excel worksheets (Microsoft Corp., Redmond, WA, USA) and then exported to Stata 15.0 (StataCorp, 2017, College Station, Texas, USA) for statistical analysis. Descriptive statistics were computed for all the variables of interest. Variables were checked for more than 30% missing values, a case in which they were not considered for further analysis. Categorical variables with multiple answers were dichotomized according to risk and distribution. A complex design analysis was conducted according to a cluster effect by district and the stratified nature of the study using the *Survey* command. Univariable analysis was performed to assess unconditional associations between the outcome (MAP-herd status) and each independent predictor using simple logistic regression. Associations with a P ≤ 0.20 were considered for inclusion in the multivariable logistic regression model. Conditional associations were explored. Evaluation of potential confounders

was then performed by assessing the change in the β-coefficient of the variables of the adjusted model compared to the non-adjusted model. Confounders were only retained if a change greater than 15% was observed, regardless of the significance of the coefficient of the confounding variable in the model. The variables to be explored as confounders (i.e. herd size, predominant breed) were considered according to literature. Biologically plausible interactions were studied between significant variables from the multivariable models, as well as the 2-way interactions between significant predictors with a significant unconditional association with the dependent variable. Selection of the independent variables included in the final model was performed based on statistical considerations using a backward stepwise procedure with P-values of entry and removal of 0.2 and 0.25, respectively. The results from the final model are presented as odds ratios (OR) with 95% CIs. The model fit was assessed using the Hosmer-Lemeshow goodness-of-fit test (Dohoo et al. 2014).

Results

Two environmental samples were collected from each of 94 dairy herds under mechanical milking parlor and pasture grazing-based systems, located in 60 districts of five municipalities of the Province of Antioquia (Colombia). None of the herds were housed or semi-housed. The 2.1% (2/94) of the herds primarily selected as potentially eligible for the study did not agree to participate when contacted by phone and a 6% of the contact phone numbers were out of service or not registered. Two herds fulfilling the inclusion criteria replaced these herds. According to the foot-and-mouth disease vaccination records, from where the sampling frame was obtained, the non-participating herds were big dairy herds (>30 milking cows) for the Colombian context and were mainly located in the municipalities of Donmatías and Entrerríos. Those who refused to participate in the study supported their decision with a reason related to the detection of bovine tuberculosis in their herds, even after the differences between diseases and causal agents were explained. After signing informed consent, all herds that agreed to participate by phone also allowed the sampling and the questionnaire fulfillment. Authors also subscribed a non-disclosure statement with owners to keep the anonymity of the name of the herd owners and herd results.

Herd characteristics

The study population was mainly composed of big herds (>30 milking cows) with access to veterinary assistance, and Holstein as the predominant breed. Most of the herds were classified as closed according to cattle purchasing and co-grazing practices in the last 10 years. The presence of other ruminants in the herd during the last 2 years was a minor practice. Most of the herds were neither tuberculosis-free nor good farming practices (GFP) certified at the time of sampling. The GFP certification explored in this study included management practices which can be considered PTB-related, such as fertilization strategies (*i.e.* organic, inorganic), other animal species in the farm (*e.g.* goats, sheep, buffaloes), and tuberculosis sanitation status (ICA, 2007). Most of the interviewed herd owners or herdsmen reported not having heard about the disease before and have not seen JD-symptomatic animals (diarrhea and progressive weight loss refractory to treatment) in their herds in the last 2 years. Herd characteristics explored and considered as predictors for the risk factor assessment are presented in Table 1.

Herd management practices

Manure spreading on pastures as a method of fertilization was found to be a common practice by most of the herds. Allowing calves to spend extended time with the dams (≥ 2 days after calving) was found in most of the cases. Nose-to-nose contact between adult cattle and ≤ 6 months-old calves was not allowed in most of the herds. Own-dam's colostrum was the only source used to feed pre-weaned calves in the study herds, and milk without antibiotics and milk replacer represented the main feed source for the same population. Herd-level management practices explored and considered as predictors for the risk factor assessment are presented in Table 2.

Table 1. Herd characteristics in dairy herds of the Northern region in the Province of Antioquia, Colombia (2016).

Herd characteristic	Categories	Positive herds (n)	Negative herds (n)	N	Distribution (%)	OR [95% CI]	*p*-value
Herd size	≤ 30	2	15	17	18.1		
(number of lactating cows)	>30	12	65	77	81.9	1.4 [0.3-6.9]	0.514
				94			
Predominant breed	Holstein	10	71	81	86.2		
(the most common cattle breed in the herd)	Other[a]	4	9	13	13.8	3.2 [0.8-12.2]	0.096*
				94			
Veterinary assistance	Yes	12	73	85	90.4		
(availability of veterinarian assistance in the farm)	No	2	7	9	9.6	0.6 [0.1-3.1]	0.871
				94			
Purchasing practices	Yes	4	37	41	43.6		
(use of external replacement cattle in the last 10 years)	No	10	43	53	56.4	0.5 [0.1-1.6]	0.226
				94			
Outsider animals grazing in own pastures	Yes	1	1	2	2.1		
	No	13	79	92	97.9	6.1 [0.4-103.3]	0.262
				94			
Own animals grazing in non-proper pastures	Yes	0	4	4	4.3		
	No	14	76	90	95.7	0	Inestimable
				94			
Mixed farming in the last 2 years	Yes	3	17	20	21.3		0.938
(cattle co-existence with goats, sheep, and/or buffaloes in the last 2 years)	No	11	63	74	87.7	1.0 [0.3-4.0]	
				94			

Type of ruminants co-existing with the cattle in the last 2 years	Goats	2	8	10	10.6	5.7 [1.5-20.9]	0.099
	Sheep	0	8	8	8.5	0	Inestimable
	Sheep and goats	1	1	2	2.2	1.4 [0.2-11.6]	0.773
	Not applicable	11	63	74	78.7		
				94			
Good farming practices status (herd certified by the ICA as a GFP certified herd)	Yes	9	33	42	44.7		
	No	5	47	52	55.3	2.6 [0.8-8.4]	0.111*
				94			
Bovine tuberculosis status (herd certified by the ICA as free from bovine tuberculosis)	Yes	11	57	68	72.3		
	No	3	23	26	27.7	1.5 [0.4-5.8]	0.572
				94			
Producer´s knowledge about the disease	Some[b]	4	0	14	14.9		
	Never heard about it before	10	70	80	85.1	0.4 [0.1-1.4]	0.119*
				94			
PTB-compatible symptoms´ history (report of animals with compatible JD symptoms in the herd)	Yes[c]	2	15	17	18.1		
	Never	12	65	77	81.9	1.4 [0.3-6.9]	0.688
				94			

ICA: Instituto Colombiano Agropecuario. OR: Odds Ratio. CI: Confidence interval.

[a] Includes: Jersey, Swedish red, and crossbreeds.

[b] Includes: Recognize the name only, some basics, and fairly knowledgeable.

[c] Includes: At present and/or in the last 2 years.

* Variables used for the multivariable analysis ($p \leq 0.20$).

Table 2. Herd management practices in dairy herds of the Northern region in the Province of Antioquia, Colombia (2016).

Herd management practice	Categories	Positive herds (n)	Negative herds (n)	N	Distribution (%)	OR [95%CI]	*p*-value
Manure spreading	Yes	14	77	91	96.8		
(use of cow manure as a fertilizer	No	0	3	3	3.2	0	Inestimable
in the pastures)				94			
Typical time of separation	≤ 1	4	17	21	22.3	0.68 [0.2-2.4]	0.544
(separation of the newborn calf	≥ 2	10	63	73	77.4		
from their dam after birth, in days)				94			
Calves ≤ 6 months old sharing	Yes	2	10	12	12.8		
spaces with adult cows	No	12	70	82	87.2	1.2 [0.2-6.0]	0.854
(nose-to-nose contact)				94			
Colostrum fed to calves	From multiple cows	0	0	0	-	0	Inestimable
(source)	From its own dam	14	80	94	100.0		
				94			
Milk fed to unweaned calves	Unsalable milk	5	30	35	37.2		
(source)	Other sources[a]	9	50	59	67.8	1.08 [0.3-3.5]	0.899
				94			

OR: Odds Ratio. CI: Confidence interval.

[a] Includes: Milk without antibiotic (salable milk) and milk replacer.

Herd-level apparent prevalence

Fourteen herds out of 94 were positive to the IS*900*-qPCR (14.9%; 95% CI: 7.7-22.1). Apparent prevalence at municipality-level, ranging from 0 to 33.3%, is shown in Table 3.

Table 3. *Mycobacterium avium* subsp. *paratuberculosis*-apparent prevalence at municipality-level in the Province of Antioquia, Colombia (2016) using qPCR on environmental samples.

Municipality	**Sample weight*** **(%)**	**Herds of study (n)**	**N of positive herds (n) [%]**
San Pedro de los Milagros	41.5	39	4 [10.3]
Entrerríos	26.6	25	6 [24.0]
Santa Rosa de Osos	16.0	15	0 [-]
Donmatías	12.7	12	4 [33.3]
Belmira	3.2	3	0 [-]
Total	100	94	14 [14.9]

*According to foot-and-mouth disease vaccination records (Fedegán 2015).

Risk factors assessment

Among the 17 risk factors explored in the univariable analysis (12 herd characteristics and five management practices), three were associated with the IS*900*-qPCR MAP-herd status ($p \leq 0.20$; Tables 1 and 2). These variables were selected for the multivariable analysis.

The variables "*own animals grazing in foreign pastures*", "*manure spreading*", and "*colostrum fed to calves*" were excluded from all logistic regressions. These variables had an average of "0" among herds with a MAP positive status and the maximum likelihood estimation procedure in the logistic regression analysis would not converge if the variable was included. The variables "*herd size*" and "c*attle purchasing practices*" were considered as potential confounders. The interaction between significant predictors in the final model was also explored. The relative change in the coefficients was >15%, so none of the confounders considered herein were furtherly explored. A final model was built (Table 4). In the best fit of the model ($p = 0.70$), having

a predominant breed other than Holstein in the herd was significantly associated with a positive MAP-herd status as determined by qPCR performed on environmental samples (OR = 3.7; 95% CI: 1.1-15.2).

Table 4. Final multivariable logistic regression model to identify herd characteristics and management practices associated with a positive *Mycobacterium avium* subsp. *paratuberculosis* status determined by IS*900*-qPCR on environmental samples in 94 herds located in five municipalities of the Province of Antioquia, Colombia (2016).

Risk factor	**OR [95% CI]**	**Standard error**	***p*-value**
Predominant breed			
Holstein	Reference		
Other	3.7 [1.1-15.2]	2.7	0.045
GFP status			
No	Reference		
Yes	2.6 [0.8-9.1]	1.7	0.172
Producer´s knowledge about the disease			
Some	Reference		
Never heard about it before	0.4 [0.1-1.6]	0.3	0.183

OR: Odds Ratio. CI: Confidence interval.

Discussion

This study was carried out to determine MAP herd-level prevalence according to environmental samples in 94 herds. In addition, the study was carried out to explore the herd-level risk factors associated with MAP infection in dairy herds under mechanical milking parlor and pasture grazing-based systems in the Northern Antioquia, Colombia. To the authors´ knowledge, this is the first approach using a regional-scale environmental sampling and MAP-qPCR to determine herd-level infection status in the country so far, considering the milking system as an inclusion and differential criterion for the study population. In the present study, all herds found positive to MAP-qPCR were considered as infected, based on the fact that a MAP-elimination source leads

to environmental fecal contamination, and therefore to the risk of ingestion by susceptible cattle (Elliott et al. 2015).

Our study found that farms where other than Holstein breeds were predominant (namely Jersey, Jersey×Holstein crossbreeds, and Jersey×Swedish red crossbreeds) were more likely to be MAP-qPCR positively infected using environmental sampling compared to those on which Holstein was predominant (OR = 3.7; $p = 0.045$). An apparent higher susceptibility to JD for other than Holstein-breed dairy cows have been reported in previous studies (NcNab et al. 1991; Çetinkaya et al. 1997; Jakobsen et al. 2000). Contrastively, Jaramillo-Moreno et al. (2017) found an association between MAP-seropositive findings and breed on a JD-endemic dairy herd in the municipality of San Pedro de los Milagros, were the 87.2% (72/83) of the adult population were Holstein and the rest of the herd were from other than Holstein breeds (not specified). However, such results are difficult to compare to ours, given the limited population considered of the latter study.

Correa-Valencia et al. (2019; unpublished results), found that herds with a history of mixed farming of cattle with other ruminants had higher odds of being MAP infected than herds without (OR = 3.9), it can be said that no explanation can be sustained about that respect, given the familiarity that the authors have with the population and the productive system of the present report. The practice of having other ruminants (preferably small species such as goats or sheep) and the breed tendency reported herein are general phenomena for both milking systems.

Some variables that we hypothesized to be of significant risk and previously identified as such by other studies for the herd-level assessment using different diagnosis approaches, were not significant in the logistic regression analysis, including "*own animals grazing in foreign pastures*", "*manure spreading*", and "*colostrum fed to calves*". Nevertheless, special attention should be given to the fact that the dairies with a manure storage lagoon (all of our study herds) have a greater tendency to practice fertilization of pastures with cow manure —in our specific case, the 96.8% of the herds (91/94), since they have the storage structure for that end.

Although previous studies have reported that the highest probability of a MAP positive-herd is observed in large herds, no relationship was found in our study. However, the role of "*herd size*", as well of "c*attle purchasing practices*" as confounders were investigated by fitting models considering MAP-IS*900*-qPCR positive results with and without these variables (independently included). The purchase of infected cattle is considered the primary way of JD transmission between herds (Sweeney 1996). Therefore, several studies have reported an association between purchase policies and MAP-herd-level status (Wells and Wagner 2000; Hirst et al. 2004; Pillars et al. 2009; Ridge et al. 2010; Vilar et al. 2015; Puerto-Parada et al. 2018). Having cattle purchasing from other herds without knowledge of their disease status as a frequent herd practice, increased the risk for MAP culture-positive environmental samples (Wolf et al. 2016). This practice was explored from three points of view in the present study ("*foreign animals grazing in own pastures*", "*own animals grazing in foreign pastures*", and "*cattle purchasing practices*") and were grouped as part of the classification of the herd as *open* or *closed,* being the second group the most representative one, factor that could be consider as protective in this specific case.

In our study, 10 producers that had not heard of JD and 12 reporting never have had clinical cases of the disease had MAP-qPCR positive environmental samples, suggesting that clinical disease was not occurring, or at least was not recognized in their herds. Moreover, only two of the 14 positive herds do not have access to veterinary assistance, and then, may not have information available when compatible cases of the disease were presented in the herd.

Milk and colostrum can be contaminated with MAP, either through fecal contamination of teats or shedding from the udder (Nielsen et al. 2008). From a local point of view, Fernández-Silva et al. (2016) reported that the odds of being a seropositive herd were lower in those feeding calves with pooled colostrum from several cows compared to those to herds feeding calves with colostrum from their own dams. This previous study was carried out on 14 dairies, located in the municipalities of Belmira and San Pedro de los Milagros (Province of Antioquia), two of the five municipalities included in our study. Their results are in contrast to previous knowledge of the risk of being seropositive represented by the use of colostrum from multiple cows *vs* own dam´s

(Nielsen et al. 2008). Our results reported that all the colostrum given to the calves is from their own dams (100% of the herds). Other studies have related feeding antibiotic-contaminated or other waste milk to calves to be a significant risk factor for MAP transmission (Ridge et al. 2005). Our results indicated that milk replacers and salable milk (without antibiotics) were the main sources used to feed unweaned calves. Nevertheless, according to the authors´ experience, it is still a common practice to use discarded milk to feed the calves. Feeding practices in dairies are closely related to postpartum husbandry practices, such as the practice of leaving a cow with her calf after birth, which was also representative of the herds of our study and has been previously reported as a risk factor (Goodger et al. 1996; Obasanjo et al. 1997; Ansari-Lari et al. 2009), increasing the odds of within-herd transmission of MAP.

The apparent MAP herd-level prevalence of 14.9% estimated in the present study (ranging from 0-33.3% at municipality-level), seems to be lower than the prevalence found in cattle by other authors in European, North American, and Latin American and Caribbean countries (Wells and Wagner 2000; Nielsen and Toft 2009; Fernández-Silva et al. 2014). Scientific literature hypothesizes that up-to-date MAP-herd-level prevalence is increasing in some countries, such as Colombia, that do not have mandatory control programs (Salem et al. 2012) but not enough data can support that statement. Similarly, results from a recent study carried out in the same region, found an apparent prevalence of 4.1% (Correa-Valencia et al. 2019; unpublished results). Differences in both prevalence estimations could be due, hypothetically, to a higher metabolic load and consequent stress for individuals, which must walk at least twice a day to and from the milking parlor. This could be translated into a compromised immunity that could favor the success of intestinal colonization by MAP, the formation of granulomatous lesions, and the consequent elimination of the agent to the environment (Clarke 1997; Nielsen et al. 2002; Fecteau and Whitlock 2010). In addition, the higher apparent prevalence could be due to a higher probability of detection of positive herds in the environment when two samples of each are collected, as followed in our case (Wolf et al. 2014; Donat et al. 2015). These proposed arguments need further research approaches.

A well-defined place known as suitable to yield a herd-level MAP-positive result is the manure storage lagoon (Lombard et al. 2006; Pillars et al. 2009). Its considerations in the study region seem not to be a representative feature of the local dairy systems, taking into account that only 1 of 4 dairies in the Province has this kind of building (Fedegán 2015). Nevertheless, they were a representative source of the positive findings, since 8 of the 14 herds found as MAP-qPCR positive were detected using the samples from the manure storage lagoons, whereas five of the positives were from the adult cattle concentration and/or high traffic area, and one from both sampling places. This may be an additional explanation when comparing our prevalence result with that reported by Correa-Valencia et al. (2019; unpublished results), as previously mentioned.

Misclassification biases of herds with low within-herd prevalence as negative are not likely to occur because it was controlled considering the Se and Sp of the PCR method used and because the laboratory procedures were done by the same trained person (as well as DNA extraction). Selective entry bias may be presented since only registered herds, according to foot-and-mouth disease vaccination records (Fedegán 2015), and following *a priori* inclusion criteria were considered as the source for eligible herds. Nevertheless, considering that this vaccination is mandatory in the country, it is unlikely that any herd remains outside the registry. Selection biases were not likely to occur because of randomization and representativeness of the target population in the source and study populations; non-response biases, by the replacement sampling selection; detection biases, by inclusion and exclusion criteria established; and, missing data biases, by the fact we applied a face-to-face interview at the moment of sampling, assuring the complete fulfilling of the questionnaire. Inter-observer variability could induce bias. However, the impact of this bias was considered low, as all interviewers received standardized training and sample collection followed a strict protocol outlined in an instruction sheet that accompanied every sampling kit. The effect of the Sp of the method is important in this case because of the low prevalence found. No duplicated run or confirmations to PCR were available because of budget restrictions.

In conclusion, the apparent prevalence found in the herds of study was 14.9%. In addition, our study found that farms where other than Holstein breeds were predominant (namely, Jersey, Jersey×Holstein crossbreeds, and Jersey×Swedish red crossbreeds) were more likely to be MAP-qPCR positively infected using environmental sampling than those on which Holstein was predominant. This feature could be considered for JD´s control, particularly in typical dairies in Colombia under the same facilities and management practices than the ones considered herein.

Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

This research was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act number 71, June 15th, 2011).

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References

Alinovi CA, Ward MP, Lin TL, Moore GE, Wu CC (2009) Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. *paratuberculosis*. Vet Microbiol 14, 136:177–179. https://10.1016/j.vetmic.2008.10.012

Aly SS, Mangold BL, Whitlock RH, Sweeney RW, Anderson RJ, Jiang J, Schukken YH, Hovingh E, Wolfgang D, Van Kessel JA, Karns JS, Lombard JE, Smith JM, Gardner IA (2010) Correlation between Herrold egg yolk medium culture and real-time quantitative polymerase chain reaction results for *Mycobacterium avium* subspecies *paratuberculosis* in pooled fecal and environmental samples. J Vet Diagn Invest 22(5):677–683.

Ansari-Lari M, Haghkhah M, Bahramy A, Novin Baheran AM (2009) Risk factors for *Mycobacterium avium* subspecies *paratuberculosis* in Fars province (Southern Iran) dairy herds. Trop Anim Health Prod 41:553–557. https://doi.org/10.1007/s11250-008-9221-7

Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten VPMG, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ, Mackintosh CG, Manning EJ, Weber MF, Heuer C, Forde TL, Ritter C, Roche S, Corbett CS, Wolf R, Griebel PJ, Kastelic JP, De Buck J (2017) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transbound Emerg Dis 65(Suppl. 1):1–24. https://doi.org/10.1111/tbed.12723

Benavides B, Ángela B, Arteaga V, Carlos C, Montezuma A (2016) Estudio epidemiológico de paratuberculosis bovina en hatos lecheros del sur de Nariño, Colombia. Rev. Med. Vet. (Bogota). 31:57–66.

Bolton MW, Pillars RB, Kaneene JB, Mauer WA, Grooms DL (2011) Detection of *Mycobacterium avium subspecies paratubercu*losis in naturally exposed dairy heifers and associated risk factors. J Dairy Sci 94:4669–4675. https://doi.org/10.3168/jds.2011-4158

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: Minimum Information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797

Çetinkaya B, Erdogan H, Morgan K (1997) Relationships between the presence of Johne's disease and farm and management factors in dairy cattle in England. Prev Vet Med 32:253–266. https://doi.org/10.1016/S0167-5877(97)00028-7

Clarke CJ (1997) The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Pathol 116: 217–261.

Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ (2006) Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. J Am Vet Med Assoc 229:1912–1919. https://doi.org/10.2460/javma.229.12.1912

Corbett CS, Naqvi SA, Bauman CA, De Buck J, Orsel K, Uehlinger F, Kelton DF, Barkema HW (2018a) Prevalence of *Mycobacterium avium* ssp. *paratuberculosis* infections in Canadian dairy herds. J Dairy Sci 101(12):11218–11228. https://doi.org/10.3168/jds.2018-14854

Corbett CS, Naqvi SA, De Buck J, Kanevets U, Kastelic JP, Barkema HW (2018b) Environmental sample characteristics and herd size associated with decreased herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis*. J Dairy Sci 101(9):8092–8099. https://doi: 10.3168/jds.2018-14661.

Correa-Valencia NM, Ramírez NF, Olivera M, Fernández-Silva JA (2016) 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. Trop Anim Health Prod 48:1191–1200. https://doi.org/10.1007/s11250-016-1074-x

Daniels MJ, Hutchings MR, Allcroft DJ, McKendrick J, Greig A (2002) Risk factors for Johne's disease in Scotland--the results of a survey of farmers. Vet Rec 150:135–139.

Dieguez FJ, Arnaiz I, Sanjuan ML, Vilar MJ, Yus E (2008) Management practices associated with *Mycobacterium avium* subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. Vet Rec 162:614–617. https://doi.org/10.1136/vr.162.19.614

Dohoo I, Martin W, Stryhn H (2014) Veterinary Epidemiologic Research. 8 Berkeley Way, Charlottetown, Prince Edward Island, Canada, VER Inc.

Donat K, Kube J, Dressel J, Einax E, Pfeffer M, Failing K (2015) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiol Infect 143:975–985. https://doi.org/10.1017/S0950268814002465

Doré E, Paré J, Côté G, Buczinski S, Labrecque O, Roy JP, Fecteau G (2012) Risk factors associated with transmission of *Mycobacterium avium* subsp. *paratuberculosis* to calves within dairy herd: A systematic review. J Vet Intern Med 26:32–45. https://doi.org/10.1111/j.1939-1676.2011.00854.x

Douarre PE, Cashman W, Buckley J, Coffey A, O'Mahony JM (2010) Isolation and detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from cattle in Ireland using both traditional culture and molecular based methods. Gut Pathog 2:1–7. https://doi.org/10.1186/1757-4749-2-11

Eamens GJ, Whittington RJ, Marsh IB, Turner MJ, Saunders V, Kemsley PD, Rayward D (2000) Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. Vet Microbiol 77:357–367. https://doi.org/10.1016/S0378-1135(00)00321-7

Elliott GN, Hough RL, Avery LM, Maltin CA, Campbell CD (2014) Environmental risk factors in the incidence of Johne's disease. Crit Rev Microbiol 7828:1–20. https://doi.org/10.3109/1040841X.2013.867830

Elliott GN, Hough RL, Avery LM, Maltin CA, Campbell CD (2015) Environmental risk factors in the incidence of Johnes disease. Crit Rev Microbiol 41:488–507. https://doi.org/10.3109/1040841X.2013.867830

Fecteau M, Whitlock R (2010) Paratuberculosis in cattle. In: Paratuberculosis: Organism, disease, control. CAB International,, Oxfordshire, England, pp. 144–156.

Fedegán. Registro de vacunación, primer ciclo (2015). Official restricted access material.

Fernández-Silva J, Ramirez N, Correa N (2016) Factors associated with *Mycobacterium avium* subsp. *paratuberculosis* in dairy cows from Northern Antioquia, Colombia. Rev Colomb Ciencias Pecu 30:48–59. https://doi.org/10.17533/udea.rccp.v30n1a06

Fernández-Silva JA, Abdulmawjood A, Akineden Ö, Bülte M (2011) Serological and molecular detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle of dairy herds in Colombia. Trop Anim Health Prod 43:501–1507. https://doi.org/10.1007/s11250-011-9833-1

Fernández-Silva JA, Correa-Valencia NM, Ramírez NF (2014) Systematic review of the prevalence of paratuberculosis in cattle, sheep, and goats in Latin America and the Caribbean. Trop Anim Health Prod 46:1321–1340. https://doi.org/10.1007/s11250-014-0656-8

ICA. Instituto Colombiano Agropecuario (2007) Resolución 0002341 de 2007. URL: http://www.ica.gov.co/getattachment/0b5de556-cb4a-43a8-a27a-cd9a2064b1ab/2341.aspx. Accessed April 30 2018.

Lombard JE, Sweeney R, Smith DR, Gavalchin J, Eda S (2011) Consensus-based reporting standards for diagnostic test accuracy studies for paratuberculosis in ruminants. Prev Vet Med 101:18–34. https://doi.org/10.1016/j.prevetmed.2011.04.002

Gobernación de Antioquia, 2016. Fichas municipales de Antioquia (2015-2016) Colombia. http://www.antioquia.gov.co/planeacion/fichas_municipales_web/index.html Accessed September 8 2018.

Goodger WJ, Collins MT, Nordlund K, Eisele C, Pelletier J, Thomas CB, Sockett DC (1996) Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle. J Am Vet Med Assoc 208(11):1877–1881.

Hirst HL, Garry FB, Morley PS, Salman MD, Dinsmore RP, Wagner BA, McSweeney KD, Goodell GM (2004) Seroprevalence of *Mycobacterium avium* subsp *paratuberculosis* infection among dairy cows in Colorado and herd-level risk factors for seropositivity. J Am Vet Med Assoc 225:97–101. https://doi.org/10.2460/javma.2004.225.97

Inokuma H, Parola P, Raoult D, Brouqui P (2001) Molecular survey of *Ehrlichia* infection in ticks from animals in Yamaguchi Prefecture, Japan. Vet Parasitol 99:335–339. https://doi.org/10.1016/S0304-4017(01)00470-8

Jakobsen MB, Alban L, Nielsen SS (2000) A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. Prev Vet Med 46:15–27. https://doi.org/10.1016/S0167-5877(00)00138-0

Jaramillo-Moreno S, Montoya-Zuluaga MA, Uribe-Santa JS, Ramírez-Vásquez NF, Fernández-Silva JA (2017) Seroprevalencia de paratuberculosis (*Mycobacterium avium* subsp.*paratuberculosis*) en un hato de lechería especializada del altiplano norte de Antioquia, Colombia. Vet y Zootec 11:24–33. https://doi.org/10.17151/vetzo.2017.11.2.3

Jorgenson JB (1977) Survival of *Mycobacterium paratuberculosis* in slurry. Nord Vet Med 29(6):267–270.

Kruze J, Monti G, Schulze F, Mella A, Leiva S (2013) Herd-level prevalence of Map infection in dairy herds of southern Chile determined by culture of environmental fecal samples and bulk-tank milk qPCR. Prev Vet Med 111:319–324. https://doi.org/10.1016/j.prevetmed.2013.05.011

Kudahl AB, Østergaard S, Sørensen JT, Nielsen SS (2007) A stochastic model simulating paratuberculosis in a dairy herd. Prev Vet Med 78(2):97–117. https://doi.org/10.1016/j.prevetmed.2006.05.015

Logar K, Kopinč R, Bandelj P, Starič J, Lapanje A, Ocepek M (2012) Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in subclinically infected dairy cattle: Comparison with faecal culture, milk real-time PCR and milk ELISA. BMC Vet Res 8:1–10. https://doi.org/10.1186/1746-6148-8-49

Lombard JE, Wagner BA, Smith RL, McCluskey BJ, Harris BN, Payeur JB, Garry FB, Salman MD (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. J Dairy Sci 89:4163–4171. https://doi.org/10.3168/jds.S0022-0302(06)72461-4

McAloon CG, Whyte P, More SJ, Green MJ, O'Grady L, Garcia A, Doherty ML (2016) The effect of paratuberculosis on milk yield—A systematic review and meta-analysis. J Dairy Sci 99:1449–1460. https://doi.org/10.3168/jds.2015-10156

Muskens J, Elbers ARW, van Weering HJ, Noordhuizen JPTM (2003) Herd management practices associated with paratuberculosis seroprevalence in Dutch dairy herds. J Vet Med B Infect Dis Vet Public Health 50:372–7. https://doi.org/10.1046/j.1439-0450.2003.00697.x

NcNab WB, Meek AH, Duncan JR, Martin SW, Van Dreumel AA (1991) An epidemiological study of paratuberculosis in dairy cattle in Ontario: study design and prevalence estimates. Can J Vet Res 55(3):246–251.

Nielsen SS, Bjerre H, Toft N (2008) Colostrum and milk as risk factors for infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. J Dairy Sci 91:4610–4615. https://doi.org/10.3168/jds.2008-1272

Nielsen SS, Enevoldsen C, Gröhn YT (2002) The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev Vet Med 54:1–10. https://doi.org/10.1016/S0167-5877(02)00008-9

Nielsen SS, Toft N (2009) A review of prevalences of paratuberculosis in farmed animals in Europe. Prev Vet Med 88:1–14. https://doi.org/10.1016/j.prevetmed.2008.07.003

Nielsen SS, Toft N (2008) *Ante mort*em diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet Microbiol 22;129(3-4):217–235. https://doi.org/10.1016/j.vetmic.2007.12.011

Obasanjo IO, Gröhn YT, Mohammed HO, Grohn YT, Mohammed HO (1997) Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in dairy herds on the New York State Paratuberculosis Control Program. Prev Vet Med 32:243–251. https://doi.org/10.1016/S0167-5877(97)00027-5

Ott SL, Wells SJ, Wagner BA (1999) Herd-level economic losses associated with Johne's disease on US dairy operations. Prev Vet Med 40, 179–192. https://doi.org/10.1016/S0167-5877(99)00037-9

Pillars RB, Grooms DL, Kaneene JB (2009) Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project. Can Vet J 50; 1039–1046.

Pillars RB, Grooms DL, Woltanski JA, Blair E (2009) Prevalence of Michigan dairy herds infected with *Mycobacterium avium* subspecies *paratuberculosis* as determined by environmental sampling. Prev Vet Med 89:191–196. https://doi.org/10.1016/j.prevetmed.2009.02.022

Pinedo PJ, Buergelt CD, Donovan GA, Melendez P, Morel L, Wu R, Langaee TY, Rae DO (2009) Candidate gene polymorphisms (BoIFNG, TLR4, SLC11A1) as risk factors for paratuberculosis infection in cattle. Prev Vet Med 91(2-4):189–196 https://doi.org/10.1016/j.prevetmed.2009.05.020

Puerto-Parada M, Arango-Sabogal JC, Paré J, Doré E, Côté G, Wellemans V, Buczinski S, Roy JP, Labrecque O, Fecteau G (2018) Risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* herd status in Québec dairy herds. Prev Vet Med 152:74–80. https://doi.org/10.1016/j.prevetmed.2018.02.010

Raizman EA, Wells S, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE (2004) The Distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J Dairy Sci 87:2959–2966. https://doi.org/10.3168/jds.S0022-0302(04)73427-X

Rangel SJ, Paré J, Doré E, Arango JC, Côté G, Buczinski S, Labrecque O, Fairbrother JH, Roy JP, Wellemans V, Fecteau G (2015) A systematic review of risk factors associated with the introduction of *Mycobacterium avium* spp. *paratuberculosis* (MAP) into dairy herds. Can Vet J 56:169–177. https://doi.org/10.1016/S0167-5877(97)00027-5

Ridge SE, Baker IM, Hannah M (2005) Effect of compliance with recommended calf-rearing practices on control of bovine Johne's disease RIDGE2005. Aust Vet J 83:85–90. https://doi.org/10.1111/j.1751-0813.2005.tb12204.x

Ridge SE, Heuer C, Cogger N, Heck A, Moor S, Baker IM, Vaughan S (2010) Herd management practices and the transmission of Johne's disease within infected dairy herds in Victoria, Australia. Prev Vet Med 95:186–197. https://doi.org/10.1016/j.prevetmed.2010.05.001

Salem M, Heydel C, El-Sayed A, Ahmed SA, Zschöck M, Baljer G (2012) *Mycobacterium avium* subspecies *paratuberculosis*: An insidious problem for the ruminant industry. Trop Anim Health Prod 45:351–366. https://doi.org/10.1007/s11250-012-0274-2

Sorge US, Lissemore K, Godkin A, Jansen J, Hendrick S, Wells S, Kelton DF (2012) Risk factors for herds to test positive for *Mycobacterium avium* ssp. *paratuberculosis*-antibodies with a commercial milk enzyme-linked immunosorbent assay (ELISA) in Ontario and Western Canada. Can Vet J 53:963–970.

Soumya MP, Pillai RM, Antony PX, Mukhopadhyay HK, Rao VN (2009) Comparison of faecal culture and IS900 PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faecal samples. Vet Res Commun 33:781–791. https://doi.org/10.1007/s11259-009-9226-3

StataCorp (2017) Stata Statistical Software: Release 15. 2017. https://doi.org/10.2307/2234838

Stevenson K (2010) Diagnosis of Johne's disease: Current limitations and prospects. Cattle Pract 18, 104–109.

Sweeney RW (1996) Transmission of paratuberculosis. Vet Clin North Am Food Anim Pract 12(2):305–312. https://doi.org/10.1016/S0749-0720(15)30408-4

Sweeney RW, Collins MT, Koets AP, Mcguirk SM, Roussel AJ (2012) Paratuberculosis (Johne's disease) in cattle and other susceptible species. J Vet Intern Med 26:1239–1250. https://doi.org/10.1111/j.1939-1676.2012.01019.x

Tavornpanich S, Johnson WO, Anderson RJ, Gardner IA (2008) Herd characteristics and management practices associated with seroprevalence of *Mycobacterium avium* subsp *paratuberculosis* infection in dairy herds. Am J Vet Res 69(7):904–911. https://doi: 10.2460/ajvr.69.7.904.

Tiwari A, VanLeeuwen JA, Dohoo IR, Keefe GP, Haddad JP, Scott HM, Whiting T (2009) Risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* seropositivity in Canadian dairy cows and herds. Prev Vet Med 88:32–41. https://doi.org/10.1016/j.prevetmed.2008.06.019

USDA (2010) Uniform Program Standards for the Voluntary Bovine Johne´s Disease Control Program. Washington D.C. United States, Department of Agriculture-USDA, Animal and Plant Health Inspection Service-APHIS. https://johnes.org/handouts/files/USDA_Program_Standards_Sept-2010.pdf

Vega A (1947) Relación entre el diagnóstico de la paratuberculosis bovina por el examen coprológico y de la prueba alérgica de termorreación aviar por vía subcutánea, Universidad Nacional de Colombia.

Vilar ALT, Santos CSAB, Pimenta CLRM, Freitas TD, Brasil AWL, Clementino IJ, Alves CJ, Bezerra CS, Riet-Correa F, Oliveira TS, Azevedo SS (2015) Herd-level prevalence and associated risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in cattle in the State of Paraíba, Northeastern Brazil. Prev Vet Med 121:49–55. https://doi.org/10.1016/j.prevetmed.2015.06.003

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Wells SJ, Wagner BA (2000) Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J Am Vet Med Assoc 216:1450–1457. https://doi.org/doi:10.2460/javma.2000.216.1450

Whittington RJ, Marsh IB, Reddacliff LA (2005) Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. Appl Environ Microbiol 71(9):5304-5308. https://doi.org/10.1128/AEM.71.9.5304-5308.2005

Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA (2004) Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl Environ Microbiol 70:2989–3004. https://doi.org/10.1128/AEM.70.5.2989-3004.2004

Windsor PA, Whittington RJ (2010) Evidence for age susceptibility of cattle to Johne's disease. Vet J 184:37–44. https://doi.org/10.1016/j.tvjl.2009.01.007

Wolf R, Barkema HW, De Buck J, Orsel K (2015) Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results. J Dairy Sci 98:275–287. https://doi.org/10.3168/jds.2014-8676

Wolf R, Barkema HW, De Buck J, Orsel K (2016) Dairy farms testing positive for *Mycobacterium avium* ssp. *paratuberculosis* have poorer hygiene practices and are less cautious when purchasing cattle than test-negative herds. J Dairy Sci 99:4526–4536. https://doi.org/10.3168/jds.2015-10478

Wolf R, Barkema HW, De Buck J, Slomp M, Flaig J, Haupstein D, Pickel C., Orsel K (2014) High herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Western Canadian dairy farms, based on environmental sampling. J Dairy Sci 97:6250–6259. https://doi.org/10.3168/jds.2014-8101

Chapter three

The present article was constructed to accomplish the specific objectives 2 (isolate MAP by means of fecal culture of real-time PCR-positive samples) and 3 (genotype MAP isolates by means of PCR-based methods (MIRU-VNTR, MLSSR) in dairy herds of the Northern region of the Province of Antioquia (Colombia). The manuscript is under review by Tropical Animal Health and Production journal (submitted in May 2019).

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Molecular diversity of Mycobacterium avium subsp. paratuberculosis in dairy cattle herds of Antioquia, Colombia

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Abstract

Paratuberculosis is an economically important, chronic, and incurable disease in ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Understanding the genetic variability of MAP strains is an important issue in diagnosis, epidemiological research, and in the design of strategies for prevention and control of the disease, both at regional and country-level. The aim of the study was the determination of MAP molecular diversity. Environmental samples from 25 IS*900*-qPCR MAP-positive dairy herds were cultured by duplicate in Herrold´s egg yolk medium with mycobactin J to obtain isolates. Suspicious colonies were confirmed by MAP-IS*900*-qPCR. Positive DNA was sub-typed using mycobacterial interspersed repetitive units-variable number of tandem repeat (MIRU-VNTR) and multilocus short sequence repeats (MLSSR) techniques to analyze the genetic difference(s) between the isolates. Sub-typing revealed two different genotypes by MIRU-VNTR (INMV 2 and INMV 36). MLSSR was carried out to increase the discriminatory power from what was obtained by MIRU-VNTR, but no differences were observed among the isolates recovered. MAP genotypes INMV 2 and INMV 36 circulate in the study region. No further discrimination was achieved by MLSSR. Our study represents an important approach to the knowledge on MAP epidemiological status in the study population.

Keywords: *genetics, genotyping, Johne's disease, MLSSR, MIRU-VNTR.*

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an extremely slow-growing, acid-fast, mycobactin dependent multispecies pathogen. Infection with this bacterium leads to a chronic granulomatous enteritis in cattle and other wild and domestic ruminants known as paratuberculosis (PTB) or Johne´s disease (Clarke, 1997; Harris and Barletta, 2001). Clinical signs of PTB include diarrhea, weight loss, decreased milk production, and mortality, leading to important economic losses (McAloon et al., 2016). A major concern with MAP is the ease with

which the bacterium spreads, since subclinical or clinically infected animals shed MAP in feces and milk, enabling dissemination to susceptible calves, the environment, and retail milk (Sweeney, 1996; Fecteau and Whitlock, 2010; Sweeney et al., 2012). MAP-containing milk and meat are of particular concern because the bacterium has been suggested as possibly associated with Crohn's disease in humans (Kuenstner et al., 2017).

Molecular discrimination of MAP field isolates become a crucial tool to complement the general knowledge on MAP, its distribution, behavior, and characteristics (Sohal et al., 2009; Douarre et al., 2011; Rónai et al., 2015). The use of molecular subtyping methods of MAP has increased in the last two decades (Motiwala et al., 2006; Sohal et al., 2009). MAP sub-typing is a useful tool in epidemiological research, offering a better understanding of the MAP-infection origin, dynamics, associated risk factors, transmission profiles, pathogenesis, among other related features, allowing a rational design of adequate control measures, diagnosis improvement, and vaccine development (Motiwala et al., 2006; Sohal et al., 2010). Nevertheless, subtyping of MAP strains presents a challenge since they are genetically monomorphic and traditional molecular techniques have limited discriminatory power. The advances and availability of whole-genome sequencing have extended possibilities for the characterization of MAP, providing a phylogenetic context to facilitate global epidemiology studies (Ahlstrom et al., 2016).

Sub-typing techniques include mycobacterial interspersed repetitive units–variable number of tandem repeat (MIRU-VNTR), which are based on the polymorphism of repetitive elements, and multilocus short sequence repeats (MLSSR), consisting on the detection of simpler tracts of 2-5 bp tandem repeats (Amonsin et al., 2004; Thibault et al., 2007). The combination of methods targeting different genomic MAP-structures (*i.e.* MIRU-VNTR, MLSSR) has been reported to increase the discriminatory ability of combined methods compared to the discriminatory ability of each method used separately (Thibault et al., 2008; Castellanos et al., 2009; Douarre et al., 2011).

Some knowledge about PTB has been achieved over time in Colombia, however, information about the impact and distribution of the disease, as well as its molecular epidemiology in the

country is still limited. Therefore, the aim of the study was to sub-type MAP isolates obtained from dairy herds located in the Northern region of the Province of Antioquia (Colombia) by MIRU-VNTR and MLSSR techniques.

Materials and methods

Study herds

The present study was carried out in 25 dairy herds already detected as MAP-infected by IS*900*-qPCR on environmental samples. Infected herds were selected from a population of 386 dairy herds located in 62 districts of six different municipalities in the Northern region of the Province of Antioquia (Colombia), during December 2017. The 25 MAP-IS*900*-qPCR positive herds of study were located in six different districts in three different municipalities. The environmental sampling strategy and the molecular analysis (DNA extraction and molecular detection of IS*900* region from MAP) were carried out as previously reported. Briefly, each participating herd was visited once during the study period to collect two composite environmental samples. The first one contained material from at least six different sites (subsamples) of concentration of adult cattle and/or high traffic areas (e.g. paddocks, areas surrounding waterers and feeders, alleyways, gutters, milking parlor holding areas). The second one contained manure from the milking parlor collected in the manure storage lagoon, after mixing its content for at least 5 min. The six subsamples from the second place were obtained from different places of the perimeter of the lagoon by submerging the sampling container up to 10 cm beneath the surface. The DNA extraction was carried out using a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, CA, USA). The protocol included a bead-beating *prior* step (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA). The extracted DNA was analyzed using a duplex IS*900*-qPCR (Bactotype MAP PCR Kit®, Qiagen, Leipzig, Germany).

Culture

Environmental samples from the IS*900*-qPCR-positive herds were decontaminated with 0.75% (w/v) hexadecylpyridinium chloride solution (HPC) for 24 h, according to standard procedures

(Jorge Arturo Fernández-Silva et al., 2011). All culture media were incubated at 37 °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, tending to heap up slightly and becoming dull light yellow with wrinkling of the surface; Whittington, 2010). The colonies were subcultured in HEYM and those resembling MAP were considered presumptively positive. All isolates were subjected to confirmatory IS*900*-qPCR (Bactotype MAP PCR Kit®, Qiagen, Leipzig, Germany) in order to confirm the identity of the isolates. Decontamination, as well as culture procedures, were carried out in the Diagnostic Unit at the Facultad de Ciencias Agrarias, Universidad de Antioquia, in Medellín, Colombia.

MAP sub-typing

For the molecular characterization of MAP isolates, a combination of two different sub-typing methods, both based on PCR-amplification of repetitive elements of MAP genome, was applied.

MIRU-VNTR. The procedure was carried out by amplifying eight MIRU-VNTR loci 3, 7, 10, 25, 32, 47, 292, and X3 (alias 1658) using the PCR conditions previously reported by Thibault et al. (2007) with slight modifications. The PCR mixture was composed of 2 µL from tenfold-diluted DNA solution was added to a final volume of 50 µL containing 0.25 µL of GoTaq® DNA polymerase (Promega; 5 U/µL), 2 µL of betaine (Sigma), dTTP (Qiagen, Leipzig, Germany), 5 µL of PCR buffer (supplied by the manufacturer), 1 µM of primers, and 2 mM of $MgCl_2$. The reactions were carried out using a SimpliAmp thermal cycler (Applied Biosystems, Darmstadt, Germany). The PCR conditions included 1 cycle of 3 min at 95 °C, 1 cycle of 1 min at 95 °C, 30 cycles of 1 min s at 58 °C, 1 cycle of 1 min at 72 °C, and 10 min at 72 °C. To determine the molecular weight (MW) of each PCR product and to estimate the number of tandem repeats present in each loci, 10 µl of PCR product was loaded in a 2% agarose gel. In addition, 50 bp ladder was used to determine the MW. To digitalize the gel, the Gel Doc TM imager (BioRad) was used. The results were expressed by an octal code and the genotype pattern (INMV) was determined using the international free access on-line database (http://mac-inmv.tours.inra.fr/).

MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus. MAP K10 strain was used as the reference control.

MLSSR. The procedure was carried out by amplification of four short sequence repeats (SSR) loci, locus 1 (g-repeats), locus 2 (g-repeats), locus 8 (ggt-repeats), and locus 9 (tgc-repeats) using PCR conditions as previously reported by Amonsin et al. (2004) with slight modifications. The selection was carried out by selecting the loci with the highest discriminatory index. The PCR mixture was composed of 2 µL from tenfold-diluted DNA solution added to a final volume of 50 µL containing 0.5 µL of GoTaq® DNA polymerase (Promega; 5 U/µL), dTTP (Qiagen, Leipzig, Germany), 10 µL of PCR buffer (supplied by the manufacturer), 1 µM of primers, and 2 mM of MgCl2. The reactions were carried out using a SimpliAmp thermal cycler (Applied Biosystems, Darmstadt, Germany). The PCR conditions included 1 cycle of 3 min at 95 °C, 1 cycle of 1 min at 95 °C, 35 cycles of 1 min s at 60 °C, 1 cycle of 2 min at 72 °C, and 7 min at 72 °C. PCR products were analyzed by electrophoresis using 1.5% agarose gels (agarose electrophoresis grade; TransGen Biotech). All amplicons of every locus were purified using the MinElute® PCR Purification Kit (Qiagen, Leipzig, Germany) and sequenced independently (Genomics unit, Biotechnology Laboratory, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina). The quality of sequencing and the number of short repeat units to identify the alleles were analyzed using the Sequencing Analysis Software v5.4 (Applied Biosystems). MLSSR genotypes were expressed as the combination of the number of repeats found in the four loci amplified by PCR. If the number of g-repeats at locus 2 was higher than 11, g-repeats for such locus were denoted as >11g as previously suggested (Thibault et al., 2008). Sub-typing procedures were carried out at the Institute of Agrobiotechnology and Molecular Biology (IABIMO)-CONICET, INTA in Buenos Aires, Argentina.

Results

Of the 25 MAP-IS*900*-qPCR positive environmental samples, 13 showed MAP-compatible growth in one or both HEYM slants, six of which were confirmed as MAP by IS*900*-qPCR. All

isolates grew within 8-15 weeks of incubation. In total, three MAP isolates recovered from environmental samples collected from the wastewater lagoon from three different herds were suitable for MAP sub-typing. The three isolates revealed two different genotypes by MIRU-VNTR [INMV 2 (numerical code: 32332228) and INMV 36 (numerical code: 32342228)]. MLSSR was carried out to increase the discriminatory power, but no differences were observed, as it can be seen in Table 1. Nevertheless, MLSSR profile obtained from herd 3 may correspond to MLSSR 50 (numerical code: 711555455455) according to Thibault et al. (2008).

The herd characteristics, as well as herd management practices of the three herds of the sub-typing report, are shown in Tables 2 and 3, respectively.

Discussion

In the present study, MIRU-VNTR and MLSSR sub-typing techniques were applied aiming to achieve an epidemiological analysis of MAP in one of the most representative dairy regions of Colombia. This tropical country has an animal productive system and husbandry practices that can vary when compared to other regions of the world. Therefore, molecular epidemiology of MAP in the country is expected to show particular patterns from that of other seasonal and tropical countries.

Although comparisons with other studies are very difficult because of the use of different loci for analysis, MIRU-VNTR profiles 1 (INMV 1) and 2 (INMV 2) has been previously reported as the most common genotypes found in isolates from other Latin-American countries (Thibault et al., 2007; Fernández-Silva et al., 2012; Gioffré et al., 2015; Imperiale et al., 2017), as well as from European isolates (Stevenson et al., 2009; Douarre et al., 2011; Biet et al., 2012; de Kruijf et al., 2017). Nonetheless, our findings cannot be directly compared with those reported by Fernández-Silva et al. (2012), since we considered eight MIRU-VNTR loci and four SSRs and they used eleven loci (1, 3, 7, 4, 10, 25, 32, 47, 259, 292, and X3 —alias 1658) and three SSRs (1, 2, and 8), respectively.

Table 1 MIRU-VNTR and MLSSR profiles obtained from MAP-positive environmental isolates in dairy herds in the Province of Antioquia, Colombia.

Herd/ Isolate	Municipality	District	Number of copies of MIRU-VNTR								INMV profile	Number of copies of SSR loci			
			3	7	10	25	32	47	292	X3 (1658)		1 (g)	2 (g)	8 (ggt)	9 (tgc)
1	Entrerríos	Toruro	2	2	2	3	8	4	3	2	36	7	10	5	4
2	San Pedro de los Milagros	Santa Bárbara	2	2	2	3	8	4	3	2	36	7	10	5	4
3	San Pedro de los Milagros	San Francisco	2	2	2	3	8	3	3	2	2	7	11	5	4

MIRU-VNTR genotyping and INMV profile performed according to Thibault et al. (2007). MLSSR genotyping performed according to Amonsin et al. (2004).

Table 2 Herd-level characteristics of the dairy herds of study were sub-typed isolates were obtained from (Province of Antioquia, Colombia, 2016).

Herd	Herd size	Predominant cattle breed	Access to veterinary assistance	Open-purchasing practices	Open-grazing practices	Mixed farming	GFP certified-status	Bovine tuberculosis free-status	Producer´s knowledge about the disease	PTB-compatible signs´ history
1	30-60	Holstein	Yes	No	No	No	No	No	Never heard of the disease	In the last 2 years
2	30-60	Holstein	Yes	No	No	Yes (sheep)	Yes	Yes	Never heard of the disease	Nowadays
3	>60	Holstein	Yes	Yes	No	Yes (goats)	Yes	Yes	Never heard of the disease	Never

Herd size refers to the number of lactating cows; open-purchasing practices to the practice of external replacement cattle in the last 10 years; open-grazing practices include allowing foreign animals to graze in own pastures and/or own animals to graze in foreign pastures; mixed farming refers

to cattle co-existence with goats, sheep, and/or buffaloes in the last 2 years; good farming practices (GFP)-status to a herd certified by the Instituto Colombiano Agropecuario (ICA) as such; bovine tuberculosis-status to a herd certified by the ICA as free from bovine tuberculosis; PTB-compatible signs´ history in the herd as the account of animals within the last 2 years; typical time of separation refers to the separation of newborn calf from their dam after birth; and, calves ≤ 6 months old sharing spaces with adult cows relates to such practice in the herds, allowing nose-to-nose contact.

Table 3 Management practices of the dairy herds of study were sub-typed isolates were obtained from (Province of Antioquia, Colombia, 2016).

Herd	Cow manure spreading as fertilizer	Typical time of separation (in days)	Calves ≤ 6 months old sharing spaces with adult cows	Colostrum fed to calves (source)	Milk fed to unweaned calves (source)
1	Yes	1	No	From their own dam	Discarded milk
2	Yes	3	No	From their own dam	Salable milk (without antibiotic)
3	Yes	5	No	From their own dam	Salable milk (without antibiotic)

On the other hand, some reports using the same loci/SSRs we used have been previously published. Correa et al. (2013) in Mexico, reported the INMV 2 profile in an isolate from cattle feces. Gioffré et al. (2015), reported seven different INMV profiles, including INMV 2 in cattle and goats from Argentinian and Mexican isolates. Imperiale et al. (2017), reported the INMV 2 as the second most common profile found in stool and/or intestinal mucosa from cattle and humans in Argentina, also concluding that INMV 2 was the original clone from which the others derive. According to MLSSR profiles reported herein, the sub-types isolated in our study are commonly found in cattle and other species in different countries (Ghadiali et al., 2004; Thibault et al., 2008; Fernández-Silva et al., 2012). Interestingly, a bovine isolate from Colombia's neighbor country Venezuela has shown a different genotype by SSR (11g-10g-5ggt-5ggt), suggesting strain diversity in the Northern part of South America (Thibault et al., 2008).

At a national scale, Fernández-Silva et al. (2011) reported a molecular characterization by MIRU-VNTR and MLSSR of eight MAP isolates (obtained from feces, tissue, and wastewater lagoons) from five dairy herds in the Northern region of Antioquia, already defined as MAP-infected according to direct and indirect testing. Authors revealed two different combined-strain profiles (1A and 2B), being the first molecular characterization of MAP in Colombia to that date. Our findings cannot be compared with those reported by Fernández-Silva et al. (2011), since we considered eight MIRU-VNTR loci and they used twelve (1, 2, 3, 4, 7, 10, 25, 32, 47, 259, 292, and X3 —alias 1658). Nevertheless, authors considered the same four SSRs we did, reporting the MLSSR-genotype B (7-g, 10-g, 5-ggt, 4-tgc), obtained from tissue and wastewater lagoon.

As a conclusion, and based on the sub-typing patterns of the MAP isolates obtained herein, from 386 dairy herds located in the Northern dairy region of Antioquia (Colombia), we found different MAP genotypes circulating in the study region: INMV 2 and INMV 36 according to MIRU-VNTR analysis and no discrimination among common INMV profiles according to MLSSR results.

Our findings lead to important epidemiological implications with regard to control and prevention of PTB in Colombia. It is suggested that newer typing methods, such as single nucleotide polymorphism (SNP), which is capable of detecting differences among major types may be used

to obtain further discernment into the epidemiological features of MAP in the country, including the influence of culture media, the role played by the local wildlife, the diversity of agro-ecosystems, and the crossbreeding of imported and indigenous animals to be taken into account in the analysis as possible sources of genomic diversity of MAP.

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Statement of animal rights

This research was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act number 71, June 15th, 2011).

Conflict of interest statement

The authors are not aware of any financial or personal relationships with other people or organizations that could inappropriately influence the work reported in this paper. The study

sponsors (CODI- Universidad de Antioquia) had no direct role in developing the study design, data collection analysis or interpretation.

References

Ahlstrom, C., Barkema, H. W. and De Buck, J. 2016. Relative frequency of 4 major strain types of *Mycobacterium avium* ssp. *paratuberculosis* in Canadian dairy herds using a novel single nucleotide polymorphism-based polymerase chain reaction, Journal of Dairy Science, 99, 8297-303. https://doi.org/10.3168/jds.2016-11397

Amonsin, A., Li, L.L., Zhang, Q., Bannantine, J.P., Motiwala, A.S., Sreevatsan, S. and Kapur, V., 2004. Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis* strains 3. Journal of Clinical Microbiology, 42, 1694–1702. https://doi.org/10.1128/JCM.42.4.1694

Biet, F., Sevilla, I.A., Cochard, T., Lefrançois, L.H., Garrido, J.M., Heron, I., Juste, R.A., McLuckie, J., Thibault, V.C. and Supply, P., 2012. Inter-and Intra-subtype genotypic differences that differentiate *Mycobacterium avium* subspecies *paratuberculosis* strains. BMC Microbiology, 12, 264.

Castellanos, E., Aranaz, A., Gould, K.A., Linedale, R., Stevenson, K., Alvarez, J., Dominguez, L., De Juan, L., Hinds, J. and Bull, T.J., 2009. Discovery of stable and variable differences in the *Mycobacterium avium* subsp. *paratuberculosis* type I, II, and III genomes by pan-genome microarray analysis. Applied Environmental Microbiology, 75, 676–686. https://doi.org/10.1128/AEM.01683-08

Clarke, C.J., 1997. The pathology and pathogenesis of paratuberculosis in ruminants and other species. Journal of Comparative Pathology, 116, 217–261.

Correa, M., Medina, G. and Rentería, T., 2013. Caracterización molecular de *Mycobacterium avium* subespecie *paratuberculosis* en bovinos y ovinos de Mexicali , Baja California. Revista Mexicana de Ciencias Pecuarias, 4(4), 489-500.

Correa-Valencia, N., García-Tamayo, Y.M. and Fernández-Silva, J.A. 2018. *Mycobacterium avium* subsp. *paratuberculosis* in Colombia (1924-2016): A review. Revista Colombiana de Ciencias Pecuarias, 31(3),165-179. https://doi.org/10.17533/udea.rccp.v31n3a01

de Kruijf, M., Lesniak, O.N., Yearsley, D., Ramovic, E., Coffey, A. and O'Mahony, J., 2017. Low genetic diversity of bovine *Mycobacterium avium* subspecies *paratuberculosis* isolates detected by MIRU-VNTR genotyping. Veterinary Microbiology, 203, 280-285. https://doi.org/10.1016/j.vetmic.2017.03.029

Douarre, P.E., Cashman, W., Buckley, J., Coffey, A. and Mahony, J., 2011. Molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* using multi-locus short sequence repeat (MLSSR) and mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing methods. Veterinary Microbiology 149, 482–487. https://doi.org/10.1016/j.vetmic.2010.12.001

Fecteau, M. and Whitlock, R., 2010. Paratuberculosis in cattle. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 144-156.

Fernández-Silva, Jorge Arturo, Abdulmawjood, A., Akineden, Ö. and Bülte, M., 2012. Genotypes of *Mycobacterium avium* subsp. *paratuberculosis* from South American countries determined by two methods based on genomic repetitive sequences. Tropical Animal Health and Production, 44, 1123–1126. https://doi.org/10.1007/s11250-011-0060-6

Fernández-Silva, Jorge Arturo, Abdulmawjood, A., Akineden, Ö. and Bülte, M., 2011. Serological and molecular detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle of dairy herds in Colombia. Tropical Animal Health and Production, 43, 1501–1507. https://doi.org/10.1007/s11250-011-9833-1

Fernández-Silva, J. A., Abdulmawjood, A., Akineden, Ö., Dräger, K., Klawonn, W. and Bülte, M., 2012. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* at a regional scale in Germany. Research in Veterinary Science, 93, 776–782. https://doi.org/10.1016/j.rvsc.2011.12.005

Fernández-Silva, J. A., Abdulmawjood, A. and Bülte, M., 2011. Diagnosis and molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* from dairy cows in Colombia. Veterinary Medicine International, 2011, 1–12. https://doi.org/10.4061/2011/352561

Ghadiali, A.H., Strother, M., Naser, S.A. and Manning, E.J.B., 2004. *Mycobacterium avium* subsp . *paratuberculosis* strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. Society, 42, 5345–5348. https://doi.org/10.1128/JCM.42.11.5345

Gioffré, A., Muñoz, M.C., Alvarado Pinedo, M.F., Vaca, R., Morsella, C., Fiorentino, M.A., Paolicchi, F., Ruybal, P., Zumárraga, M., Travería, G.E. and Romano, M.I., 2015. Molecular typing of Argentinian *Mycobacterium avium* subsp. *paratuberculosis* isolates by multiple-locus variable number-tandem repeat analysis. Brazilian Journal of Microbiology, 46, 557–564. https://doi.org/10.1590/S1517-838246220140283

Harris, N.B. and Barletta, R.G., 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clinical Microbiology Reviews, 14(3), 489-512. https://doi.org/10.1128/CMR.14.3.489-512.2001

Imperiale, B.R., Moyano, R.D., Di Giulio, A.B., Romero, M.A., Alvarado Pinedo, M.F., Santangelo, M.P., Travería, G.E., Morcillo, N.S. and Romano, M.I., 2017. Genetic diversity of *Mycobacterium avium* complex strains isolated in Argentina by MIRU-VNTR. Epidemiology & Infection, 145, 1382–1391. https://doi.org/10.1017/S0950268817000139

Kuenstner, J.T., Naser, S., Chamberlin, W., Borody, T., Graham, D.Y., McNees, A., Hermon-Taylor, J., Hermon-Taylor, A., Dow, C.T., Thayer, W., Biesecker, J., Collins, M.T., Sechi, L.A., Singh, S.V., Zhang, P., Shafran, I., Weg, S., Telega, G., Rothstein, R., Oken, H., Schimpff, S., Bach, H., Bull, T., Grant, I., Ellingson, J., Dahmen, H., Lipton, J., Gupta, S., Chaubey, K., Singh, M., Agarwal, P., Kumar, A., Misri, J., Sohal, J., Dhama, K., Hemati, Z., Davis, W., Hier, M., Aitken, J., Pierce, E., Parrish, N., Goldberg, N., Kali, M., Bendre, S., Agrawal, G., Baldassano, R., Linn, P., Sweeney, R.W., Fecteau, M., Hofstaedter, C., Potula, R., Timofeeva, O., Geier, S., John, K., Zayanni, N., Malaty, H.M., Kahlenborn, C., Kravitz, A., Bulfon, A., Daskalopoulos, G., Mitchell, H., Neilan, B., Timms, V., Cossu, D., Mameli, G., Angermeier, P., Jelic, T., Goethe, R., Juste, R.A. and Kuenstner, L., 2017. The Consensus from the *Mycobacterium avium* ssp. *paratuberculosis* (MAP) Conference 2017. Frontiers in Public Health, 5, 1–5. https://doi.org/10.3389/fpubh.2017.00208

McAloon, C.G., Whyte, P., More, S.J., Green, M.J., O'Grady, L., Garcia, A. and Doherty, M.L., 2016. The effect of paratuberculosis on milk yield—A systematic review and meta-analysis. Journal of Dairy Science, 99, 1449–1460. https://doi.org/10.3168/jds.2015-10156

Motiwala, A.S., Li, L., Kapur, V. and Sreevatsan, S., 2006. Current understanding of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis*. Microbes and Infection, 8, 1406–1418. https://doi.org/10.1016/j.micinf.2005.12.003

Rónai, Z., Csivincsik, A., Gyuranecz, M., Kreizinger, Z., Dán, A. and Jánosi, S., 2015. Molecular analysis and MIRU-VNTR typing of *Mycobacterium avium* subsp. *paratuberculosis* strains from various sources. Journal of Applied Microbiology, 118, 275–283. https://doi.org/10.1111/jam.12702

Sohal, J.S., Singh, S. V., Subodh, S., Sheoran, N., Narayanasamy, K., Singh, P.K., Singh, A.V. and Maitra, A., 2009. *Mycobacterium avium* subspecies *paratuberculosis* diagnosis and geno-typing: Genomic insights. Microbiology Research, 164, 330–337. https://doi.org/10.1016/j.micres.2007.03.005

Sohal, J.S., Singh, S. V, Singh, P.K. and Singh, A.V., 2010. On the evolution of "Indian Bison type" strains of *Mycobacterium avium* subspecies *paratuberculosis*. Microbiology Research, 165, 163–171. https://doi.org/10.1016/j.micres.2009.03.007

Stevenson, K., Alvarez, J., Bakker, D., Biet, F., De Juan, L., Denham, S., Dimareli, Z., Dohmann, K., Gerlach, G.F., Heron, I., Kopecna, M., May, L., Pavlik, I., Sharp, J.M., Thibault, V.C., Willemsen, P., Zadoks, R.N. and Greig, A., 2009. Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* across host species and European countries with evidence for transmission between wildlife and domestic ruminants. BMC Microbiology, 9, 1–13. https://doi.org/10.1186/1471-2180-9-212

Sweeney, R.W., 1996. Transmission of paratuberculosis. Veterinary Clinics of North America: Food Animal Practice, 12(2), 305–312. https://doi.org/10.1016/S0749-0720(15)30408-4

Sweeney, R.W., Collins, M.T., Koets, A.P., Mcguirk, S.M. and Roussel, A.J., 2012. Paratuberculosis (Johne's Disease) in cattle and other susceptible species. Journal of Veterinary Internal Medicine, 26, 1239–1250. https://doi.org/10.1111/j.1939-1676.2012.01019.x

Thibault, V.C., Grayon, M., Boschiroli, M.L., Hubbans, C., Overduin, P., Stevenson, K., Gutierrez, M.C., Supply, P. and Biet, F., 2007. New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. *paratuberculosis* and M. avium strains: Comparison with IS900 and IS1245 restriction fragment length polymorphism typing. Journal of Clinical Microbiology, 45, 2404–2410. https://doi.org/10.1128/JCM.00476-07

Thibault, V.C., Grayon, M., Boschiroli, M.L., Willery, E., Allix-Béguec, C., Stevenson, K., Biet, F. and Supply, P., 2008. Combined multilocus short-sequence-repeat and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing of *Mycobacterium avium* subsp. *paratuberculosis* isolates. Journal of Clinical Microbiology, 46, 4091–4094. https://doi.org/10.1128/JCM.01349-08

Whittington, R., 2010. Cultivation of *Mycobacterium avium* subsp. *paratuberculosis*. In: Behr MA, Collins DM, editors. Paratuberculosis: Organism, disease, control. First edition. Cambridge, MA: Editorial Cabi International; 2010. p. 244-260.

Chapter four

The present article was constructed to accomplish the specific objective 5 (describe the spatial distribution and the environmental variables related to MAP using geostatistical analysis) in dairy herds of the Northern region of the Province of Antioquia (Colombia). The manuscript is under review by Spatial and Spatio-Temporal Epidemiology journal (submitted in May 2019).

URL for the Author Guidelines (*Spatial and Spatio-Temporal Epidemiology*):
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Spatial and environmental analyses of the distribution of Mycobacterium avium subsp. paratuberculosis-infected dairy herds of the Province of Antioquia (Colombia)

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Abstract

The spatial distribution and the environmental conditions related to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) survival and persistence in Colombian infected herds are unknown. This study aimed to describe the spatial distribution and the environmental variables related to MAP in 386 dairy herds, located in six municipalities in the Province of Antioquia (Colombia).

Participant herds were visited once to collect one composite environmental sample, analyzed by a real-time PCR method. Rainfall trends, day and nighttime surface temperature, and vegetation cover index were taken as environmental references of the physical background of the study area. An overall high rainfall regime was found in the study area. The daytime and nighttime surface temperature showed important variations during sampling months. No evidence of management of the vegetation cover was found.

Keywords: *environment, geostatistics, Johne´s disease, rainfall, vegetation.*

1. Introduction

Paratuberculosis (PTB) is on the World Organization for Animal Health (OIE) list of diseases. PTB refers to a chronic infection affecting cattle and other ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The main clinical symptoms of PTB are diarrhea and weight loss (Clarke, 1997), leading to economic losses for the dairy productive sector due to reduced milk yield and the inevitable death of infected animals (Nielsen and Toft, 2009). MAP is also suspected of being involved in the development of Crohn's disease in humans (Behr, 2010). The factors favoring spread among animals and transmission to humans are currently under investigation (Kuenstner et al., 2017). Infectious animals shedding MAP in feces and thereby, and thereby spreading the bacterium to susceptible hosts from an environmental source is the main strategy of transmission (Sweeney, 1996). MAP is thought to be an obligate parasitic pathogen of mammals, so the bacterium can persist in soil and water but does not multiply outside the host (Manning, 2001).

Prevention and control of PTB demands a high level of knowledge about the magnitude of the disease, but also about factors influencing its entrance and perpetuation in herds. It is also well known that herd practices can increase or decrease the probability of MAP to enter or to maintain in a given cattle population, and that these practices vary not only between countries or agro-ecological zones but also between regions and even herds (Fernández-Silva, Correa-Valencia

and Ramírez, 2014). Therefore, it is necessary to carry out more extensive and strategically designed samplings considering the information available on PTB and its causal agent in the country.

Renewed global commitment has led to an impulse to attain comprehensive data on animal infectious diseases distribution and intensity. Once survey data have been collected, they can be integrated into a Geographical Information System (GIS) for mapping and further analysis, thus helping guide available resources to be most rationally and cost-effectively deployed. At the same time, research into the spatial distribution of diseases has become a standard requirement of government agencies, since modern methods of disease mapping allow to infer if there are *high-risk* areas (Law *et al.*, 2004).

There are several spatial data analysis tools. Spatial statistics´ spectrum can range from the locations of points or objects —probabilistic modeling, up to the measurements made in random variables, considering specific spatial locations (random fields) (Cressie, 1992; Illian et al., 2008). Among them, geostatistics has been consolidated as one of the most solid and reliable tools for the analysis of this kind of data, especially when the concepts of classical statistics are insufficient or when dealing with not-necessarily random variables, as often happens in the case of observational data (Matheron, 1963).

The geostatistical mapping —a subset of statistics, is focused on the analysis and interpretation of georeferenced geographic data (Hengl, 2009), where a summary data extraction process is carried out from a spatial context, and is then compared to theoretical models that trend to explain how the spatial pattern originates and develops (Dimitriou-Fakalou, 2010). Data is subjected to an interpolation process, which highlights kriging and simulation routes, particularly when there is a difficulty or a considerable cost in obtaining data is required (Strebelle, 2006). The conditional simulation aims to present one of the possible insights of a random function, with the same characteristics of spatial variability as reality, by reproducing the first two experimental moments of the real data [mean and covariance C (h) or semivariograms γ (h)] or, in simpler words, the main characteristics of dispersion of the real phenomenon are reproduced,

conditioned to the field data (Blöschl, 2002). Results deriving from geostatistical mapping and the conditional simulation that accompanies it can point to an environmental exposure characterization, being also helpful in the identification of geographical patterns and in the exploration of herd-level infection predictors for MAP.

In the Colombian case, the identification of areas where MAP seems to be concentrated or potentially detected could allow policymakers to implement targeted screenings and interventions since it is a notifiable disease in Colombia since 2015. However, the spatial distribution of MAP in Colombia has never been examined.

Therefore, the objectives of this study were (a) to describe the spatial distribution of MAP in dairy herds, and (b) to describe environmental variables taken as reference of the physical background of the study area, specifically those related to MAP-qPCR positive herds located in six municipalities of the Northern region of the Province of Antioquia (Colombia), based on environmental sampling and qPCR analysis.

2. Materials and methods

Study design and data

This study was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act number 71, June 15th, 2011).

The data was collected during a randomized cross-sectional study carried out in the Northern region of the Province of Antioquia (Colombia). Herds of study were distributed in districts inside six municipalities known to participate in the 70% of the milk production in the Province (San José de La Montaña, Belmira, Santa Rosa de Osos, Entrerríos, San Pedro de Los Milagros, and Donmatías). The herd was considered as the unit of analysis. Optimum and proportional allocation both at municipality and district-level were considered in the study design. The sample

size was defined according to the formula for prevalence estimation from a finite population (Dohoo et al., 2014). From 7,794 herds registered in the six municipalities of interest (according to foot-and-mouth disease vaccination records; Fedegán, 2015), 386 herds in 63 districts were randomly selected.

The study area is located between 1,090 and 2,979 m.a.s.l. and the temperature ranges from 12 to 16°C during the year. According to the Caldas-Lang climate classification, the study areas are classified as cold-wet and cold very humid. The study area belongs to the Medio Cauca/Alto Nechí watershed, which has a bimodal rainfall regime, with a dry season slightly marked in the mid-year and a second semester (September to November) with a higher rainfall level (IDEAM, 2012).

Environmental sample collection and laboratory analysis

The participating herds were visited once and environmental sampling was done in each herd of study, from July to October 2016. The distribution of samplings between months was as follows: July (53.2%; 205/386), August (14.8%; 57/386), September (23.4%; 90/386), and October (8.6%; 33/386). Each environmental sample contained samples from a single pool of at least six different sites of concentration of adult cattle, high traffic areas (*e.g.* areas surrounding waterers and feeders, areas surrounding the current mobile milking-unit place), and wastewater storage lagoons —when it was available at the herd, as previously reported by the literature (Raizman et al., 2004; Collins et al., 2006; Pillars et al., 2009; USDA, 2010; Kruze et al., 2013; Donat et al., 2015; Wolf et al., 2015), considering differences in the sampling methodology due to differences in management systems and facilities in the region of study, as well, as budget restrictions. Identification of MAP was achieved using a duplex quantitative real-time PCR method (qPCR; Bactotype MAP PCR Kit®, Qiagen). Procedures were carried out following the instructions of the manufacturer. A sample was considered positive when it reported a cycle threshold (Ct) ≤ 40 and a sigmoid-pattern curve result. Test wells with Cts greater than 40 were disregarded (according to MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments; Bustin et al., 2009).

A herd was considered as MAP infected if the environmental sample was found positive by the qPCR.

Spatial analysis

A polygon shapefiles for Colombia administrative boundaries at the district level was obtained from the Instituto Geográfico Agustín Codazzi (IGAC) website (https://www.igac.gov.co/). The boundaries of the districts required for the study were selected. All of the extracted data were linked and georeferenced with the district polygons using QGIS software (v.2.18.13, Las Palmas).

MAP-related variables. The study area was subdivided into fixed spatial units (lattices), in which disease counts are observed in a fixed time period (sampling months from July to October 2016). The dependent variable was the number of MAP-qPCR positive herds in each municipality, based on an anti-logarithmically transformed Ct values, reported for each herd. District-level location data (latitude, longitude) were included as independent variables. These variables were registered during sampling when researchers visited each herd to collect the environmental samples. A semivariogram analysis was used to investigate the spatial structure and spatial autocorrelation in the data (Bailey and Gatrell, 1995). In order to provide a continuous description of the covariance structure, a spherical spatial model was fitted to empirical variogram points using nonlinear least squares (Kaluzny et al., 1998). All the analysis were carried out using GS+™ software (v.10, Gamma Design Software, Plainwell, MI, USA). The modeling was done with the values of the inverse of the Ct (1/Ct) to facilitate the interpretation, based on the conditional simulation method, adjusted by the anisotropic variogram, and performing cross-validation of the results. One-thousand simulations were used through the conditional simulation analysis, using different seeds and at least three multigrid refinements. The interpolation range was adjusted to the set of municipalities sampled.

Day and nighttime surface temperatures. Maps of temperature variation were constructed according to interpolations made based on semivariograms of the mean of the values at each

sampling point with MAP-qPCR positive herds. The interpolation method was 2×2 ordinary krigeage of the isotropic model. The processing of the information was carried out with the GS+™ software (v.10, Gamma Design Software, Plainwell, MI, USA).

Vegetation cover index. Once the image was chosen, the processing was carried out to obtain the Normalized Vegetation Index (NDVI), which is used to estimate the quantity, quality, and development of the vegetation based on the measurement of the intensity of the vegetation and radiation of certain bands of the electromagnetic spectrum; its reading goes from -1 to 1, where values below zero (0) indicate the presence of water or soils saturated by humidity, and those between 0-1 indicates different densities of vegetation cover (Yengoh et al., 2015). As a complement to the analysis, the Normalized Water Differential Index (NDWI) was also obtained. Such index is used as a measure of the amount of water the vegetation has or the level of moisture saturation hat the soil has (Gao, 1996); its reading goes from -1 to 0, mean “brilliant surface without vegetation or water content”, values greater than 0 to + 1 mean water content (USGS, 2017).

Environmental analysis

In order to identify potential environmental conditions related to the presence of MAP in environmental samples analyzed by qPRC, this study included measures of rainfall trends, day and nighttime surface temperatures, and vegetation cover index providing a physical background of the whole study area. The selection of these variables was exploratory according to previously published studies on the role of environmental and soil factors´ influencing MAP survival outside the host, as well as its availability to the public.

Rainfall trends. The analysis of rainfall trends in the region considered data available from the tropical rainfall measuring mission (TRMM) with a spatial resolution of 4.3 Km, and GPM core observatory (spatial resolution of 11.1 Km). The analysis was performed at a general level and specified according to the months of sampling and for November, which served as a benchmark for the analysis of the vegetation cover index.

Day and nighttime surface temperatures. Defined as the air column found between the ground and up to about 2 m high. Data from the moderate resolution imaging spectroradiometer (MODIS) sensors and the TRMM mission, between July and November (2016), were used. Historical data from November were taken as reference for the spatial analysis of the vegetation cover index, whose set of satellite images correspond to that month, according to the portal of the Laboratório de Sensoriamento Remoto Aplicado à Agricultura e Floresta (LAF) of the Intitudo de Pesquisas Espaciales (INPE) of Brazil, which makes them available for South America (Freitas, 2011). The MODIS data (MOD11 —temperature and emissivity, spatial resolution of 1.0 Km) are available from the Land Processes Distributed Active Archive Center (LPDAAC/U.S. geological survey –USGS), Earth Resources Observation and Science (EROS) center (https://lpdaac.usgs.gov). The TRMM data (3B43V6; spatial resolution: 4.3 Km) are available from the GES DISC distributed active DAAC file system as part of the Data and Information Services Center (DISC) of NASA's Goddard Earth Sciences (GES). The GPM core observatory provides a precipitation data product, available through the Giovanni portal of NASA (https://pmm.nasa.gov/data-access/downloads/gpm).

Images from remote sensors. A satellite image of the region that fulfilled minimum requirements of cloudiness (less than 10% in the area of interest) and of approximate date to the range of months sampled, was searched. The chosen scene was a one from the collection LANDSAR 7 ETM + (level 1), obtained through the portal U.S. geological survey (USGS; https://earthexplorer.usgs.gov/). The best set of images found, close to the sampling dates was November 21st, 2016. The set of images chosen was the one cataloged as LE07_L1TP_009055_20161121_20170112_01_T1, and LE07_L1TP_009056_20161121_20170112_01_T1.

All data on environmental variables 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, and significant differences (p<0.05) were assessed using a Student´s t-test.

3. Results

The IS*900*-qPCR produced 25 positive (Ct ≤ 40) and 361 negative (Ct>40 or not detected/reported by the software), results from 386 environmental samples examined. Twenty-four of the 25 herd-level positive results were from the samples collected during July 2016, while one of the positive results was from the samples collected during September 2016. Overall and by-municipality frequencies are shown in Table 1. The study area, as well as the distribution of MAP-positive and negative dairy herds, are shown in Figure 1.

Table 1. Frequency of *Mycobacterium avium* subsp. *paratuberculosis* in 386 dairy herds sampled in the Northern region, Province of Antioquia, Colombia (2016).

Municipality	**Sample weight*** **(%)**	**Study herds (n)**	**Positive herds n (%)**
San José de la Montaña	4.4	17	2 (8)
Belmira	8.8	34	2 (8)
Santa Rosa de Osos	38.2	147	1 (4)
Entrerríos	15.1	58	6 (28)
San Pedro de los Milagros	23.6	91	8 (28)
Donmatías	9.9	39	6 (24)
Total	100	386	25 (6.5)

*According to foot-and-mouth disease vaccination records (Fedegán, 2015).

The semivariogram obtained from the spatial distribution of the MAP-qPCR positive herds indicates a natural phenomenon that only complies with the hypothesis of intrinsic stationarity, pointing that the spatial distribution of the positive data is better adjusted to an anisotropic model (for this case, spherical), with the following results: An effect nugget of 5.3×10^{-3} and a structural variance (Sill) of 1.75×10^{-2}. The model has a low coefficient of determination (r2 = 0.146), but an acceptable ratio between Nugget and Sill (0.30). Theoretically, it is accepted that the model interprets reality well if the difference between Nugett and Sill does not exceed 50% (0.5; Giraldo, 2011).

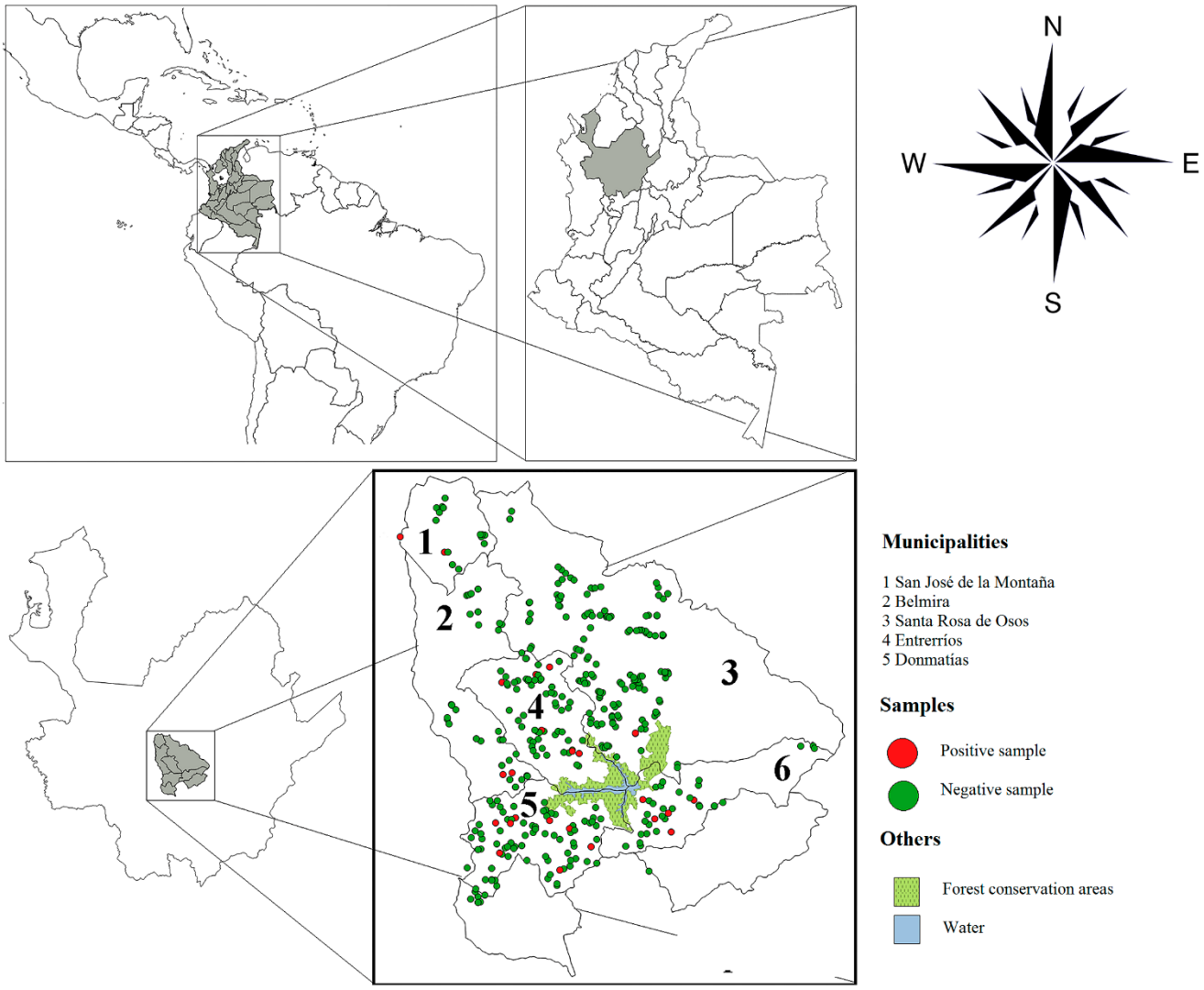

Figure 1. The geographic location of the six municipalities and the 386 dairy herds sampled in the Northern region, Province of Antioquia, Colombia (2016). Green dots refer to the herds found to be negative while red dots refer to those found positive to *Mycobacterium avium* subsp. *paratuberculosis* by IS*900*-qPCR (MAP-qPCR positive herds) based on environmental sampling.

With anisotropy included in the model, the estimated range was between 279.4 Km (118º azimuth in the lowest range), and 502.3 Km (33º azimuth in the highest range), considering each grade of range in the semivariogram as equivalent to 111,045 Km; 2,516 and 4,523 ° (in the four directions North-West, North-East, East, South-East). The lowest range corresponds to the

lowest average variance (3.125×10^{-3}), and in the highest range, the highest average spatial variance (9.882×10^{-3}). According to both sub-figures seen in Figure 2, there is an increase in positive reports in the North-West / South / South-East directions.

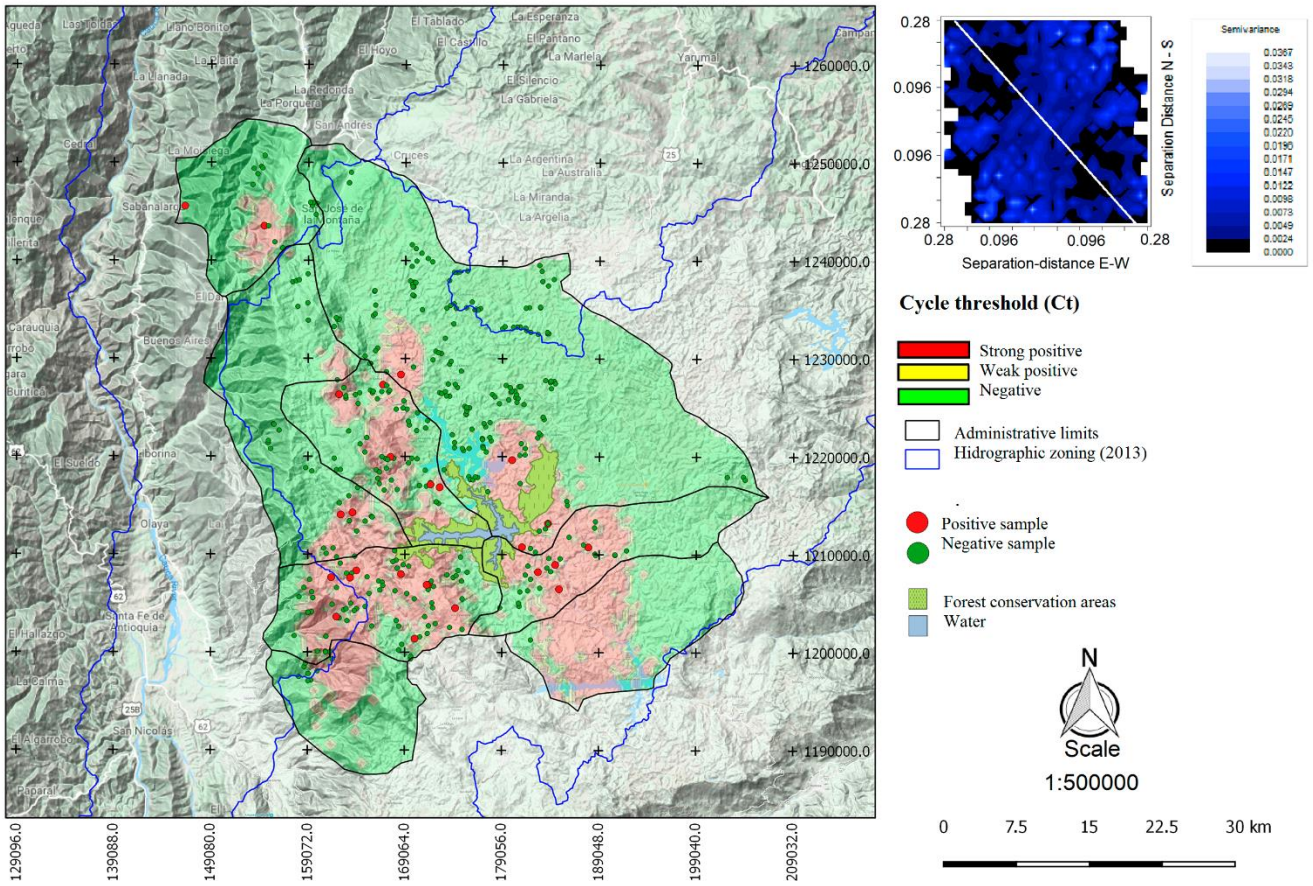

Figure 2. Simulation map of predicted distribution for MAP-qPCR positive herds (July to October 2016). The white line of the semivariance anisotropic map shows the flux direction with the lowest variance.

Figure 3 shows the maps of the spatial variation for day and nighttime surface temperature, according to the average at each point, considering the reports of positive herds and the values observed between July and October 2016. It is observed that the highest day-time surface temperature areas (between 20 and 20.8 ºC) also present the highest nighttime temperature values (between 5.07 and 9.17 ºC). However, an area to the West —where the average surface

temperature falls to negative values, a moderate average values were present during the day (18.6 and 19.3ºC). These findings suggest a thermal anomaly in such area. However, with the available data and the characteristics of this work, it is not possible to confirm such situation.

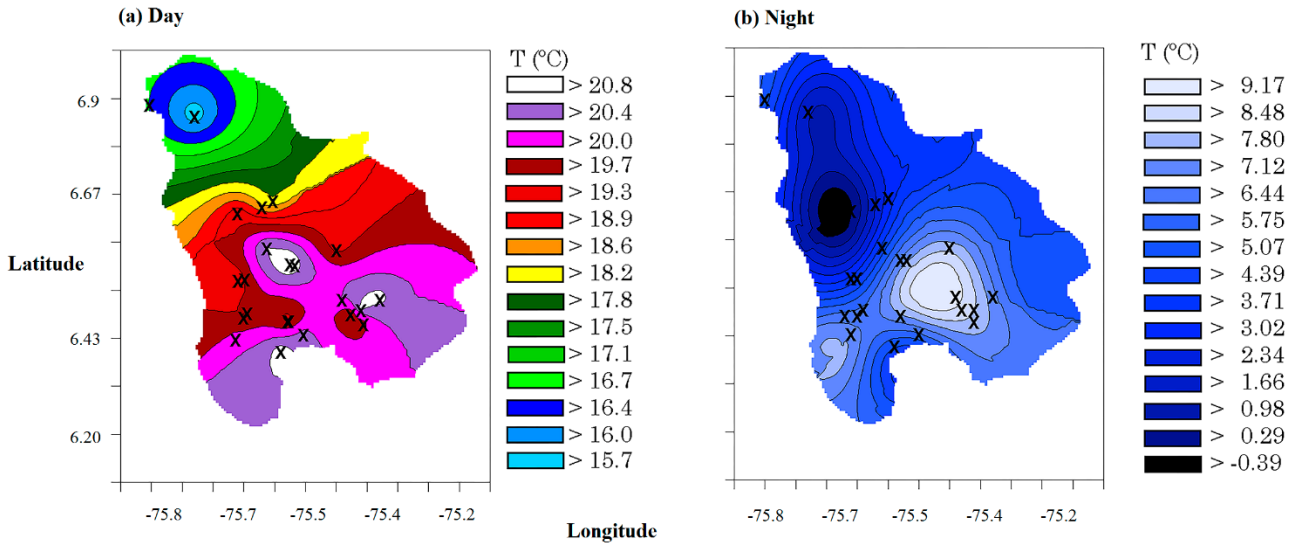

Figure 3. Spatial variation of the surface temperature (ºC) of the six municipalities and the 386 dairy herds sampled in the Northern region, Province of Antioquia, Colombia (2016). (a) Day-time spherical model; residual SS = 10.2; R^2 = 0.098; proportion (C/[Co+C]) = 0.999. ; (b) Nighttime spherical model; nugget variance = 0.27; sill = 18.83; range = 0.586; residual SS = 49.7; R^2 = 0.402; proportion (C/[Co+C]) = 0.986.

Figure 4 shows the spatial distribution of the vegetation cover (Figure 4a), obtained by applying the NDVI (Figure 4b) and the NDWI (Figure 4c). The *false-color image* (Figure 4a) allows visualizing the current condition of the vegetation cover, where there is no evidence of pasture management nor of natural areas that allow some degree of soil protection. With the first index (Figure 4b) it is observed that the native vegetation is practically nonexistent and that the pastures are immersed in a matrix of exposed soil. When comparing the humidity between the different types of vegetation cover (Figure 4b *vs* Figure 4c), it can be observed that the areas with the highest grass density are precisely those with the lowest moisture density, while the wetter areas correspond better with the *right areas* (exposed soil/pastures).

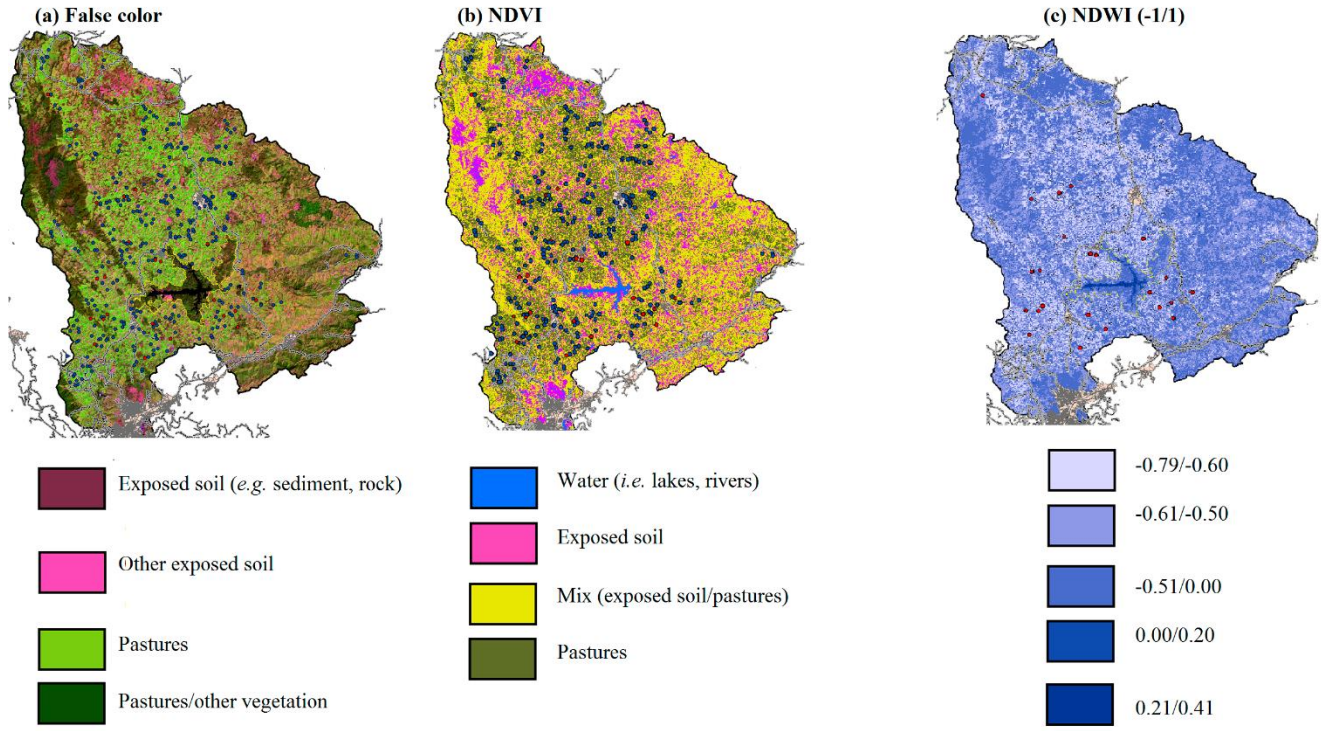

Figure 4. Vegetation cover (NDVI) and Moisture Saturation Index (NDWI) of the six municipalities and the 386 dairy herds sampled in the Northern region, Province of Antioquia, Colombia (2016).

According to Table 2, the average rainfall trends for the MAP-qPCR positive herds was *high* (average of 233.3 mm/month), as shown by the time series of the 2002-2017 monthly accumulated index. In addition, the sampling months (July to October 2016) presented high values compared to the historical trend of the mean. In general, it is accepted that the rainfall conditions of November 2016 fit well with those observed during the sampling months. This is important when interpreting the condition of the vegetation, carried out, as previously mentioned, by a set of satellite images of that month. September stands out with the maximum monthly-accumulated (306.4 mm), and July with the minimum monthly-accumulated (185.8 mm). The values of skewness are presented with a normal distribution (at a range +0.5), except for the intervals of November (2002-2016) and July-November (2016), which presented a positive asymmetry. Concerning the kurtosis, July stands out with a curve that can be considered as strongly leptokurtic (its values are strongly concentrated around the mean). In terms of

precipitation, July presents the mean and the lowest minimum value when compared with the other sampling months.

Table 2. Descriptive results of the rainfall trends at the MAP-qPCR positive dairy herds (2002-2017) in the Northern region, Province of Antioquia, Colombia (2016).

Variable	Valid N	Mean	Min	Max	SD	Confidence SD -95%	Confidence SD +95%	Skewness	Kurtosis
All months (2002-2017)	5,039	201.64	8.10	530.90	93.66	91.87	95.53	0.19	-0.56
July-November (2002-2017)	2,040	238.98	85.90	458.20	71.69	69.56	73.96	0.19	-0.58
November (2002-2016)	408	222.32	131.70	458.20	68.52	64.12	73.58	0.92	0.77
July-November (2002-2017)	2,040	238.98	85.90	458.20	71.69	69.56	73.96	0.19	-0.58
July-November (2016)	120	229.44	185.80	306.40	30.34	26.93	34.76	0.56	0.00
July (2016)	24	203.30	185.80	260.20	20.39	15.85	28.61	1.51	1.97
August (2016)	24	216.44	189.10	265.00	23.37	18.17	32.79	0.34	-0.52
September (2016)	24	266.99	249.30	306.40	23.66	18.39	33.19	1.02	-0.83
October (2016)	24	246.45	228.20	266.60	14.81	11.51	20.77	0.11	-1.46
November (2016)	24	214.01	201.10	242.00	10.58	8.22	14.84	0.31	0.44

SD: Standard deviation.

Table 3 presents the results of the statistical test applied to the rainfall-related independent samples. When the average of the set of historical values (2002-2017) of the sampling months (July to October 2016) are compared with the values presented from each month in the sampling year (2016), it is observed that only July differs significantly from that average. Considering the bimodal rainfall regime of the region, July is the month in which the second dry season of the year is theoretically presented. November —the reference month for the vegetation analysis—, presents significant historical differences with September-only, although in the year of sampling-only did not present significant differences with August. However, all sampling months showed significant differences in monthly-accumulated precipitation.

According to Table 4, the day-time surface temperature for the MAP-qPCR positive herds varied significantly from one day to the next in a given month during the sampling months (July to October 2016; between 9.55 and 26.83 ºC). The temperature difference between the minimum and the maximum historical record (2002-2016) was even wider (from 5.25 to 28.63 ºC).

Table 3. Student's *t*-test for the rainfall trends-related independent samples at the MAP-qPCR positive dairy herds (2002-2017) in the Northern region, Province of Antioquia, Colombia (2016).

Group 1 *vs* Group 2	***t*-value**	**df**	***p*-value**	**F-ratio**	***p*-variances**
July-November (2002-2016) *vs* November (2002-2016)	**4.31**	**2,446**	**0.00**	**1.09**	**0.25**
July-November (2002-2016) *vs* July-November (2016)	1.45	2,158	0.15	5.58	0.00
July-November (2002-2016) *vs* July (2016)	**2.44**	**2,062**	**0.01**	**12.36**	**0.00**
July-November (2002-2016) *vs* August (2016)	1.54	2,062	0.12	9.41	0.00
July-November (2002-2016) *vs* September (2016)	-1.91	2,062	0.06	9.18	0.00
July-November (2002-2016) *vs* October (2016)	-0.51	2,062	0.61	23.44	0.00
July-November (2002-2016) *vs* November (2016)	1.71	2,062	0.09	45.95	0.00
November (2002-2016) *vs* July-November (2016)	-1.11	526	0.27	5.10	0.00
November (2002-2016) *vs* July (2016)	1.35	430	0.18	11.29	0.00
November (2002-2016) *vs* August (2016)	0.42	430	0.68	8.59	0.00
November (2002-2016) *vs* September (2016)	**-3.18**	**430**	**0.00**	**8.39**	**0.00**
November (2002-2016) *vs* October (2016)	-1.72	430	0.09	21.41	0.00
November (2002-2016) *vs* November (2016)	0.59	430	0.55	41.98	0.00
July-November (2016) *vs* July (2016)	**4.04**	**142**	**0.00**	**2.21**	**0.03**
July-November (2016) *vs* August (2016)	1.98	142	0.05	1.69	0.15
July-November (2016) *vs* September (2016)	**-5.72**	**142**	**0.00**	**1.64**	**0.17**
July-November (2016) *vs* October (2016)	**-2.68**	**142**	**0.01**	**4.20**	**0.00**
July-November (2016) *vs* November (2016)	**2.46**	**142**	**0.02**	**8.23**	**0.00**
July (2016) *vs* August (2016)	**-2.07**	**46**	**0.04**	**1.31**	**0.52**
July (2016) *vs* September (2016)	**-9.99**	**46**	**0.00**	**1.35**	**0.48**
July (2016) *vs* October (2016)	**-8.39**	**46**	**0.00**	**1.90**	**0.13**
July (2016) *vs* November (2016)	**-2.28**	**46**	**0.03**	**3.72**	**0.00**
August (2016) *vs* September (2016)	**-7.45**	**46**	**0.00**	**1.02**	**0.95**
August (2016) *vs* October (2016)	**-5.31**	**46**	**0.00**	**2.49**	**0.03**
August (2016) *vs* November (2016)	0.46	46	0.64	4.88	0.00
September (2016) *vs* October (2016)	**3.61**	**46**	**0.00**	**2.55**	**0.03**
September (2016) *vs* November (2016)	**10.01**	**46**	**0.00**	**5.00**	**0.00**
October (2016) *vs* November (2016)	**8.73**	**46**	**0.00**	**1.96**	**0.11**

Variables were treated as independent samples. df: Degrees of freedom. Significance level: $p<0.05$ (values in bold are considered significant).

Table 4. Descriptive results of daytime surface temperature (°C) at the MAP-qPCR positive herds (2002-2017) in the Northern region, Province of Antioquia, Colombia (2016).

Variable	Valid N	Mean	Min	Max	SD	Confidence SD -95%	Confidence SD +95%	Skewness	Kurtosis
July-October (2002-2016)	2,503	20.36	5.25	28.63	3.01	2.92	3.09	-0.44	0.84
July-October (2016)	185	19.66	9.55	26.83	2.77	2.51	3.09	-0.32	0.53
July (2016)	48	20.09	13.73	24.23	2.29	1.91	2.87	-0.61	0.50
August (2016)	47	19.34	12.45	24.19	2.15	1.79	2.70	-0.80	1.58
September (2016)	48	21.02	13.51	26.83	2.85	2.37	3.57	-0.40	0.41
October (2016)	42	17.95	9.55	24.05	2.90	2.39	3.70	0.04	1.05

SD: Standard deviation.

Table 5 presents the results of the statistical test applied to the day-time surface temperature values in the region of study, during the sampling months. According to the table, there are no significant differences between average temperatures among July and August and July and September. Data analysis shows that what makes statistically significant differences are those in the minimum values registered, which can reach 4.18 ºC (difference in the minimum values of July and October). The difference between the maximum values reaches at most 0.18 ºC (the difference between the maximum values of July and October). The difference between the average values of July and October reaches 2.14 ºC, that is, in the region, a difference of 2.14 ºC is already significant with respect to the day-time surface temperature.

Table 6 presents the descriptive statistics for nighttime surface temperature for the MAP-qPCR positive herds during the sampling months (July to October 2016). A visual comparison between Tables 5 and 4 (in a timely manner) allows noticing that the temperature can fall extremely from day to night, reaching even, negative values. However, in general terms, the nighttime temperature (although it decreases a lot) usually stays above 0 ºC. Independently, the difference in the monthly averages during the sampling months does not exceed 1.1 ºC. On the other hand, according to the recent historical data (2002-2016), there may be a difference of up to 34 ºC between a minimum and a maximum surface temperature registered between one date and another. In the year of sampling, the biggest difference was seen in July (31.84 ºC) and the lowest in October (20.06 ºC).

Table 5. Student´s *t*-test for the daytime surface temperature (°C)-related independent samples at the MAP-qPCR positive dairy herds (2002-2017) in the Northern region, Province of Antioquia, Colombia (2016).

Group 1 *vs* Group 2	***t*-value**	**df**	***p*-value**	**SD Group 1**	**SD Group 2**	**F-ratio variances**	***p*-variances**	**Levene F(1,df)**	**df Levene**	***p*-Levene**
July-October (2002-2016) *vs* July-October (2016)	**4.66**	**3056**	**0.00**	**3.70**	**2.77**	**1.78**	**0.00**	**8.80**	**3056.00**	**0.00**
July-October (2002-2016) *vs* July (2016)	1.60	2919	0.11	3.70	2.29	2.60	0.00	7.46	2919.00	0.01
July-October (2002-2016) *vs* August (2016)	**2.97**	**2918**	**0.00**	**3.70**	**2.15**	**2.96**	**0.00**	**9.48**	**2918.00**	**0.00**
July-October (2002-2016) *vs* September (2016)	-0.14	2919	0.89	3.70	2.85	1.68	0.03	2.18	2919.00	0.14
July-October (2002-2016) *vs* October (2016)	**5.23**	**2913**	**0.00**	**3.70**	**2.90**	**1.62**	**0.05**	**2.45**	**2913.00**	**0.12**
July-October (2016) *vs* July (2016)	-1.01	231	0.32	2.77	2.29	1.46	0.13	2.59	231.00	0.11
July-October (2016) *vs* August (2016)	0.72	230	0.47	2.77	2.15	1.66	0.04	4.47	230.00	0.04
July-October (2016) *vs* September (2016)	**-3.02**	**231**	**0.00**	**2.77**	**2.85**	**1.06**	**0.76**	**0.00**	**231.00**	**0.95**
July-October (2016) *vs* October (2016)	**3.57**	**225**	**0.00**	**2.77**	**2.90**	**1.10**	**0.67**	**0.03**	**225.00**	**0.86**
July (2016) *vs* August (2016)	1.65	93	0.10	2.29	2.15	1.14	0.66	0.22	93.00	0.64
July (2016) *vs* September (2016)	-1.76	94	0.08	2.29	2.85	1.55	0.14	1.82	94.00	0.18
July (2016) *vs* October (2016)	**3.91**	**88**	**0.00**	**2.29**	**2.90**	**1.60**	**0.12**	**1.11**	**88.00**	**0.29**
August (2016) *vs* September (2016)	**-3.23**	**93**	**0.00**	**2.15**	**2.85**	**1.76**	**0.06**	**3.15**	**93.00**	**0.08**
August (2016) *vs* October (2016)	**2.59**	**87**	**0.01**	**2.15**	**2.90**	**1.82**	**0.05**	**2.10**	**87.00**	**0.15**
September (2016) *vs* October (2016)	**5.05**	**88**	**0.00**	**2.85**	**2.90**	**1.03**	**0.91**	**0.03**	**88.00**	**0.86**

Variables were treated as independent samples. df: Degrees of freedom. SD: Standard deviation. Significance level: $p<0.05$ (values in bold are considered significant).

Table 6 presents the descriptive statistics for nighttime surface temperature for the MAP-qPCR positive herds during the sampling months (July to October 2016). A visual comparison between Tables 5 and 4 (in a timely manner) allows noticing that the temperature can fall extremely from day to night, reaching even, negative values. However, in general terms, the nighttime temperature (although it decreases a lot) usually stays above 0 ºC. Independently, the difference in the monthly averages during the sampling months does not exceed 1.1 ºC. On the other hand, according to the recent historical data (2002-2016), there may be a difference of up to 34 ºC between a minimum and a maximum surface temperature registered between one date and another. In the year of sampling, the biggest difference was seen in July (31.84 ºC) and the lowest in October (20.06 ºC).

Table 6. Descriptive results of nighttime surface temperature (°C) at the MAP-qPCR positive herds (2002-2017) in the Northern region, Province of Antioquia, Colombia (2016).

Variable	Valid N	Mean	Min	Max	SD	Confidence SD -95%	Confidence SD +95%	Skewness	Kurtosis
July-October (2002-2016)	2450	6.77	-22.99	12.93	4.12	4.01	4.24	-2.91	12.68
July-October (2016)	180	6.04	-19.37	12.47	6.04	5.47	6.73	-2.44	6.39
July (2016)	40	5.80	-19.37	12.47	9.46	7.75	12.15	-1.97	2.44
August (2016)	47	6.64	-15.57	11.47	4.51	3.75	5.67	-3.13	12.97
September (2016)	45	5.66	-14.59	10.43	5.23	4.33	6.61	-2.15	5.08
October (2106)	48	5.99	-8.23	11.83	4.28	3.56	5.36	-1.50	2.55

SD: Standard deviation.

Table 7 presents the results of the statistical test applied to compare the nighttime surface temperature monthly means. A significant mean-difference between July and October in the recent historical records was reported. However, during the sampling year this difference was not manifested, so, data were collected under similar circumstances.

Table 7. Student´s *t*-test for the nighttime surface temperature (°C)-related independent samples at the MAP-qPCR positive dairy herds (2002-2017) in the Northern region, Province of Antioquia, Colombia (2016).

Group 1 *vs* Group 2	*t*-value	df	*p-value*	SD Group 1	SD Group 2	F-ratio variances	*p*-variances	Levene F(1, df)	*p*-Levene
July-October (2002-2016) *vs* July-October (2016)	**2.21**	**2628**	**0.03**	**4.12**	**6.04**	**2.14**	**0.00**	**27.30**	**0.00**
July-October (2002-2016) *vs* July (2016)	1.43	2488	0.15	4.12	9.46	5.27	0.00	63.59	0.00
July-October (2002-2016) *vs* August (2016)	0.20	2495	0.84	4.12	4.51	1.20	0.34	0.01	0.91
July-October (2002-2016) *vs* September (2016)	1.78	2493	0.08	4.12	5.23	1.61	0.01	4.18	0.04
July-October (2002-2016) *vs* October (2016)	1.28	2496	0.20	4.12	4.28	1.08	0.67	1.57	0.21
July-October (2016) *vs* July (2016)	0.20	218	0.84	6.04	9.46	2.46	0.00	10.49	0.00
July-October (2016) *vs* August (2016)	-0.65	225	0.52	6.04	4.51	1.79	0.02	3.17	0.08
July-October (2016) *vs* September (2016)	0.39	223	0.70	6.04	5.23	1.33	0.27	0.22	0.64
July-October (2016) *vs* October (2016)	-0.55	85	0.59	9.46	4.51	4.39	0.00	13.56	0.00
July (2016) *vs* August (2016)	0.08	83	0.93	9.46	5.23	3.27	0.00	7.68	0.01
July (2016) *vs* September (2016)	-0.13	86	0.90	9.46	4.28	4.89	0.00	11.70	0.00
July (2016) *vs* October (2016)	0.97	90	0.33	4.51	5.23	1.34	0.32	1.47	0.23
August (2016) *vs* September (2016)	0.72	93	0.47	4.51	4.28	1.11	0.72	0.64	0.43
August (2016) *vs* October (2016)	-0.34	91	0.73	5.23	4.28	1.49	0.18	0.34	0.56

Variables were treated as independent samples. df: Degrees of freedom. SD: Standard deviation. Significance level: $p<0.05$ (values in bold are considered significant).

4. Discussion

Our purpose was to describe the spatial distribution of MAP-infected herds in the study area, as well as to approximate to the *appropriate* environmental conditions that would potentially lead to an effective detection of MAP, considering the features defined in our methodology, thus proposing the ideal sampling time of the year in tropical countries as Colombia.

According to our spatial analysis on MAP results, there was an increase in positive herds in the North-West (Entrerríos municipality), South (San Pedro de Los Milagros municipality), and in the South-East (Donmatías municipality) of the study area. In this respect, more in-depth studies are needed aiming to define differential characteristics of these municipalities and even more, for the natural areas represented.

Each environmental sample collected during our study contained material from different sites of concentration of adult cattle as well as from wastewater storage lagoons (when available). These collection locations are, directly exposed to environmental conditions (for the most part), as well as adult cattle high-traffic areas, being the main reason why the exploration of factors such as rainfall, surface temperature, and vegetation cover was included herein.

MAP is an acid-fast bacterium with a unique cell wall structure, supporting a not-sporogenic increased persistence in tough environments and the ability to survive for extended periods outside the host (Whittington et al., 2004; Whittington et al., 2005; Grewal et al., 2006). In addition, MAP is taxonomically classified as an obligate pathogen and is not believed capable of replicating outside a suitable host (Manning, 2001). So, in theory, it can be eradicated by removing all infected animals. However, this organism can survive for long periods outside the host, enabling it to persist and spread in the grassland environment and to resist a periodic lack of susceptible hosts (Thorel, Krichevsky and Lévy-Frébault, 1990). Thus, PTB eradication may be affected by long-term reservoirs of shed, viable MAP, and therefore infection, in both the immediate farm and wider natural environments. Although the improvement of on-farm management measures has been established to be the only approach to reduce herd prevalence

of PTB (Kudahl et al., 2007; Kudahl et al., 2008), the potential for infection via the wider environments needs to be considered for effective disease eradication, if it is the case.

Knowledge of the spatial epidemiology of infection is an important issue from the perspectives of both planning surveys and scientific understanding of the agent transmission and epidemiology (Hess et al., 2002), regardless of the host, agent, or the disease. In addition, identifying spatial and environmental patterns is important in the study of diseases expected to have ecologic causes (or at least, to be related to them). First, because the animal attributes could depend on the level of the environmental and ecologic risk of the farm; and, second, because when comparing whole-herd management characteristics made on one farm with those made on other farms, similar decisions may be considered for neighboring herds and similar risks may be explored by the common environmental and ecologic risks.

The transmission of MAP in animal feces was recognized early in the last century, and the question of how long pastures remain infective was raised as early as 1912. It has been suggested that at least 6 months to a year is required to render pastures safe after grazing by infected cattle (Lovell et al., 1944; Reddacliff et al., 2003). According to Barkema et al. (2017), this approach requires further effort in MAP research, since one important knowledge gap on the topic is the influence of soil and pasture characteristics on the agent´s survival outside the host, considering that if a cow is shedding MAP in the feces, her manure is infectious and can remain in the environment for at least 1 year (Whittington *et al.*, 2004).

Other published data on the survival of MAP in naturally exposed substrates, mimicking the natural environment, and in various laboratory models —both in a trend toward prolonged environmental viability, are available (Lovell et al., 1944; Larsen et al., 1956; Jorgenson, 1977; Richards and Thoen, 1977; Richards, 1981; Olsen et al., 1985; Katayama et al., 2000; Collins et al., 2001; Katayama et al., 2001; Eamens et al., 2001; Salgado et al., 2013). Survival of MAP in the environment has been suggested to depend on many factors, including soil type, pH and moisture, fecal content, and concentrations of macro and micronutrients (*e.g.* calcium, iron, molybdenum, copper) (Lovell et al., 1944; Larsen et al., 1956; Johnson-Ifearulundu and

Kaneene, 1999; Rowe and Grant, 2006). Other possibilities include surface water, shade, temperature, and exposure to sunlight (Whittington et al., 2001; Katayama et al., 2004; Whittington et al., 2005; Elliott et al., 2015).

Nevertheless, the results obtained so far from the studies on MAP survival in the environment are very difficult to generalize to the already varied environmental conditions (Elliott *et al.*, 2014) and due to MAP strain types (Whittington *et al.*, 2004). In addition, most studies have been carried out as an experimental model and not as a field study, which does not correspond to real circumstances. And, also, most of these data (not to say all) come from the Northern hemisphere, where livestock are commonly housed indoors during winter on straw bedding, and where climates tend to be milder than in the tropical-temperate grazing regions of Colombia.

Other unknown aspects are related to the dose of viable MAP that would lead to infection in susceptible animals of different age groups, also, whether ingestion of the surviving concentration of MAP bacteria would actually lead to a MAP infection, whether environmental factors only influence survival and/or virulence of MAP or may have an influence on the host, through (in)direct effects of these factors on the immune system (Lugton, 2004), or whether management practices related to MAP transmission may be associated with environmental factors such as soil type, exposure to sunlight, and humidity (Barkema *et al.*, 2017).

Our results showed that an overall *high* rainfall regime (average of 233.3 mm/month) could favor the detection of the agent in environmental samples of dairy herds infected by MAP in Colombia. July 2016 —the month where the vast majority of positive samples were collected, showed the lowest rainfall regime accumulated of the sampling period (185.8 mm), even though it was still *rainy* when it was expected to be a *dry* month, according to historical records and to the theoretical bimodal rainfall regime base. September 2016 —the month where only one of the positive samples was collected, showed the highest rainfall regime accumulated of the sampling period (306.4 mm), being historically considered as a *rainy* month.

Salgado et al. (2011) reported that the evaluated rainfall amount has a positive effect on the fate of MAP applied to the soil surface with manure slurry. Under high rainfall regimen (2,000 mm/year), MAP was detected more often from lysimeters with loamy soil than from sandy soil. From these findings, authors concluded that MAP tends to move slowly through soils (faster through sandy soil) and tends to remain on the grass and in the upper layers of pasture soil, representing a clear infection hazard for grazing livestock and a potential for the contamination of runoff after heavy rains. Nevertheless, Whittington et al. (2004) reported no effect of the soil moisture on MAP survival.

It can be considered that the bimodal rainfall cycle of the region of study is irrelevant in terms of dispersion of MAP, due to the high values of rainfall that occur even in the *dry* seasons. Nevertheless, such precipitation could contribute to the dispersion of the bacterium, due to the opportunity of dilution offered by water, and therefore, of dispersion of the contaminated feces, difficulting biosecurity measures considered in the control of MAP spread, in and between herds.

Our results indicated that the daytime surface temperature presented important variations during sampling months (from 9.55 to 26.83 °C) without any tendency to significant differences in and between. July and September 2016 —months where all positive samples were collected, were the ones with the highest daytime surface temperatures during the sample period, with differences of 2.14 and 3.07 °C compared to the coldest month (October 2016). Nighttime surface temperature showed important variations (-19.37 to 12.47 °C), especially during July 2016.

A visual comparison between daytime and nighttime surface temperatures results (Tables 5 and 4) showed extreme changes from day to night, reaching even, negative values, but the difference in the monthly averages during the sampling months did not exceed 1.1 ºC. The biggest difference was seen in July 2016 (31.84 ºC) —the month where the vast majority of positive samples were collected. Spatial analysis results showed that areas that showed the highest daytime surface temperature also presented the highest nighttime temperature. Nevertheless, considering our data, no hypothetical approaches should be details about this respect.

When comparing the monthly averages of daytime temperatures (*t*-test), some proximity was observed between the means and the maximum temperatures, but not between the minimum values observed. This tendency is mainly due to abrupt changes in temperature between day and night and vice versa, which is commonly seen in places where there is not enough vegetation cover or where there is no guarantee of heat retention, which seems to be happening with the grasslands of the study area, according to data. However, nighttime surface temperature offers a kind of homogeneous environment for mycobacteria exposed outside the host, regardless of their resistance to temperature changes during the day and in the day-to-night transition.

Scientifically described, the surface temperature is the radiant temperature of the Earth's surface, including grass, bare ground, roads, sidewalks, buildings, and trees, to name a few. The surface temperature can be observed using the electromagnetic spectrum derived from every object depending on its temperature. All the aspects of the total energy balance in nature contribute or are influenced by the surface temperature. The time of day influences the surface temperature, increased in the morning and reached its maximum one or two hours after noon. The sunlight, the season, and the amount of vegetation and moisture in the surface also influence the surface temperature. When there is no humidity available on the surface, like in a desert or in an asphalt surface, there is no evaporation to cool the surface, and the temperature increases more during the day. Importantly, there is a direct relationship between the increase in the surface temperature and the rainfall regime in a region, since the first results in the condensation of the superficial water, leading to the formation of clouds (Trenberth and Shea, 2005). We consider this variable to be analyzed since, in several aspects, it directly affects the survival, interactions, and maintenance of soil microbiome (Conant *et al.*, 2011).

The effect of daytime temperature variation or day-to-night temperature transition on the persistence and dispersion of MAP both locally and regionally is not known. For example, whether such environmental factors favor or restrict the routes of transmission of the bacterium, or the burden of MAP on the host, or whether it influences the basic reproductive number (R0) of the mycobacterium, among other questions whose answers are not yet included in the

available scientific literature. In other words, given these circumstances, the fact that MAP has been detected in the region does not necessarily mean that its metapopulation is progressing. With the information available, it is only possible to launch some speculations that can serve as a working hypothesis for future research.

Even though MAP infection presents a long incubation period inside the host, there is a stage where the bacterium is undoubtedly exposed to the environment, which in general appears to be stressful (considering that the highest daytime surface temperatures areas also present the highest nighttime temperatures, according to the spatial analysis carried out herein). Visually comparing MAP-qPCR positive *vs* negative herds, it can be inferred that, with respect to the general thermal environment, MAP has mechanisms of resilience to some extent efficient, for the survival of some local populations, although it does not seem to be enough for a massive dispersion of its metapopulation. This is an aspect that can only be solved through a study design based on the analysis of MAP metapopulation on a monthly-time-series basis, covering its entire biological cycle, with the inherent limitations of such an approach.

According to our results, the study region is characterized by no evidence of management of the vegetation cover, in both pastures and areas with native vegetation, except for a conservancy area (which currently does not make significant differences in terms of density and functional type of vegetation cover with respect to the areas for livestock use). Such vegetation cover features lead to exposed soil, indifferently to the productive purpose of it. The specific areas with MAP-qPCR positive herds were observed with relatively low humidity (based on the surrounding areas), but a clear pattern in that sense is not highlighted. Our results can be considered as a start point for further designs to solve if the differential vegetation patterns of the study area could increase or diminish MAP population in the environment inside and around the farms.

The solution to this knowledge gap is an important issue considering what is reported by the scientific literature regarding the effect of shading on MAP environmental populations (Larsen et al., 1956). Whittington et al. (2004) reported an increased MAP survival time (up to 55 weeks) in a dry and fully-shaded environment (soil and pastures). Lack of shade was shown to decrease

survival, presumably because of infrared wavelengths (UV radiation) and temperature flux (mainly diurnal). This evidence suggests that MAP survives for long periods in common housing-dairy farm environments, different from those seen in typical Colombian dairies. Whittington et al. (2005) reported a MAP survival time up to 48 and 36 weeks in water and/or sediment in shaded and semi-exposed location, respectively. Survival in sediment was 12 to 26 weeks longer than survival in the water column. Survival in soil and fecal material in the terrestrial environment in the shaded location was only 12 weeks. According to the authors, water may be a significant reservoir of MAP infection.

Then, what is the *appropriate* natural environment for MAP outside the host, guaranteeing its persistence and virulence? According to the literature, is a combination of direct and indirect effects of the three variables evaluated in our study, which seems to assure MAP persistence outside the host, promising an appropriate environment. It appears that humid soils (Whitman, 2009), low surface temperature (Shroen et al., 2000), and shaded areas (Whittington et al., 2004; Whittington et al., 2005) positively affect MAP survival. Considering our results, it can be inferred that the study area represents a convenient environment for the persistence of MAP outside the host. Soil moisture is a major factor, determining the extent to which a unit of net solar radiation increases the temperature of a unit of soil (Whitman, 2009); additionally, it helps to prevent microbial desiccation (Aly and Mangold, 2010). However, soils are complex structures since their composition is influenced by climate, parent material, landscape position, living organisms, and human activity, and not only by their water composition. Soils with similar properties can present the same characteristic behavior in terms of agricultural use, regardless of the geographic location of the soil. Their properties are numerous and varied, including texture, slope, drainage class, permeability, pH, and frost potential (USDA, 2003). And, analogous to other agricultural variables, the survival of microorganisms is likely to be influenced by more than one soil property (Ward and Perez, 2004).

Seems to be that the same environmental characteristics hypothetically needed for MAP survival are the same needed for free-living amoebae (FLA), protozoa found in the same environmental niches as MAP. These microorganisms remain in nature, fulfilling their life cycle without an

intermediate host, feeding mainly on bacteria and fungi from the environment (Rodríguez-Zaragoza, 1994). The genus *Acanthamoeba* encysts under unfavorable situations, such as lack of food, desiccation, accumulation of waste products, and chemical agents (Schuster and Visvesvara, 2004). Several studies have suggested that FLA —found in water and soil, might be a potential environmental host (indeed, reservoir and vector) for MAP. Samba-Louaka et al. (2018) evaluated *in vitro* interactions between several strains of MAP and *Acanthamoeba castellanii*. The results indicated that the bacteria were able to grow within the amoeba (blocking, to a certain extent, the normal acidification process of its phagosomes) and survive up to 7 days within it, having no deleterious effects on amoebal viability. This stability could indeed favor MAP resilience in the environment, providing shelter against harsh conditions, as well as a potential substrate source for mycobacterial multiplication (Caire-Brändli et al., 2014; Barisch et al., 2015; Delafont et al., 2017).

The FLA mainly feed on environmental bacteria, shaping such community composition (Jürgens and Matz, 2002). However, it has been shown that some bacteria may resist FLA´s digestion and could persist or even grow within amoebae (Greub and Raoult, 2004). Importantly, these resistant bacteria are generally also more resistant to phagocytic immune cells, such as macrophages, as have been reported for *Legionella pneumophila* and for various *Mycobacterium* species in interaction with *A. castellanii*. The latter is the most common species in water and the best-known FLA. As a consequence, FLA is considered as a training ground for pathogenic bacteria, including some *Mycobacterium* species (Molmeret *et al.*, 2005; Salah, Ghigo and Drancourt, 2009). The chance of internalized mycobacteria can drastically vary according to the species, ranging from digestion (*e.g. Mycobacterium bovis*) and survival without replication (*e.g. Mycobacterium tuberculosis*) to intra amoebal multiplication (*e.g. Mycobacterium abscessus, Mycobacterium chelonae*) (Drancourt, 2014). No reports on MAP are available about this aspect, mainly because the MAP-amoebae interaction has been poorly described (Mura et al., 2006; Whan et al., 2006; White et al., 2010; Salgado et al., 2015). Actually, these studies used a very limited range of strains and none of them has clearly demonstrated the presence of MAP inside environmental FLA. Another question that would arise from the interaction of these two microorganisms is the dynamics of entry and perpetuation of

MAP inside FLA under adverse conditions for the same, a situation in which it would encyst in *pro* of its own survival.

A big issue in the interpretation of the results in most epidemiological studies is that the establishment of the study areas tends to be arbitrary. Such studies focus on their effects in communes, political-administrative units, health areas, and other systems, which is a natural context is of limited relevance. In this sense, it is highly recommended that a spatial context should be taken into account during the sampling design, being this approach significant and representative of the study subject. For example, in the case of bovine PTB, a previous spatial analysis of the distribution, size and, most of all, connectivity of the grazing areas crossed with the bovine population density, would be ideal to better measure the scope of the disease and its potential of dispersion from the positive points of infection previously detected. Our simulation results suggest the existence of spatial correlation in most of the observed cases, leading to the possibility of establishing *hot spots* for a future sampling of the agent in the study area.

In conclusion, our study referred to an exploratory, non-experimental observational study carried out on an uncontrolled tropical and a real dynamic environment. Our purpose was to describe the general conditions of the environmental context where the detection of positive herds is most likely to happen, considering the same (or a very approximate) sample collection and handling, and molecular detection method. In the future, study designs on this disease (or others similar) should be based on spatial probabilistic maps, increasing the chances of finding the causal agent in the environment using with fewer resources.

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7. Declarations of interest

None.

8. References

Aly, S., and Mangold, B. (2010) 'Correlation between Herrold egg yolk medium culture and real-time quantitative polymerase chain reaction results for *Mycobacterium avium* subspecies', Journal of Veterinary Diagnostic Investigation 683, pp. 677–683. URL: http://vdi.sagepub.com/content/22/5/677.short

Bailey, T. C., and Gatrell, A. C. (1995) 'Interactive spatial data analysis', Interactive spatial data analysis. URL: http://www.scopus.com/inward/record.url?eid=2-s2.0-0029502393&partnerID=tZOtx3y1

Barisch, C., Paschke, P., Hagedorn, M., Maniak, M., and Soldati, T. (2015) 'Lipid droplet dynamics at early stages of *Mycobacterium marinum* infection in *Dictyostelium*', Cellular Microbiology. http://doi/10.1111/cmi.12437

Barkema, H. W., Orsel, K., Nielsen, S. S., Koets, A. P., Rutten, V. P. M. G., Bannantine, J. P., Keefe, G. P., Kelton, D. F., Wells, S. J., Whittington, R. J., Mackintosh, C. G., Manning, E. J., Weber, M. F., Heuer, C., Forde, T. L., Ritter, C., Roche, S., Corbett, C. S., Wolf, R., Griebel, P. J., Kastelic, J. P., and De Buck, J. (2017) 'Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection', Transboundary and Emerging Diseases, (May), pp. 1–24. http://doi/10.1111/tbed.12723

Behr, M. A. (2010) 'Paratuberculosis and Crohn 's Disease', in Paratuberculosis: Organism, Disease, Control, pp. 40–49.

Blöschl, G. (2002) 'Geostatistics for Environmental Scientists.', Vadose Zone Journal. http://doi/10.2136/vzj2002.0321

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. (2009) 'The MIQE guidelines: Minimum Information for publication of quantitative real-time PCR experiments', Clinical Chemistry, 55(4), pp. 611–622. http://doi/10.1373/clinchem.2008.112797

Caire-Brändli, I. B., Papadopoulos, A., Malaga, W., Marais, D., Canaan, S., Thilo, L. and De Chastelliera, C. (2014) 'Reversible lipid accumulation and associated division arrest of *Mycobacterium avium* in lipoprotein-induced foamy macrophages may resemble key events during latency and reactivation of tuberculosis', Infection and Immunity. http://doi/10.1128/IAI.01196-13

Clarke, C. J. (1997) 'The pathology and pathogenesis of paratuberculosis in ruminants and other species', Journal of Comparative Pathology, 116(1906), pp. 217–261.

Collins, M. T., Gardner, I. A., Garry, F. B., Roussel, A. J., and Wells, S. J. (2006) 'Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States', Journal of the American Veterinary Medical Association, 229(12), pp. 1912–1919. http://doi/10.2460/javma.229.12.1912

Conant, R. T., Ryan, M. G., Ågren, G. I., Birge, H. E., Davidson, E. A., Eliasson, P. E., Evans, S. E., Frey, S. D., Giardina, C. P., Hopkins, F. M., Hyvönen, R., Kirschbaum, M. U. F., Lavallee, J. M., Leifeld, J., Parton, W. J., Megan Steinweg, J., Wallenstein, M. D., Martin Wetterstedt, J. Å., and Bradford, M. A. (2011) 'Temperature and soil organic matter decomposition rates - synthesis of current knowledge and a way forward', Global Change Biology. http://doi/10.1111/j.1365-2486.2011.02496.x

Cressie, N. (1992) 'Statistics for spatial data', Terra Nova. http://doi/10.1111/j.1365-3121.1992.tb00605.x

Delafont, V., Samba-Louaka, A., Cambau, E., Bouchon, Di., Moulin, L., and Héchard, Y. (2017) '*Mycobacterium llatzerense*, a waterborne *Mycobacterium*, that resists phagocytosis by *Acanthamoeba castellanii*', Scientific Reports. http://doi/10.1038/srep46270

Dimitriou-Fakalou, C. (2010) 'Statistical inference for spatial auto-linear processes', Journal of Statistical Theory and Practice. http://doi/10.1080/15598608.2010.10411991

Dohoo, I., Martin, W., and Stryhn, H. (2014). Veterinary Epidemiologic Research. 8 Berkeley Way, Charlottetown, Prince Edward Island, Canada, VER Inc.

Donat, K., Kube, J., Dressel, J., Einax, E., Pfeffer, M., and Failing, K. (2015) 'Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture', Epidemiology and Infection, 143(5), pp. 975–985. http://doi/10.1017/S0950268814002465

Drancourt, M. (2014) 'Looking in amoebae as a source of mycobacteria', Microbial Pathogenesis. http://doi/10.1016/j.micpath.2014.07.001

Eamens, G. J., Spence, S. A., and Turner, M. J. (2001) 'Survival of *Mycobacterium avium* subsp *paratuberculosis* in amitraz cattle dip fluid', Australian Veterinary Journal. http://doi/10.1111/j.1751-0813.2001.tb10676.x

Elliott, G. N., Hough, R. L., Avery, L. M., Maltin, C. A., and Campbell, C. D. (2015) 'Environmental risk factors in the incidence of Johnes disease', Critical Reviews in Microbiology, 41(4), pp. 488–507. http://doi/10.3109/1040841X.2013.867830

Elliott, G. N., Hough, R. L., Avery, L. M., Maltin, C., and Campbell, C. D. (2014) 'Environmental risk factors in the incidence of Johne's disease.', Critical reviews in microbiology, 7828(November 2015), pp. 1–20. http://doi/10.3109/1040841X.2013.867830

Fedegán. Registro de vacunación, primer ciclo, 2015. Colombia (confidential material).

Fernández-Silva, J. A., Correa-Valencia, N. M., and Ramírez, N. F. (2014) 'Systematic review of the prevalence of paratuberculosis in cattle, sheep, and goats in Latin America and the Caribbean', Tropical Animal Health and Production, 46(8), pp. 1321–1340. http://doi/10.1007/s11250-014-0656-8

Freitas, R. M. (2011) 'Virtual laboratory of remote sensing time series: visualization of MODIS EVI2 data set over South America', Journal of Computational Interdisciplinary Sciences. http://doi/10.6062/jcis.2011.02.01.0032

Gao, B. C. (1996) 'NDWI - A normalized difference water index for remote sensing of vegetation liquid water from space', Remote Sensing of Environment. http://doi/10.1016/S0034-4257(96)00067-3

Giraldo, R. (2011) 'Teoría y Aplicación', Introducción a la geoestadistica.

Greub, G., and Raoult, D. (2004) 'Microorganisms resistant to free-living amoebae', Clinical Microbiology Reviews. http://doi/10.1128/CMR.17.2.413-433.2004

Grewal, S. K., Rajeev, S., Sreevatsan, S., and Michel, F. C. (2006) 'Persistence of *Mycobacterium avium* subsp. *paratuberculosis* and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure', Applied and Environmental Microbiology. http://doi/10.1128/AEM.72.1.565-574.2006

Hengl, T. (2009) A Practical guide to geostatistical mapping of environmental variables, Office. http://doi/10.1016/0277-9390(86)90082-8

Hess G.R, Randolph S.E, Arneberg P, Chemini C, Furlanello C, Harwood J, Roberts M.G., and Swinton J (2002) 'Spatial aspects of disease dynamics', The Ecology of Wildlife Diseases. http://doi/10.1080/1066568000330103

IDEAM (2012). Mapa de clasificación climática de Caldas-Lang, república de Colombia, año 2012. Bogotá, DC., Instituto de Hidrología, Meteorología y Estudios Ambientales (IDEAM)**:** Clasificación climática de Lang, 2012/Colombia. URL: http://www.ideam.gov.co/documents/21021/21789/climas+%5BModo+de+compatibilidad%5D.pdf/d8c85704-a07a-4290-ba65-f2042ce99ff9

Illian, J., Penttinen, A., Stoyan, H., and Stoyan, D. (2008) Statistical analysis and modelling of spatial point patterns, statistical analysis and modelling of spatial point patterns. http://doi/10.1002/9780470725160

Johnson-Ifearulundu, Y. J., and Kaneene, J. B. (1997). Relationship between soil type and *Mycobacterium paratuberculosis*. J. American Veterinary Medicine Association 210, 1735–1740.

Jorgenson, J. B. (1977) 'Survival of *Mycobacterium paratuberculosis* in slurry', Nordisk Veterinaer Medicin.

Jürgens, K., and Matz, C. (2002) 'Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria', Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology. http://doi/10.1023/A:1020505204959

Kaluzny, S. P., Vega, S. C., Cardoso, T. P., and Shelly, A. A. (1998). S+SpatialStats. User's manual for Windows and UNIX. New York: Springer-Verlag.

Katayama, N., Tanaka, C., Fujita, T., Saitou, S., Suzuki, S., and Onouchi, E. (2000) 'Effect of ensilage on inactivation of *M. avium* subsp. *paratuberculosis'*. Grass and Forage Science 46:282–288.

Katayama, N., Tanaka, C., Fujita, T., Suzuki, T., Watanabe, S., and Suzuki, S. (2001) 'Effects of silage fermentation and ammonia treatment on activity of *Mycobacterium avium* subsp. *paratuberculosis'*, Grassland Science.

Katayama, N., Suzuki, T., Shibata, M., Ootake, M., Kamata, S., and Yokomizo, Y. (2004) Influence of ultraviolet-B (UV-B) on viability of *Mycobacterium avium* subsp. *paratuberculosis*, Grass and Forage Science 50, 336-340.

Kruze, J., Monti, G., Schulze, F., Mella, A., and Leiva, S. (2013) 'Herd-level prevalence of MAP infection in dairy herds of southern Chile determined by culture of environmental fecal samples and bulk-tank milk qPCR', Preventive Veterinary Medicine. Elsevier B.V., 111(3–4), pp. 319–324. http://doi/10.1016/j.prevetmed.2013.05.011

Kudahl, A. B., Nielsen, S. S., and Østergaard, S. (2008) 'Economy, efficacy, and feasibility of a risk-based control program against paratuberculosis', Journal of Dairy Science. http://doi/10.3168/jds.2008-1257

Kudahl, A. B., Østergaard, S., Sørensen, J. T., and Nielsen, S. S. (2007) 'A stochastic model simulating paratuberculosis in a dairy herd', Preventive Veterinary Medicine. http://doi/10.1016/j.prevetmed.2006.05.015

Kuenstner, J. T., Naser, S., Chamberlin, W., Borody, T., Graham, D. Y., McNees, A., Hermon-Taylor, J., Hermon-Taylor, A., Dow, C. T., Thayer, W., Biesecker, J., Collins, M. T., Sechi, L. A., Singh, S. V., Zhang, P., Shafran, I., Weg, S., Telega, G., Rothstein, R., Oken, H., Schimpff, S., Bach, H., Bull, T., Grant, I., Ellingson, J., Dahmen, H., Lipton, J., Gupta, S., Chaubey, K., Singh, M., Agarwal, P., Kumar, A., Misri, J., Sohal, J., Dhama, K., Hemati, Z., Davis, W., Hier, M., Aitken, J., Pierce, E., Parrish, N., Goldberg, N., Kali, M., Bendre, S., Agrawal, G., Baldassano, R., Linn, P., Sweeney, R. W., Fecteau, M., Hofstaedter, C., Potula, R., Timofeeva, O., Geier, S., John, K., Zayanni, N., Malaty, H. M., Kahlenborn, C., Kravitz, A., Bulfon, A., Daskalopoulos, G., Mitchell, H., Neilan, B., Timms, V., Cossu, D., Mameli, G., Angermeier, P., Jelic, T., Goethe, R., Juste, R. A., and Kuenstner, L. (2017) 'The Consensus from the *Mycobacterium avium* ssp. *paratuberculosis* (MAP) Conference 2017', Frontiers in Public Health, 5(September), pp. 1–5. http://doi/10.3389/fpubh.2017.00208

Larsen, A. B., Merkal, R. S., and Vardaman, T. H. (1956) 'Survival time of *Mycobacterium paratuberculosis*.', American Journal of Veterinary Research.

Law, D. C. G., Serre, M. L., Christakos, G., Leone, P. A., and Miller, W. C. (2004) 'Spatial analysis and mapping of sexually transmitted diseases to optimise intervention and prevention strategies.', Sexually Transmitted Infections, 80(4), pp. 294–9. http://doi/10.1136/sti.2003.006700

Lovell, R., Levi, M., and Francis, J. (1944) 'Studies on the survival of Johne's bacilli', Journal of Comparative Pathology. http://doi/10.1016/S0368-1742(44)80013-3

Lugton, I. W. (2004) 'Review of possible links between the clinical expression of paratuberculosis and deficiency of macro and micronutrients', Australian Veterinary Journal. http://doi/10.1111/j.1751-0813.2004.tb11167.x

Manning, E. J. B. (2001) '*Mycobacterium avium* subspecies *paratuberculosis*', Journal of Zoo and Wildlife Medicine, 32(3), pp. 293–304.

Matheron, G. (1963) 'Principles of geostatistics', Economic Geology. http://doi/10.2113/gsecongeo.58.8.1246

Molmeret, M., Horn, M., Wagner, M., Santic, M., and Kwaik, Y. A. (2005) 'Amoebae as training grounds for intracellular bacterial pathogens', Applied and Environmental Microbiology. http://doi/10.1128/AEM.71.1.20-28.2005

Mura, M., Bull, T. J., Evans, H., Sidi-Boumedine, K., McMinn, L., Rhodes, G., Pickup, R., and Hermon-Taylor, J. (2006) 'Replication and long-term persistence of bovine and human strains of *Mycobacterium avium* subsp. *paratuberculosis* within Acanthamoeba polyphaga', Applied and Environmental Microbiology. http://doi/10.1128/AEM.72.1.854-859.2006

Nielsen, S. S., and Toft, N. (2009) 'A review of prevalences of paratuberculosis in farmed animals in Europe', Preventive Veterinary Medicine, 88(1), pp. 1–14. http://doi/10.1016/j.prevetmed.2008.07.003

Olsen, J. E., Jørgensen, J. B., and Nansen, P. (1985) 'On the reduction of *Mycobacterium paratuberculosis* in bovine slurry subjected to batch mesophilic or thermophilic anaerobic digestion', Agricultural Wastes. http://doi/10.1016/0141-4607(85)90052-6

Pillars, R. B., Grooms, D. L., and Kaneene, J. B. (2009) 'Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project.', The Canadian Veterinary Journal. La revue Veterinaire Canadienne, 50(10), pp. 1039–1046.

Raizman, E. A., Wells, S., Godden, S. M., Bey, R. F., Oakes, M. J., Bentley, D. C., and Olsen, K. E. (2004) 'The Distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the Environment Surrounding Minnesota Dairy Farms', Journal of Dairy Science, 87(9), pp. 2959–2966. http://doi/10.3168/jds.S0022-0302(04)73427-X

Reddacliff, L. A., Nicholls, P. J., Vadali, A., and Whittington, R. J. (2003) 'Use of growth indices from radiometric culture for quantification of sheep strains of *Mycobacterium avium* subsp. *paratuberculosis'*, Applied and Environmental Microbiology. http://doi/10.1128/AEM.69.6.3510-3516.2003

Richards, W. D. (1981) 'Effects of physical and chemical factors on the viability of *Mycobacterium paratuberculosis'*, Journal of Clinical Microbiology.

Richards, W. D., and Thoen, C. O. (1977) 'Effect of freezing on the viability of *Mycobacterium paratuberculosis* in bovine feces', Journal of Clinical Microbiology.

Rodríguez-Zaragoza, S. (1994) 'Ecology of free-living amoebae', Critical Reviews in Microbiology. http://doi/10.3109/10408419409114556

Salah, I. B., Ghigo, E., and Drancourt, M. (2009) 'Free-living amoebae, a training field for macrophage resistance of mycobacteria', Clinical Microbiology and Infection. http://doi/10.1111/j.1469-0691.2009.03011.x

Salgado, M., Aleuy, O. A., Sevilla, I. A., Troncoso, E., Salgado, M., Aleuy, O. A., Sevilla, I. A., and Troncoso, E. (2015) 'Detection of *Mycobacterium avium* subsp. *paratuberculosis* in a cattle/pudu interface', Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 67(5), pp. 1205–1209. http://doi/10.1590/1678-4162-7530

Salgado, M., Alfaro, M., Salazar, F., Troncoso, E., Mitchell, R. M., Ramirez, L., Naguil, A., Zamorano, P., and Collins, M. T. (2013) 'Effect of soil slope on the appearance of *Mycobacterium avium* subsp. *Paratuberculosis* in water running off grassland soil after application of contaminated slurry', Applied and Environmental Microbiology. http://doi/10.1128/AEM.00610-13

Salgado, M., Collins, M. T., Salazar, F., Kruze, J., Bölske, G., Söderlund, R., Juste, R., Sevilla, I. A., Biet, F., Troncoso, F., and Alfaro, M. (2011) 'Fate of *Mycobacterium avium* subsp. *paratuberculosis* after application of contaminated dairy cattle manure to agricultural soils', Applied and Environmental Microbiology, 77(6), pp. 2122–2129. http://doi/10.1128/AEM.02103-10

Samba-Louaka, A., Robino, E., Cochard, T., Branger, M., Delafont, V., Aucher, W., Wambeke, W., Bannantine, J. P., Biet, F., and Héchard, Y. (2018) 'Environmental *Mycobacterium avium* subsp. *paratuberculosis* hosted by free-living amoebae', Frontiers in Cellular and Infection Microbiology, 8. http://doi/10.3389/fcimb.2018.00028

Schuster, F. L., and Visvesvara, G. S. (2004) 'Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals', International Journal for Parasitology. http://doi/10.1016/j.ijpara.2004.06.004

Schroen, C., Kluver, P. W. M., Butler, K. L., and Condron, R. A. H. (2000). Survival of *Mycobacterium paratuberculosis* in the environment. Meat and Livestock Australia Ltd, North Sydney.

Strebelle, S. B. (2006) 'Sequential simulation for modeling geological structures from training images', in Stochastic modeling and geostatistics: Principles, methods, and case studies, volume II: AAPG Computer Applications in Geology 5. http://doi/10.1306/1063812CA53231

Sweeney, R. W. (1996) 'Transmission of paratuberculosis.', The Veterinary clinics of North America. Food animal practice, pp. 305–312. http://doi/10.1016/S0749-0720(15)30408-4

Thorel, M. F., Krichevsky, M., and Lévy-Frébault, V. V. (1990) 'Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. s', International Journal of Systematic Bacteriology, 40(3), pp. 254–60. http://doi/10.1099/00207713-40-3-254

Trenberth, K. E., and Shea, D. J. (2005) 'Relationships between precipitation and surface temperature', Geophysical Research Letters. http://doi/10.1029/2005GL022760

USDA (United States Department of Agriculture)–Natural Resources Conservation Service. From the surface down. An introduction to soil surveys for agronomic use. URL: www.soils.usda.gov/education/resources

USDA (United States Department of Agriculture) (2010). Uniform Program Standards for the Voluntary Bovine Johne´s Disease Control Program. Washington D.C. United States, Department of Agriculture-USDA, Animal and Plant Health Inspection Service-APHIS. URL: https://johnes.org/handouts/files/USDA_Program_Standards_Sept-2010.pdf

USGS. (2017). "Normalized Difference Water Index (NDWI)." from https://deltas.usgs.gov/fm/data/data_ndwi.aspx.

Vega-Morales, A. (1947) Relación entre el diagnóstico de la paratuberculosis bovina por el examen coprológico y de la prueba alérgica de termorreacción con la tuberculina aviaria por vía subcutánea. Bogotá, Colombia, Universidad Nacional de Colombia, diss.

Ward, M. P., and Perez, A. M. (2004) 'Association between soil type and paratuberculosis in cattle herds', American Journal of Veterinary Research. http://doi/10.2460/ajvr.2004.65.10

Whan, L., Grant, I. R., and Rowe, M. T. (2006) 'Interaction between *Mycobacterium avium* subsp. *paratuberculosis* and environmental protozoa', BMC Microbiology. http://doi/10.1186/1471-2180-6-63

White, C. I., Birtles, R. J., Wigley, P., and Jones, P. H. (2010) '*Mycobacterium avium* subspecies *paratuberculosis* in free-living amoebae isolated from fields not used for grazing', Veterinary Record. doi: 10.1136/vr.b4797.

Whitman, W. B. (2009) 'Modern Soil Microbiology, second ed.', Agricultural Systems. doi: 10.1016/j.agsy.2008.12.004.

Whittington, R. J., Marsh, I. B. and Reddacliff, L. a. (2005) 'Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment', Applied and environmental Microbiology, 71(9), pp. 5304–5308. http://doi/10.1128/AEM.71.9.5304

Whittington, R. J., Marshall, D. J., Nicholls, P. J., Marsh, I. B., and Reddacliff, L. A. (2004) 'Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment', Applied and Environmental Microbiology, 70(5), pp. 2989–3004. http://doi/10.1128/AEM.70.5.2989-3004.2004

Wolf, R., Barkema, H. W., De Buck, J., and Orsel, K. (2015) 'Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results', Journal of Dairy Science, 98(1), pp. 275–287. http://doi/10.3168/jds.2014-8676

Yengoh, G. T., Dent, D., Olsson, L., Tengberg, A. E., and Tucker III, C. J. (2015) Use of the normalized difference vegetation index (ndvi) to assess land degradation at multiple scales: Current status, future trends, and practical considerations, Springer.

General Conclusions

According to the literature research presented herein on paratuberculosis and its causative agent in Colombia, 20 original studies have been carried out so far in four different animal species, mainly using ELISA, and predominantly in the Provinces of Antioquia and Cundinamarca. In general, the results reported by the original studies done so far in the country are still insufficient to accurately reflect the epidemiologic situation about MAP or its economic and public health impact in Colombia.

The present study found a herd-level prevalence of 4.1% and “having a history of mixed farming of cattle with other ruminants (*i.e.* sheep, goats) in the last 2 years” as a risk factor for MAP infection in 292 dairies under mechanical milking parlor and pasture grazing-based systems. On the other hand, a prevalence of 14.9% and “having other than Holstein breeds were predominant” (namely, Jersey, Jersey×Holstein crossbreeds, and Jersey×Swedish red crossbreeds) as a risk factor in 94 dairies with in-paddock milking facilities were found. Such features could be considered for PTB´s control, particularly in typical dairies in Colombia under the same facilities and management practices than the ones considered herein.

Based on the sub-typing patterns of the MAP isolates obtained from environmental samples in the 386 dairy herds of study, two different MAP genotypes were found: INMV 2 and INMV 36 according to MIRU-VNTR analysis, and no discrimination among common INMV profiles according to MLSSR results was found. The present findings lead to important epidemiological implications with regard to control and prevention of PTB in the country.

General conditions of the environmental context, where the detection of MAP-positive herds is most likely to happen, considering the same (or a very approximate) sample collection and handling, and the molecular detection method used herein, using a spatial and environmental-analytic approach were reported herein. It seems that the appropriate natural environment for MAP outside the host is a combination of direct and indirect effects of the three variables evaluated in our study: Humid soils, low surface temperature, and shaded areas.

Considering the present results, it can be inferred that the study area represents a convenient environment for the persistence of MAP outside the host. The implications regarding the transmission potential of the bacterium to susceptible hosts should be considered as a starting point in the definition of a strategic and coupled control program, in view of the environmental conditions of our country.

Recommendations

Future research approaches should consider the determination of MAP herd and cow-level prevalences in other representative populations, including other municipalities, regions and provinces 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 beef and double-purpose farms, with different characteristics and under different management practices.

In the future, study designs on this disease (or others similar) should be based on spatial probabilistic maps, increasing the chances of finding the causal agent in the environment using fewer resources. In the particular case of bovine PTB, a previous spatial analysis of the distribution, size and, most of all, connectivity of the grazing areas crossed with the bovine population density, would be ideal to better measure the scope of the disease and its potential of dispersion from the positive points of infection previously detected.

It is suggested that newer typing methods, such as single nucleotide polymorphism (SNP), which is capable of detecting differences among major types, may be used to obtain further discernment into the epidemiological features of MAP in the country, including the influence of culture media, the role played by the local wildlife, the diversity of agro-ecosystems, and the crossbreeding of imported and indigenous animals to be taken into account in the analysis as possible sources of genomic diversity of MAP.

All the information reported herein will help to the establishment of a cost-effective basis for PTB control at a herd and regional-level, considering the recent concern about disease notification in Colombia.

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