Prevalence and risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep located at some regions in the Province of Antioquia, Colombia

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“Not everything that can be counted counts, and not everything that counts can be counted”

-William Bruce Cameron-

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List of abbreviations and acronyms

AFB	Acid Fast Bact
AGID	Agar Gel Immune Diffusion
bp	Base Pairs
CD	Crohn´s Disease
CF	Complement Fixation
CFU	Colony Forming Units
CI	Confidence Intervals
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunoabsorbent Assay
e.g.	exempli gratia (for example)
et al.	Et al (and others)
FC	Fecal Culture
g	Gram
h	hour
HEYM	Herrold´s Egg Yolk Medium

HPC	Hexadecyl Pyridinium Chloride
IAC	Internal Amplification Control
i.e.	id est (that is)
IS	Insertion Sequence
JD	Johne´s Disease
L	Liter
M.	Mycobacterium
MAC	*Mycobacterium avium* Complex
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
min	Minute
ml	Milliliter
MLSSR	Multilocus Short Sequence Repeats
mm	Millimeter
MIRU-VNTR	Mycobacterial Interspersed Repeats Units-Variable Number of Tandem Repeats
OD	Optical Density
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction

PFC	Pooled Fecal Culture
PTB	Paratuberculosis
qPCR	Quantitative (real-time PCR)
S/P	Value of the sample / Value of the positive control
SD	Standard Deviation
Se	Sensitivity
Sp	Specificity
subsp.	subspecies
U	Unit
µl	Microliter
µM	Micromolar
w/v	Weight/Volume
%	Percentage

General Summary

Introduction: paratuberculosis or Johne's disease is a slow–developing infectious disease characterized by chronic granulomatous enterocolitis that causes great economic losses. Johne's disease is caused by Mycobacterium avium subsp. paratuberculosis (MAP) an intracellular, Gram-positive, acid-fast, facultative and slow-growing pathogen. The reports of paratuberculosis in small ruminants in Colombia are very rare; there have been reports in sheep mainly in the Cundiboyacense Plateau and the Bogotá Savannah but the prevalence of paratuberculosis in sheep and goat populations in Colombia is unknown. **Objective:** determine the prevalence and flock level risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep located at some regions in the Province of Antioquia, Colombia. **Methods:** a total of 456 sheep over one year of age in 24 different flocks located in three regions of the Province of Antioquia, Colombia were studied. Serum samples, fecal samples, and information on flock management practices were taken to explore associations to MAP status. Serum samples were analyzed using the ELISA CATTLETYPE® MAP ab test, fecal samples were analyzed in pools by the BACTEC™ MGIT™ para TB System and an IS900 qPCR protocol. The information on risk factors was analyzed by means of descriptive statistics and logistic regression. **Results:** the seroprevalence obtain was 8% (37/456) at animal-level and 70% (17/24) at flock-level. The flock size was identified as a risk factor for the presence of MAP (p=0,019). From 90 fecal pool tested, qPCR and fecal culture detected 25 (27,7%) and 64 (71,1%) positive pools. Besides, MAP positive pools were detected in 45,8% and 83,3% of the flocks by qPCR and culture, respectively. **Conclusion:** this study demonstrates MAP presence in sheep flocks from three regions in the Province of Antioquia, Colombia and the relationship between MAP seropositivity and flock size.

Resumen general

Introducción: la paratuberculosis o enfermedad de Johne es una enfermedad infecciosa de desarrollo lento caracterizada por enterocolitis granulomatosa crónica la cual causa grandes pérdidas económicas. La enfermedad de Johne es causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP) un patógeno intracelular, Gram positivo, ácido- alcohol resistente, facultativo y de crecimiento lento. Los informes de paratuberculosis en pequeños rumiantes en Colombia son muy raros; ha habido informes en ovejas principalmente en el altiplano Cundiboyacense y la sabana de Bogotá, pero la prevalencia de paratuberculosis en las poblaciones ovina y caprina en Colombia es desconocida. **Objetivo:** determinar la prevalencia y los factores de riesgo a nivel de aprisco asociados con la infección por *Mycobacterium avium* subsp. *paratuberculosis* en ovinos localizados en algunas regiones del Departamento de Antioquia, Colombia. **Métodos:** se estudiaron un total de 456 ovejas mayores de un año de edad en 24 apriscos diferentes ubicados en tres regiones del Departamento de Antioquia, Colombia. Se tomaron muestras de suero y materia fecal e información sobre las prácticas de manejo a nivel de aprisco para explorar las asociaciones al estatus de MAP. Las muestras de suero se analizaron mediante la prueba ELISA CATTLETYPE® MAP ab, las muestras fecales se analizaron en grupos mediante el sistema BACTEC ™ MGIT ™ para TB y un protocolo IS900 qPCR. La información sobre los factores de riesgo se analizó mediante estadística descriptiva y regresión logística. **Resultados:** la seroprevalencia obtenida fue del 8% (37/456) a nivel de animal y del 70% (17/24) a nivel de aprisco. El tamaño del aprisco fue identificado como un factor de riesgo para la presencia de MAP ($p = 0,019$). De un total de 90 pooles de heces evaluados, la qPCR y el cultivo fecal detectaron 25 (27,7%) y 64 (71,1%) pooles positivos. Además, se detectaron pooles positivos a MAP en 45,8% y 83,3% de los apriscos evaluados por qPCR y cultivo, respectivamente. **Conclusión:** este estudio

demuestra la presencia de MAP en apriscos de ovejas de tres regiones del Departamento de Antioquia, Colombia y la relación entre la seropositividad a MAP y el tamaño del aprisco.

General introduction

The central problem that this project aims to solve is the lack of information on the prevalence of paratuberculosis or Johne's disease in sheep in three regions of the Province of Antioquia, as well as the factors associated with the prevalence of the disease.

Paratuberculosis ('Johne's disease') is a chronic bacterial disease of global importance in domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP, Windsor, 2015). MAP has also been associated to the human chronic enteritis known as Crohn´s disease (Waddell, 2016). Based on the knowledge that MAP has been detected in different foods, it could represent a risk for the consumers due to its zoonotic potential (Atreya, 2014). This potential zoonotic role, the human exposure to MAP by food, and the fact that this relation cannot be proved or disproved are reasons for great concern (Waddell, 2016).

The understanding of paratuberculosis should be a research objective for scientists, the sheep industry and the academy due to the devastating potential of the disease, which affects several production systems and has an impact on public health, since its zoonotic potential is widely accepted worldwide, and there are no official control measures by the Colombian animal health authorities. In addition, the knowledge of the prevalence of an infection at the herd and animal level is the key issue when decision or policy makers determine whether the infection should be considered important or not, and what measures to apply (Nielsen & Toft, 2009).

The Colombian sheep industry has identified some health problems that affect its production and demand the development of strategies that allow the prevention, control and treatment of the health

problems of this industry. Specifically, this demand seeks to know the regional and national status of diseases that can limit the marketing of meal and milk nationally and internationally (Anonymous, 2014). Paratuberculosis is one of the causes of which the sheep industry is at low level of development, limiting meat and milk commercialization at national and international level (Castellanos et al., 2010). Research on paratuberculosis in small ruminants is very limited in the world and especially in Latin America and the Caribbean. In Colombia research on this disease in small ruminants is virtually nonexistent.

This study will contribute greatly to regional, national, continental and global knowledge of the presentation and distribution of paratuberculosis. In addition, the study will provide epidemiological knowledge about the main risk factors for the disease in the Colombian productive context, give information on the applicability of diagnostic techniques in the environment of study, and provide information on the risk factors associated with the disease in the region. The study of this disease is of major interest of the proposing line of research (Epidemiology and Veterinary Public Health), because it investigates phenomena that relate animal and human health, using immune-based, PCR and culture diagnostic tests and epidemiology, as basis to achieve its goals on health improvement.

Due to the lack of information on the prevalence of the disease in the Province of Antioquia, it is expected that the flock prevalence and the seroprevalence of MAP in the study flocks under study could be around 50% at the flock and the individual level, respectively, and that at least one flock management practice is a risk factor for MAP ELISA positive results in the study flocks.

Objectives

General Objective

Determine the prevalence and flock level risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep flocks located in three regions of the province of Antioquia, Colombia.

Specific Objectives

1. Determine the presence of serum antibodies against MAP in sheep by ELISA to establish the individual seroprevalence in three regions of the Province of Antioquia, Colombia.

2. Determine the presence of MAP DNA in sheep feces to establish the flock prevalence by real-time PCR in three regions of the Province of Antioquia, Colombia.

3. Determine the presence of MAP in sheep feces to establish the flock prevalence by culture in three regions of the Province of Antioquia, Colombia.

4. Explore the main flock level risk factors associated to the positivity to MAP by ELISA in sheep in three regions of the province of Antioquia, Colombia.

Background

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (PTB) or Johne's disease (JD) which is an infectious disease characterized by chronic granulomatous enterocolitis, lymphangitis and regional lymphadenitis that lead to the typical clinical signs of a progressive weight loss (Clarke, 1997).

MAP is an intracellular, Gram-positive, acid-fast, facultative and slow-growing pathogen (Lombard, 2011). MAP belongs to the group of *Mycobacterium avium* complex (MAC), which comprises environmental saprophytes mycobacteria, opportunistic and obligate pathogens, which are of great importance in veterinary medicine and human medicine for their involvement in infections of farm animals and immunosuppressed patients (Turenne and Alexander, 2010). MAP is very resistant to environmental and chemical changes and may persist in the environment, including soil, water and fertilizer up to one year (Lombard, 2011; Salgado et al., 2013). The presence of MAP has also been described in horses, pigs, deer and alpacas, rabbits, stoats, foxes and weasels and in captive and free-living wild animals (Carta et al., 2013).

MAP was first observed by Heinrich Albert Johne and Langdon Frothingham in 1895 in bovine, where the disease has been well characterized and widely distributed worldwide, especially in dairy herds. It was first cultivated by Frederick William Twort in 1912 (Manning and Collins, 2010). Johne's disease was first recognized in cattle, then in sheep and later in goats. The disease is very frequent in domestic and wild ruminants and has a global distribution (Sonawane et al., 2016).

Under natural conditions, the fecal–oral route, especially at an early life stage of animals, is the main way to contract PTB at the individual level (Begg and Whittington, 2010; Dukkipati et al., 2016). The infection can spread via intrauterine and transmammary, but this occurs more frequently in clinically ill sheep than in those with subclinical infection (Lambeth et al., 2004). Infection can be spread vertically to the fetus and semen can be infected with the microorganism (Eppleston and Whittington, 2001; Sweeney, 1996).

Most ruminants become infected as neonates by oral ingestion of the organism, probably soon after birth from the udder from an animal that was shedding the organism, or from contaminated utensils (Lombard, 2011). Consequently, the major sources of MAP infection are infected animals and therefore the contamination of udder of the newborn´s dam, pasture, feedstuff or utensils with feces is described as the principal factor to avoid when a control of the disease in the herd is desired (Groenendaal, 2011).

Four categories or stages of disease have been determined for PTB in cattle (Fecteau and Whitlock, 2010). In the first stage or "silent" infection, animals present no clinical signs, but are possibly shedding infectious organisms undetectable with any diagnostic test. In the second stage or subclinical disease, animals do not show visible clinical signs, but they may have detectable antibodies to MAP. During this long preclinical period (2-5 years), it persists and multiplies in sub-epithelial macrophages leading to a chronic trans-mural inflammatory reaction (Manning and Collins, 2010; Stabel, 2006). In the third stage or clinical disease, most animals test positive on fecal culture (FC), PCR and have increased antibody detectable by ELISA. In the advanced clinical disease or fourth stage, animals are diarrheic, lethargic, weak, and emaciated, being culled from

the herd due to decreased milk production and severe weight loss (Losinger, 2005; McAloon et al. 2015; Ott et al.,1999).

There are two described forms of presentation of the disease in sheep: paucibacillary and multibacillary (Clarke and Little, 1996). Clarke et al. (1996) used a lesion grading system: sheep with a mean of 0–10 Acid Fast Bacilli (AFB) per macrophage were called paucibacillary whereas those with >10 were called multibacillary. Animals in both groups were emaciated and had carcass edema. The multibacillary animals were more likely to have detectable gross lesions in the intestine and associated lymph nodes. Histologically, the paucibacillary group tended to have a lymphocytic infiltrate with fewer macrophages compared with the multibacillary group, in which macrophages dominated the infiltrate (Clarke and Little, 1996).

Paratuberculosis antemortem diagnosis is complicated by the nature of the disease and some limitations of diagnostic tests. The clinical diagnosis in small ruminants is challenging, the symptoms are vague and nonspecific, and weight loss is present which also occurs in many other diseases. The disease can be diagnosed by pathology, microbiology (culture and PCR) and immunological methods (ELISA, AGID, CF). ELISA test, bacterial culture samples, and PCR are the most widely used diagnostic tests (Clark et al., 2008; Nielsen and Toft, 2008; Stevenson, 2010a; Bauman et al., 2016; Hahn et al., 2017).

The sensitivity (Se) and specificity (Sp) of diagnostic tests used to identify the presence of MAP or MAP infection has been described before. For the ELISA test, a sensitivity ranging from 15-85% and a specificity ranging from 95-99% has been reported (Timms et al., 2011). For bacterial

culture, a sensitivity ranging from 8-93% and a specificity ranging from 95-100% has been reported (Timms et al., 2011). For PCR, a sensitivity ranging from 19-96% and a specificity ranging from 96-100% also has been reported (Timms et al., 2011). However, none of these methods is highly sensitive, especially during the early stages of the disease.

The pooled fecal culture (PFC) technique developed by Whittington et al., (2000) is substantially cheaper than individual fecal culture and more sensitive and specific than serology and has been adopted as a standard test for ovine paratuberculosis control in Australia. Besides control programs, this PFC pooled test is commonly employed in ovine paratuberculosis research, primary to reduce cost of testing sheep (Dhand, N., Sergeant, E., Toribio, J., Whittington, 2010). However, the sensitivity of PFC is known only for certain pool sizes (Dhand et al., 2010; Whittington et al., 2000). The sensitivity of PFC varies according to the disease pathology: the multibacillary and paucibacillary forms of the disease. Similarly, the flock-sensitivity of PFC is also likely to vary with pool size, the prevalence of the disease, and the proportion of multibacillary cases among infected sheep (Dhand et al., 2010; Eamens et al., 2007; Mita et al., 2016).

The sensitivity and specificity of diagnostic tests vary significantly, but access to clear and reliable information about these features, product evaluations, and systematic comparisons, is difficult. The main reasons are the diversity in study designs of comparison and evaluation, test components and different pathophysiological states of animals used for diagnosis and evaluation of tests (Djonne, 2010; Lombard, 2011; Nielsen and Toft, 2009). These limitations require adequately define the purpose of diagnosis, in order to apply the most appropriate diagnostic procedures. Among the alternatives to compensate these weaknesses of diagnostic tests, the sampling of all

animals in a herd or a representative proportion of them, the use two or three diagnostic tests have been recommended for screening herds and increase the accuracy of diagnosis of MAP (Collins, 2010; Nielsen 2010; Stevenson, 2010b).

Molecular techniques for genotyping of MAP based on repetitive elements as MLSSR (Multilocus Short Sequence Repeats) and MIRU-VNTR (Mycobacterial Interspersed Repeats Units-Variable Number of Tandem Repeats) performed by PCR and sequencing have shown good results for strain differentiation of MAP in small ruminants (Castellanos et al., 2009; Möbius et al., 2009; Verdugo et al., 2014; Gioffré et al., 2015). Strain differentiation of MAP by genotyping or molecular characterization is useful for understanding the origin of infection, disease transmission, for designing adequate control measures, improve diagnosis and vaccine development (Sohal et al., 2010; Stevenson, 2015).

Isolates of MAP have been classified into two groups based on culture characteristics and host preference. Type I / III (type S of sheep) and Type II (type C of cattle, Alexander et al., 2009; Collins, 2010; Stevenson, 2010a; Campos de Souza et al., 2016; Dukkipati et al., 2016). However, strains of MAP can be isolated from a wide range of species and the species of origin is not necessarily an accurate indicator of the type of strain. Strains I / III are extremely slow-growing and have been isolated predominantly, but not exclusively, in sheep and goats, suggesting a preference for these hosts (Bauman et al.,2016). MAP type II strains are slow growing, have a very broad host range and are commonly isolated from domestic and wild species, including non-ruminants. Type II is the most commonly isolated in cattle. However, it is recognized that MAP has host preferences, but not a host exclusivity (Galiero et al., 2018).

Sheep can be infected by both groups of strains and MAP isolates obtained in small ruminants manifested genetic variability by a different number of molecular methods (Begg and Whittington, 2010).

Reports of Johne's disease in sheep have been recorded in North and South America, Australasia, the Middle East, Asia, Africa and Europe (Nielsen and Toft, 2009; Fernández-Silva et al., 2014). In a literature review carried out in Europe, the prevalence for sheep could not be estimated, while the prevalence estimated at flock level based on studies of Switzerland and Spain were greater than 20% (Nielsen, 2009). The difficulty of determining the extent of disease has been compounded by the lack of high levels of sensitivity and specificity in diagnostic tests, and the high costs involved with identifying the disease compared with the low value of a sheep.

The prevalence of MAP appears to differ between countries and geographical regions within countries. Published data on the prevalence of affected sheep flocks range from 2 to 66.8% between different countries (Bakker et al.,2000; Sergeant and Baldock, 2002; Nielsen and Toft, 2009; Coelho et al., 2010; Attili et al., 2011; Stau et al., 2012; Moron-Cedillo et al., 2013; Bauman et al., 2016). In Australia, MAP is thought to infect 2.4–4.4% of the flocks, based on data from abattoir surveillance. In New South Wales, Australia, 6–10% of the flocks have ovine Johne´s Disease, while in Western Australia less than 1% of the flocks are thought to be infected (Sergeant and Baldock, 2002). Sporadic cases of paratuberculosis have been diagnosed in sheep in Canada and, in the province of Quebec, a prevalence of 3% was detected in culled sheep (Arsenault et al., 2003). In México, Moron-Cedillo et al., (2013) found that 9,47% of the animals and 4,35-33,3%

of the flocks in the region of San Luis Potosí had reactors in a serological test. Coelho et al., (2007) carried out a study in the Northeast Region of Portugal and found that the individual prevalence of paratuberculosis was 3,7% and 46,7% of the flocks had at least one serologically positive animal. In Germany, one study revealed an individual MAP seroprevalence of 15% and a flock prevalence of 65% in flocks of sheep and goats (Stau et al., 2012). In Italy, the 6,29% of the sampled animals were positive and 73.7% flocks resulted infected according to a commercial ELISA test (Attili et al., 2011). In Ontario, Canada, the prevalence of paratuberculosis at flock-level was 68.8% and the within-flock prevalence was 48.3% in dairy sheep industries (Bauman et al., 2016). A study from Cyprus, which sampled a population of 3429 sheep and using a Bayesian method, found a sheep-flock level prevalence of 60,8% (Liapi et al., 2011).

In a systematic review, it was estimated that the prevalence of paratuberculosis in Latin America and the Caribbean for sheep could be around 16% (95% CI 7.9-24.1) at the individual level (Fernández-Silva et al.,2014). However, the low number of studies that met the inclusion criteria regarding study population, sample selection, control of bias, and the limitations in diagnostic tests used, suggests that prevalence can be much higher for the region (Fernández-Silva et al.,2014). Within-flock prevalence has not been examined in detail, but mortalities of 5–15% per year have been seen in high prevalence flocks (Reddacliff et al., 2006; Windsor, 2015;Garcia and Shalloo, 2015).

Some cow-level risk factors in cattle are the age of the animal at the time of exposure, resistance of the offspring and genetic susceptibility (Djonne, 2010). No study has comprehensively examined whether different breeds of sheep are more or less susceptible to JD, although this point

is often mentioned by flockers. In a flock with a low prevalence of MAP infection, Merino or Merino–Romney cross ewes were observed to have a significantly higher percentage of clinical ovine JD than Romney sheep (Morris et al., 2006). Another study was designed to examine the susceptibility or resistance of various breeds of sheep to MAP infection. Clinical disease occurred more frequently in the Merino (42%) and Suffolk first cross Merino (36%) compared to the Border Leicester (12%) and Poll Dorset (11%) breeds (Begg et al., 2017). Younger animals require a lower infective dose than older animals, and adult animals are unlikely to get infected unless there is extreme environmental contamination (McKenna et al., 2006).

Paratuberculosis risk factors associated with the environment and management include poor hygiene in pens and exposure of young animals with manure from older animals (Manning and Collins, 2010), consumption of milk, colostrum and contaminated food (Tiwari et al., 2008), communitary grazing and waterers (Angelidou et al., 2014). Additionally, contact between different flocks, the movement of animals of different species between flocks, poor control of intestinal parasites and proportion of purchased animals per year have also been considered risk factors associated with the disease (Dhand et al., 2007; Al-Majali, et al., 2008; Angelidou et al.,2014; Marquetoux et al., 2016; Puerto-Parada et al., 2018).

It has been suggested that MAP could be involved as part of the causal structure or as an opportunist in Crohn´s disease of humans (Chacon et al., 2004; Uzoigwe et al., 2007; Atreya et al., 2014; Waddell et al., 2015, 2016). This potential zoonotic role, the human exposure to MAP via milk, and the fact that this relation cannot be proved or disproved (Atreya et al., 2014; Sechi and Dow, 2015; Waddell et al., 2015), are reasons for great concern.

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

Paratuberculosis in sheep is widespread and is a serious threat to their production, because it tends to remain hidden, showing only indirect effects in the production (Juste and Perez, 2011). Although the perceived trade concerns differ between regions and countries, the Colombian sheep industry has identified paratuberculosis as one of the causes of the low development of the industry, limiting meat commercialization at national and international levels (Castellanos et al.,2010). Unfortunately, there is no program for prevention and control of paratuberculosis in sheep in Colombia.

In Colombia, the first diagnosis of the disease was made by the Cuban veterinarian Pérez Vigueras in 1924 from imported animals in the farm El Hato of the municipality of Usme (Province of Cundinamarca). Although the antecedents of paratuberculosis in sheep in Colombia are very scarce, there have been reports of paratuberculosis mainly in the Altiplano Cundiboyacense and the Sabana de Bogotá (Mogollon et al., 1983; Mancipe et al., 2009). The prevalence of paratuberculosis in sheep population of Colombia is unknown. The only published study that estimate the prevalence in small ruminants, reports 480 adult sheep belonging to 3 areas of the Colombian Andean region, of which a prevalence of positive reactors of 11.25 ± 2.88% ($p < 0.05$) was obtained using the complement fixation test and carbohydrate antigen (Mogollon et al., 1983). In the Province of Antioquia, a study did not found positive reactors to the ELISA test on a goat and sheep flock (n = 59) of premountain rainforest (Hernández et al., 2017).

The negative productive and reproductive effects of MAP in sheep and goats, the confirmed presence and circulation of MAP in cattle (Fernández-Silva et al.,2011; Ramírez et al.,2011; Correa-Valencia et al., 2016), the increasing consumption levels of lamb in Colombia -which increases the potential human exposure to MAP- and the absence of strategies, programs, or projects to prevent or control the disease, makes the initial estimation of the disease seroprevalence very necessary.

Prevention and control of PTB demands a high level of knowledge about the magnitude of the presentation of the disease, and about factors that influence its entrance and perpetuation in flocks. It is also well known that flock management practices can increase or decrease the probability of MAP to enter or to maintain in a population, and that these practices vary not only between countries or agroecological zones, but also between regions or even flocks. This situation leads to the need of a local determination of prevalence and specific factors associated with PTB in a specific region.

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Chapter One

The accomplishment of the specific objective 1 (Determine the presence of antibodies against MAP in sheep by ELISA to establish the individual seroprevalence in three regions of the Province of Antioquia, Colombia) and 4 (Explore the main flock level risk factors associated to the positivity to MAP by ELISA in sheep in three regions of the province of Antioquia, Colombia) *originated the presentation of a poster with the results at the 14th International Colloquium of Paratuberculosis, 2018. In addition, an original article "Seroprevalence and risk factors associated with Mycobacterium avium subsp. paratuberculosis infection in sheep flocks located at some regions in the Province of Antioquia, Colombia" has been already submitted in the Rev Colomb Cienc Pecu. The article reports the seroprevalence obtained in the study population according to ELISA results and case definition for animals and flocks, and the management risk factors detected using statistical data analysis for the ELISA positive animals.*

Seroprevalence and risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep flocks located at some regions in the Province of Antioquia, Colombia

Seroprevalencia y factores de riesgo asociados con la infección por *Mycobacterium avium* subsp. *paratuberculosis* en apriscos de ovinos ubicados en algunas regiones del Departamento de Antioquia, Colombia

Soroprevalência e fatores de risco associados à infecção por *Mycobacterium avium* subsp. *paratuberculosis* em currais de ovelhas localizadas em algumas regiões do Departamento de Antioquia, Colômbia

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Abstract

Introduction: paratuberculosis or Johne's disease is a slow–developing infectious disease characterized by chronic granulomatous enterocolitis that causes great economic losses. Johne's disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The objective of this study was to determine the presence of antibodies against MAP and explore the association between MAP serological status and flock management practices in sheep flocks in some regions of the Province of Antioquia, Colombia. **Methods:** A total of 456 sheep over one year of age in 24 different flocks were studied. A blood sample was taken to obtain blood serum and information on flock management practices was taken to explore associations to serological status. The presence of antibodies against MAP was determined by the ELISA CATTLETYPE® MAP ab test (Qiagen, Leipzig, Germany), while the information was analyzed descriptively and analytically. **Results:** the ELISA test detected 37 (8%) animals with positive results among the 456 blood serum samples analyzed and found at least one seropositive animal in 17 (70%) of the 24 flocks. The flock population was identified as a risk factor for the presence of paratuberculosis (p=0.019). **Conclusion:** Further studies at a regional and national scale are necessary to determine the distribution of MAP infection in the Colombian sheep industry.

Keywords: *ELISA, herd, Johne's disease, small ruminants.*

Resumen

Introducción: la paratuberculosis es causada por *Mycobacterium avium* subsp. *paratuberculosis* (MAP) una enfermedad infecciosa de lento desarrollo caracterizada por enterocolitis granulomatosa crónica, la cual causa grandes pérdidas económicas. El objetivo de este estudio fue determinar la presencia de anticuerpos contra MAP y explorar la asociación entre el estado serológico de MAP y los factores de manejo del aprisco en rebaños de ovejas en algunas regiones del Departamento de Antioquia, Colombia. **Métodos:** Se muestrearon 456 ovejas mayores de un año de edad en 24 apriscos diferentes. Se tomó una muestra de sangre para obtener suero sanguíneo y también se tomó información sobre los factores poblacionales para explorar asociaciones con el estado serológico. La presencia de anticuerpos contra MAP se determinó mediante la prueba ELISA CATTLETYPE® MAP ab (Qiagen, Leipzig, Alemania), mientras que la información se analizó de forma descriptiva y analítica. **Resultados:** la prueba ELISA detectó 37 (8%) animales positivos entre los 456 animales analizados y encontró al menos un animal seropositivo en 17 (70%) de los 24 apriscos estudiados. La población del aprisco se identificó como un factor de riesgo para la presencia de paratuberculosis (p=0.019). **Conclusión:** Son necesarios más estudios a escala regional y nacional para determinar la distribución de la infección MAP en la industria ovina colombiana.

Palabras clave: *ELISA, enfermedad de Johne, hato, pequeños rumiantes.*

Resumo

Introdução: a paratuberculose ou doença de Johne é uma doença infecciosa de desenvolvimento lento caracterizada por enterocolite granulomatosa crônica que causa grandes perdas econômicas. A doença de Johne é causada pelo Mycobacterium avium subsp. paratuberculosis (MAP). O objetivo deste estudo foi determinar a presença de anticorpos contra a MAP e explorar a associação entre o estado sorológico do MAP e práticas de manejo de bandos em rebanhos de ovelhas em algumas regiões da Província de Antioquia, Colômbia. **Métodos:** Um total de 456 ovelhas com mais de um ano de idade em 24 lotes diferentes foram estudados. Uma amostra de sangue foi coletada para obter soro sangüíneo e informações sobre práticas de manejo do lote foram tomadas para explorar as associações ao status sorológico. A presença de anticorpos contra a MAP foi determinada pelo teste ab de ELISA CATTLETYPE® MAP (Qiagen, Leipzig, Alemanha), enquanto a informação foi analisada descritivamente e analiticamente. **Resultados:** o teste ELISA detectou 37 (8%) animais positivos entre as 456 amostras de soro analisadas e encontrou pelo menos um animal soropositivo em 17 (70%) dos 24 rebanhos. A populaçao do lote foi identificada como fator de risco para a presença de paratuberculose (p = 0.019). **Conclusão:** Novos estudos em escala regional e nacional são necessários para determinar a distribuição da infecção por MAP na indústria ovina colombiana.

Palavras chave: *doença de Johne, ELISA, pequenos ruminantes, rebanho.*

Introduction

Paratuberculosis also known as Johne's disease is a chronic bacterial disease of global importance in domestic and wild ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (Lombard, 2011). Although it is generally assumed that the disease occurs similarly in all domestic ruminant species, there is sufficient evidence suggesting that paratuberculosis in small ruminants is different from the disease in cattle, both in the clinical form and the MAP involved strains (Clarke, 1997). In sheep, the clinical signs of paratuberculosis are limited to weight loss, premature culling and death (Begg & Whittington, 2010). Negative effects on fertility have also been reported in dairy sheep (Kostoulas, Leontides, Billinis, Amiridis, & Florou, 2006). Edema can occasionally occur, and in advanced cases animals may have hypoalbuminemia and hypocalcemia (Robbe-Austerman, 2011). Most of the sheep with paratuberculosis have normal feces, so diarrhea is not seen as a significant sign of paratuberculosis in small ruminants, except in the terminal stages of the disease (Clarke & Little, 1996).

Paratuberculosis in sheep is widespread and is a serious threat to their production, because it tends to remain hidden, showing only indirect effects in the production (Juste & Perez, 2011). Although the perceived trade concerns differ between regions and countries, the Colombian sheep industry has identified paratuberculosis as one of the causes of the low development of the industry, limiting meat commercialization at national and international levels (Castellanos *et al.*,2010). Unfortunately, there is no program for prevention and control of paratuberculosis in sheep in Colombia. The reports of paratuberculosis in small ruminants in Colombia are very rare; there

have been reports in sheep mainly in the Altiplano Cundiboyacense and the Sabana de Bogotá (Murillo-Rondón 1981, Mogollón *et al.*, 1983, Mancipe *et al.*, 2009). The prevalence of paratuberculosis in sheep and goat populations in Colombia is unknown. According to a systematic review for Latin America and the Caribbean, in which Colombian studies were not included, the prevalence of paratuberculosis in sheep is 16% at the animal level, and the prevalence in sheep at the flock level was not reported due to the lack of studies that meet the inclusion criteria (Fernández-Silva *et al.*,(2014).

The negative productive and reproductive effects of MAP in sheep and goats, the confirmed presence and circulation of MAP in cattle (Fernández-Silva et al.,2011; Ramírez et al.,2011; Correa-Valencia et al., 2016), the increasing consumption levels of lamb in Colombia -which increases the potential human exposure to MAP- and the absence of strategies, programs, or projects to prevent or control the disease, makes the initial estimation of the disease seroprevalence very necessary. The aim of this study was to determine the presence of antibodies against MAP and to explore the association between serological status and flock level risk factors in some regions of the Province of Antioquia, Colombia.

Materials and method*s*

Ethical considerations

This study was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia (Act 111, May 2017).

Flocks and animals

A cross-sectional study was undertaken in which the study population consisted in all sheep located in the Metropolitan Area region, the Northern region, and the Eastern region of the Province of Antioquia, Colombia during August to September, 2017. Sample size calculations were based on allowable error of 5%, 95% confidence, and expected prevalence of 50%, and were estimated using the equation to estimate prevalence according to Dohoo *et al* (2010). According to this, sample size was estimated in 384 animals. In the Province of Antioquia there is no official list of sheep flocks, for this reason before selecting the participating farms, a census of the farms located in the study regions was carried out. A total of 25 farms were identified but one did not agree to participate in the study for unknown reasons. In every sheep flock that agrees to participate in the study the sheep were chosen following a multistage sampling procedure, in which a constant proportion of animals were taken for each flock (Dohoo *et al.*, 2010), twenty percent of the animals older than one year of age were randomly sampled, except for flocks with less than 20 animals older than a year of age, case in which five animals were sampled. Blood samples were collected from 456 sheep in the 24 participating flocks. The only inclusion criterion to select the animals was the age (i.e. only animals older than one year of age were sampled).

Enzymed-linked immunobsorbent assay (ELISA)

Blood samples were taken from the jugular vein using Vacuette® tubes of 7 mL without anticoagulant (Greiner Bio-one, Kremsmünster, Austria) and a single 21G x 1½'' needle per animal, after local cleaning and disinfection with antiseptic alcohol. After collection, the blood samples were allowed to stand at room temperature to allow clot retraction. Subsequently, each sample was centrifuged at 2000-2500 rpm for 3-5 minutes to ease the serum extraction. The serum obtained was kept refrigerated until arrival to the Diagnostic Unit of the Facultad de Ciencias

Agrarias at the Universidad de Antioquia in Medellin where it was frozen at -20 °C until analysis by the ELISA. The presence of antibodies against MAP in the blood serum samples was determined by ELISA using the commercial diagnostic kit CATTLETYPE® MAP ab (Qiagen, Leipzig, Germany). This test included a pre–absorption step with *Mycobacterium phlei* and was performed per manufacturer's recommendations and protocol. Briefly, the samples were diluted 1:20 with dilution buffer on a pre-dilution microplate and incubated for 2 hours at 21°C. Then,100 µLof the controls and samples were transferred to the ELISA microplates and incubated for 30 minutes at 21 °C. The microplates were washed three times with 300 µL of washing solution. Then, 100 µL of the ready-to-use conjugate were aggregated into the wells and incubated for 30 minutes at 21°C. The microplates were washed three times with 300 µL of washing solution. 100 µL of the TMB substrate solution were aggregated in all microplates and incubated for 10 minutes at 21 °C in the dark. Then, 100 µL of the stop solution were aggregated into the wells and the optical intensity was read at 450 nm. To establish the ELISA result, the Optical Density (OD) of the sample (OD sample), the mean value of the Positive Control OD (ODPC), and the mean value of the Negative Control OD (ODNC) were determined. Subsequently, the mean value of the ODPC and the mean value of the ODNC were obtained. The result obtained with these calculations determined the validity of the test. Each sample was tested only once due to budget limitations.

Case definition

For each sample, the S/P was calculated by the formula S/P = OD sample - ODNC / ODPC - ODNC. Animals were assigned a MAP status (positive or negative) according to the kit manufacturer interpretation (‘kit-interpretation’), with serum results of S/P $\geq$ 0.4 classified as positive.

Data collection

In order to explore the association between flock management practices and the MAP serological status of sheep, the same day of collection of blood samples available data on flock management practices related with transmission of the infection between flocks and the maintenance and transmission of the infection within each flock was collected using a questionnaire. The information was obtained from the flock manager or flock owner. During the data collection, the questions were read out to the farmer and answers were selected from multiple closed questions or otherwise written down. The questions asked for several features, some of which have been already identified as risk factors for paratuberculosis in ruminants in previous studies: share roads between neighboring flocks (Dhand et al., 2007), presence of different species of ruminants on the same flock (Al-Majali et al., 2008), grazing of heifers with goats and/or sheep (Çetinkaya et al.,1997; Barrett *et al.*, 2011), community grazing, poor control of intestinal parasites (Angelidou et al., 2014) and trade of animals between related flocks (Marquetoux, N., Heuer, C., Wilson, P., Ridler, A., Stevenson, 2016).

Statistical analysis

The information collected using the questionnaire was stored using conventional computer programs (Excel, Microsoft Corp., Redmond, WA, USA). The results of ELISA tests were analyzed to estimate the apparent prevalence of paratuberculosis at flock level in the flocks of the municipalities located in the three regions. The information collected in the questionnaires was analyzed by descriptive and analytical statistical procedures. Bivariable and multivariable logistic regression were applied to determine the influence of multiple flock management practices and the prevalence of MAP in accordance with the diagnostic test. An unconditional mixed-effects logistic

regression analysis, grouped by flock to account for clustering, was performed. The criteria of Hosmer-Lemeshow ($p<0.25$) was used to retain variables for the multivariable model. Statistical analyzes was carried out using the Stata 12.0 software (StataCorp LP, College Station, Texas, USA).

Results

Seroprevalence

A total of 456 sheep from 24 flocks located in the Metropolitan Area region, the Northern region, and the Eastern region of the Province of Antioquia, Colombia were tested. Eight percent (37/456 IC: 5.5-10.5)) of the samples were positive and seropositive animals (one or more) were detected in 70.8% (17/24 IC: 51.2-0.90) of the flocks. The seroprevalence results among regions were 70, 100, and 74% in the Metropolitan Area region, North Region and East Region, respectively. The intraflock frequency of seropositive animals ranged from 0 to 21.4%. In the municipalities of Bello, Girardota, San Vicente, El Peñol and Rionegro belonging to different regions all tested animals were negative (Table 1).

Table 1. Seroprevalence of ovine paratuberculosis infection in 16 municipalities of the Province of Antioquia, Colombia.

Region	Municipality	Flocks	Number of animals[a]	Animal tested	animals with	Prevalence (%)

					positive results	
Metropolitan Area	Barbosa	2	130	26	3	11.5
	Bello	1	12	5	0	0
	Caldas	2	180	37	2	5.4
	Copacabana	2	386	77	4	5.19
	Envigado	1	125	25	3	12
	Girardota	1	70	14	0	0
	Medellín	1	50	10	1	10
	Subtotal	10	953	194	13	6.7
North	Santa Rosa	1	70	14	3	21.4
	San Pedro	2	240	48	2	4.16
	Subtotal	3	310	62	5	8
East	Guarne	1	20	5	1	20
	Marinilla	2	119	23	4	17.3
	Santuario	1	51	10	0	0
	San Vicente	2	69	14	2	14.28

	El Peñol	1	30	6	0	0
	Rionegro	1	100	20	0	0
	La Ceja	3	640	122	12	9.83
	Subtotal	11	1029	200	19	9.5
Total		24	2292	456	37	8.11

[a]Animals ≥ 1 year of age.

Descriptive results

The average population in the flocks was 155 animals and fifty-four percent of flocks had an area of less than 2 Has, 67.5% of the flocks had other ruminants in their facilities, mainly bovines and in 40.3% of the cases these species shared paddocks. A 61.1% of the flocks shared roads with other farms and 52.6% used manure as fertilizer for pastures. A 47.3% of the flocks actively purchase animals and in 79% of the cases they never had cases compatible with paratuberculosis (Table 2).

Table 2. Flock predictors associated with *Mycobacterium avium* subsp. *paratuberculosis* ELISA status in 456 sheep of three regions of Antioquia, Colombia.

Variable	Unit/Category	Observations	Distribution
Flock size (hectares)	< 2 has	226	49.5
	≥ 3 ≤ 5 has	151	33.1

	> 6 has	79	17.3
	≤70	107	23.4
	71-140	71	15.5
Flock population (animals > 1 year)	141-210	57	12.5
	211-280	90	19.7
	>280	131	28.7
	No	148	32.4
Presence of other ruminants	Yes	308	67.5
	No	272	59.6
Sharing paddocks	Yes	184	40.3
	No	177	38.8
Sharing roads	Yes	279	61.1
Spreading of manure on pastures	No	216	47.3
	Yes	240	52.6
	No	100	22
Use of dewormer	Yes	346	78
Mobilization between flocks	No	239	52.4

	Yes	217	47.5
Sheep purchase	No	240	52.6
	Yes	216	47.3

Factors associated with the MAP serological status

The results of the unconditional analysis revealed that the factors "flock population", "Sharing paddocks", "Sharing roads" and "use of dewormer" were eligible for their inclusion in the final model ($p<0.25$; Table 3). When the multivariable logistic regression analysis was carried out, no significant variables were obtained in the final model.

Table 3. Unconditional analysis of factors associated with the *Mycobacterium avium* subsp. *paratuberculosis* ELISA status in 456 sheep of three regions of Antioquia, Colombia.

Variable	Unit/Category	No of sampled animals	No ELISA positive animals		p
			N	%	
Flock size (hectares)	< 2 has	226	16	7	0.791

	≥ 3 ≤ 5 has	151	16	10.5	
	> 6 has	79	5	6.3	
Flock population (animals > 1 year)	≤70	107	14	13	
	71-140	71	4	5.6	0.086[a]
	141-210	57	4	7	0.191[a]
	211-280	90	7	7.7	0.172[a]
	>280	131	8	6.1	0.045[a]
Presence of other ruminants	No	148	15	10.1	0.365
	Yes	308	22	7.1	
Sharing paddocks	No	272	17	6.2	0.128[a]
	Yes	184	20	10.8	
Sharing roads	No	177	11	6.2	0.196[a]
	Yes	279	26	9.3	
Spreading of manure on pastures	No	216	14	6.4	0.260
	Yes	240	23	9.5	
Use of dewormer	No	100	5	5	0.195[a]
	Yes	346	32	9.2	

Mobilization between flocks	No	239	21	8.7	0.480
	Yes	217	16	7.3	
Sheep purchase	No	240	17	7	0.515
	Yes	216	20	9.5	

[a]Variables used for the multivariable analysis ($p<0.25$).

Discussion

In this study we report the results of a cross-sectional study carried out in three regions of the Province of Antioquia, Colombia. According to the reviewed national literature, this is the fifth study reporting results on Johne's disease in sheep in Colombia, after the studies of Murillo-Rondón (1981), Mogollón *et al.* (1983), Mancipe *et al.* (2009) and Hernández *et al.* (2017). However, this is the first epidemiological study on Johne's disease in the country performed on small ruminants aiming the determination of the seroprevalence and to identify those management factors associated with paratuberculosis seropositivity.

The results show that antibodies against MAP (8.11% of the animals and 70.8% of the flocks) are widespread in the regions of study. The flock seroprevalence obtained agrees to those reported in other international studies. In México, Moron-Cedillo *et al*. (2013) found that 9.48% of the animals located in the region of San Luís Potosí were positive in a serological test. In Germany, one study using the same commercial ELISA used the present study, revealed an individual MAP seroprevalence of 15% and a flock prevalence of 65% in flocks of sheep and goats (Stau, A.,

Seelig, B., Walter, D., Schroeder, C., Ganter, 2012). In Italy, the 6.29% of the sampled animals and 73.7% flocks were positive by a commercial ELISA test (Attili *et al.*, 2011). In Ontario, Canada the true-flock level prevalence was estimated to be 66.8% for dairy sheep (Bauman, C., Jones-Bitton, A., Menzies, P., Jansen, J. Kelton, 2016). However, Coelho *et al.* (2007) carried out a study in the Northeast Region of Portugal and found that the individual prevalence of paratuberculosis was 3.7% and 46.7% of the flocks had at least one serologically positive animal.

In this study, no seropositive animals were identified in the municipalities of Bello, Girardota, San Vicente, El Peñol and Rionegro. According to the information provided by the flock owners or flock managers during data collection of the present study, is probable that the contact between neighboring flocks and livestock be less frequent in these municipalities, since the paddocks are distant to each other or the flocks are isolated and does not allow the entry of animals. Taking into account the results of this study and previous studies (Coelho, A. C., Pinto, M. L., Silva, S., Coelho, A. M., Rodrigues, J., Juste, 2007; Stau, A., Seelig, B., Walter, D., Schroeder, C., Ganter, 2012), it seems that paratuberculosis shows a similar behavior in the regions of study, with few seropositive animals being observed within each flock, but many flocks with at least one seropositive animal (Lombard, 2011).

In this study we found a significant association between flock population and MAP seropositivity in the unconditional analysis ($p= 0.045$) according to which flocks with more than 280 animals older than a year of age had less risk of paratuberculosis in comparison with flocks with less than 70 animals ($p<0.05$). Although this association has been reported previously (Coelho, A. C., Pinto, M. L., Silva, S., Coelho, A. M., Rodrigues, J., Juste, 2007; Stau, A., Seelig, B., Walter, D.,

Schroeder, C., Ganter, 2012) our results partially differ from these studies. In the first study, Coelho *et al.* (2007) found a significant association between MAP seropositivity and flock population, being the flocks with 31-60 animals those that had more risk of paratuberculosis in comparison with flocks with less of 30 animals and more than 60 animals. The authors explained that a higher density of animals was observed in this group, which might increase the potential for exposure to contaminated feces and cause the higher prevalence in this group. In the second study, Stau *et al.* (2012), found a significant correlation between seroprevalence and flock size ($p = 0.03$) and that the percentage of positive flocks increased with flock population.

The flocks in the regions of study are very diverse. In the present study, size of flocks varies from less than 11 animals up to flocks with more than 400 animals older than a year of age, which suggests that small flocks are more likely to introduce new animals from other flocks, which increases the risk of infected asymptomatic animals entering the flock and shedding mycobacteria intermittently. According to the collected data in the present study, owners of larger flocks may not need to buy animals from other flocks and, therefore, have less risk of introducing infected animals. In addition, biosecurity measures and availability of facilities are more common is large flocks, which also reduce the risk of transmission of the disease.

The lack of associations when the multivariable logistic regression analysis was carried out in this study can be explained by the sample size, the main objective in this study was to determine the presence of antibodies against MAP and the sample size calculation were estimated using the equation to estimate prevalence not to estimate risk factors. However, we also try to explore the

association between serological status and flock level risk factors in the regions of study and found associations when the unconditional analysis was carried out.

Examining risk factors or other associations can be done using methods such as multivariate linear or logistic regression. However, when the number of observations is small and try to adjust for several factors, these methods cannot produce sensitive results or they produce unreliable results (Hackshaw, 2008; Figueiredo *et al.*, 2013). In addition to considering the total sample size, the number of positive and negative outcomes in the observed data influence the precision of the estimates of the coefficients in the model. It has been suggested that the dataset should contain a minimum of 10(k+1) positive outcomes where k is the number of predictors in the model in order to adequately fit the model (Dohoo *et al.*,2010). For this study it suggests a minimum of fifty positive results. These reasons can explain why no significant variables were obtained in the final model.

The results of this cross-sectional study indicate that the presence of antibodies against MAP is widely widespread in sheep in the regions of study. It underlines the importance of determining the current status of infection in small ruminants in Colombia, because a high prevalence is certainly connected with severe economic losses (P. A. Windsor, 2015). Apart from the economic effect, the possibility of transmitting MAP to other animals by grazing on the same pasture and to humans cannot be ignored. Further studies at a regional and national scale considering other diagnostic techniques, such as bacteriological culture and PCR are necessary to determine the distribution of MAP infection in the Colombian sheep industry.

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Conflicts of interest

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

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Chapter Two

In order to accomplish the specific objectives 2 (Determine the presence of MAP DNA in sheep feces to establish the flock prevalence by real-time PCR in some regions of the Province of Antioquia, Colombia) and 3 (Determine the presence of MAP in sheep feces to establish the flock prevalence by culture in some regions of the Province of Antioquia, Colombia), an additional chapter was included in this master's degree work. This part takes into account fecal culture and qPCR to determine de prevalence of paratuberculosis in the Metropolitan Area Region, Northern Region and Eastern Region of the province of Antioquia, Colombia.

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep flocks located in three regions of the Antioquia Province, Colombia

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Abstract

Paratuberculosis or Johne's disease is a slow–developing infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) affecting mainly domestic ruminants and producing a significant economic threat to the animal production sector. Although the perceived trade concerns differ between regions and countries, the Colombian sheep industry has identified paratuberculosis as one of the causes of its low development. In addition, the reports of paratuberculosis in small ruminants in Colombia are very scarse; there have been reports in sheep mainly in the Cundiboyacense Plateau and the Bogotá savannah, but the prevalence of paratuberculosis in sheep and goat populations in Colombia is yet unknown. The aim of the study was to detect MAP infection in order to estimate flock apparent prevalence in a sheep population located in three regions of the Province of Antioquia, Colombia. A total of 90 fecal pools from 24 different flocks were analyzed for MAP detection using both qPCR and culture. Overall, from 90 fecal pools tested, qPCR and fecal culture detected 25 (27,7%) and 64 (71,1%) pools as MAP positive, respectively. Besides, MAP positive pools were detected in 45,8% and 83,3% of the flocks by qPCR and by culture, respectively. Further prevalence studies both at a regional and national scale, as well as molecular typing of the isolates are necessary to determine the current distribution of MAP infection in the Colombian sheep industry.

Keywords: culture, flock, Johne's disease, PCR, small ruminants.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is one of the most fastidious members of the *Mycobacterium* genus. It is the causal agent of Johne's disease (also known as paratuberculosis) which is an untreatable disease characterized by granulomatous enteritis, diarrhea, loss of body weight and death (Chiodini, Coffin, Condon, Kunimoto, & McFadden, 1993).

Although it is generally assumed that the disease occurs similarly in all domestic ruminant species, there is sufficient evidence suggesting that paratuberculosis in small ruminants is different from the disease in cattle, both in the clinical form and the MAP involved strains (Clarke, 1997). The disease is responsible for significant economic losses to livestock production worldwide (Garcia & Shalloo, 2015; Raymond W. Sweeney, 2011). Additionally, a zoonotic association has been proposed since the organism has been consistently found in humans with Crohn's disease, suggesting that this agent could be zoonotic (Lee *et al.*, 2011; Chiodini *et al*., 2012).

Research on MAP infection in Colombia has been reported in small ruminants (Murillo-Rondón 1981, Mogollón *et al.*, 1983, Mancipe *et al.*, 2009). However, information on presence and distribution of this infection is still scarce and the prevalence of paratuberculosis in sheep populations in Colombia is unknown.

The lack of a prevalence estimate not only limits the capacity to assess the real impact of the disease, but also limits the capacity to allocate sufficient resources for its control precluding an adequate monitoring of the effectiveness of potential control measures. For these reasons, the estimation of this measure of frequency is relevant for any disease. Currently neither the Colombian sheep industry nor the Colombian government have estimates on the true prevalence

(TP) of infected flocks. Therefore, the aim of this study was to estimate MAP prevalence in a sheep population at a flock level in three regions of the Antioquia Province in Colombia.

Materials and methods

Selection of flocks and animals

A cross sectional study was carried out. The study population consisted in all sheep located in the Metropolitan Area region, the Northern region, and the Eastern region of the Province of Antioquia, Colombia. Flocks were located in several municipalities of the three regions. Between August and September 2017, twenty-four sheep flocks in the three regions of the Province of Antioquia, Colombia were sampled for MAP detection. The sample size calculation was done according to Dohoo *et al* (2010), allowing an error of 5%, 95% confidence, and expected prevalence of 50%. The sample size was taken following a multistage sampling procedure, in which a constant proportion of animals were taken for each flock (Dohoo et al., 2010). According to this, sample size was estimated in 384 animals. In the Province of Antioquia there is no official list of sheep flocks, for which a census of the farms located in the three study regions had to be carried out before selecting the participating farms,. A total of 25 sheep flocks were identified, but one flock did not agree to participate in the study. The only inclusion criterion to select the animals participating in the study was the age, i. e. only animals over a year of age were sampled. Therefore, in every sheep flock that agreed to participate in the study, 20% of the animals older than one year of age were randomly sampled, excepting flocks with less than 20 animals over one year of age, in which five animals were sampled to shape one single pool of the flock. Overall, fecal samples were collected from 456 sheep in the 24 participating flocks.

Collection of samples and information

Each farm was visited once. A fecal sample (2-5 gr) was taken with a new clean glove directly from the rectum of every animal selected (n=456). For this procedure, the animals were restrained in handling pens by the flock operators. No animals had been vaccinated against paratuberculosis and researchers were unaware of their paratuberculosis status at the time of the study. The same day of collection of samples, available data on flock management practices like was collected using a questionnaire. The information was obtained from the flock manager or flock owner. During the data collection, the questions were read out to the farmer and answers were selected from multiple closed questions or otherwise written down. The questions asked for several features, some of which have been already identified as risk factors for paratuberculosis in ruminants in previous studies: share roads between neighboring flocks (Dhand et al., 2007), presence of different species of ruminants on the same flock (Al-Majali et al., 2008), grazing of heifers with goats and/or sheep (Çetinkaya et al.,1997; Barrett et al., 2011), community grazing, poor control of intestinal parasites (Angelidou et al., 2014) and trade of animals between related flocks (Marquetoux, N., Heuer, C., Wilson, P., Ridler, A., Stevenson, 2016).

Fecal pooling

The samples were kept refrigerated until arrival at the Diagnostic Unit of the Facultad de Ciencias Agrarias of the Universidad de Antioquia in Medellin, Colombia where individual fecal samples of five animals from the same flock were pooled in a new sterile container (Fiorentino et al., 2012; Mita, A., Mori, Y., Nakagawa, T., Tasaki, T., Utiyama, K., Mori, 2016). The pools (n=90) were frozen at -80°C until they were processed for fecal culture and DNA isolation at the Laboratorio de Enfermedades Infecciosas of the Instituto de Medicina Preventiva Veterinaria, Universidad

Austral de Chile in Valdivia, Chile. Fecal pools were transported at 4°C from Colombia to Chile allowing the slow defrosting of samples. At arrival, samples were immediately refrigerated and processed within the following hours.

Fecal culture

Fecal pools (n=90) from all flocks were decontaminated according to the Fecal Processing Protocol for the BACTEC™ MGIT™ para TB System (BD Diagnostic Systems, Franklin, NJ, USA). Each MGIT ParaTB medium tube contained 7 ml of modified Middlebrook 7H9 broth base with mycobactin J, 500 µl of egg yolk suspension (Becton, Dickinson, Sparks, MD), 100 µl of a VAN (vancomycin, nalidixic acid, and amphotericin; Sigma-Aldrich) cocktail, and a fluorescent oxygen indicator embedded in silicon at the bottom of the tube. The final concentrations of antibiotics were 10 µg/ml vancomycin, 40 µg/ml amphotericin B, and 60 µg/ml nalidixic acid. Each inoculated MGIT tube was inserted in an MGIT 960 instrument (BD Diagnostic Systems, Franklin, NJ, USA) and incubated at 37°C for 49 days. Tubes signaling positive by day 49 were removed and tested for the presence of MAP by IS*900 q*PCR. Tubes not signaling positive by that time were considered negative.

DNA isolation

DNA isolation from positive fecal pools by culture was carried out using a protocol previously reported by Salgado *et al*. (2013a). Briefly, two grams of a fecal pool were added to a Falcon tube (Heathrow Scientific, USA) containing 50ml of sterile distilled water, centrifuged at 5,000 × g for 5 min, and left to rest for 30 min. From the middle of the tube, an aliquot of 200µL was aseptically removed and transferred to a 1.5-mL reaction tube (Eppendorf tubes; Sigma-Aldrich, USA) and

centrifuged at 5,000 × g for 5 min. The supernatant in each tube was discarded and the lid of the reaction tube was briefly touched with a clean soft paper tissue in order to remove the remaining liquid. The pellet was dispersed by pipetting with a mixture of 500 µL lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 0.6% SDS) and 2 µL proteinase K (10 µg/µL; Sigma-Aldrich). The solution was then transferred to a bead-beating tube (Biospec Products, USA) containing 200 µL of beads (0.1 mm zirconia/silica beads; Biospec Products) and incubated at 56ºC for 2 h with shaking at 600 g. The tubes were then shaken in a cell disrupter (MiniBeadbeater-8; Biospec Products) at 3,200 g for 60 sec and incubated on ice for 10 min. In order to remove foam and beads from the inner walls, the tubes were centrifuged at 5,000 × g for 30 sec. The samples were briefly vortexed to ensure that any DNA adhering to small solid particles was not loosened when the lysate was transferred. All liquid contents from the bead-beating tube were transferred to 1.5-mL reaction tubes and 500 µL of 100% ethanol were added. The tubes were left standing for 2 min at room temperature before being vortexed for 5 seconds and centrifuged at 18,000 × g for 5 min. 18°C. The supernatant was discarded and the pellet was washed once in 200 µL 70% ethanol by resuspension and centrifugation under the same conditions as mentioned above. Then, the pellet was resuspended in 50 µL of sterile distilled water. The tubes were placed in a dry heating block (Eppendorf, Hamburg, Germany) at 100°C for 5 min. The solution was briefly centrifuged at full speed (16,000 × g for 30 sec) to remove any contaminating material. Finally, a 25-µL aliquot of supernatant was placed into a new 1.5-mL reaction tube to be used as a template for PCR.

qPCR

DNA from fecal pools were tested for MAP with a real-time PCR method previously report by Salgado *et al.* (2013b). The target was the insertion element IS*900*. The PCR mixture included 5 µl DNA template, 10 µl TaqMan Universal Master Mix (Roche, Indianapolis, IN), 0.2 µM IS*900* primers, 0.1 µM probe (Roche, Indianapolis, IN), and water for a total volume of 20 µl. Primer sequences for IS*900*, which amplified a 63-nucleotide fragment of the IS*900* gene target, were 5'-GACGCGATGATCGAGGAG-3' (left) and 5'-GGGCATGCTCAGGATGAT-3' (right). The probe sequence was TCGCCGCC. The reactions were carried out in a Roche LightCycler System version 2.0 (Roche, Indianapolis, IN, USA) under the following standard conditions: one cycle at 95°C for 10 min; 45 cycles with three steps of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s; and a final cooling step at 40°C for 30 s. Negative and positive (*Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698) PCR controls were included.

Statistical analysis

The available data of the study was stored and processed using conventional computer programs (Excel, Microsoft Corp., Redmond, WA, USA). Test results were analyzed to estimate the flock apparent prevalence of MAP infection in a sheep population located several municipalities of the three regions.

Results

Descriptive results

A total of 90 fecal pools from 24 flocks located in the Metropolitan Area region, the Northern region, and the Eastern region of the Province of Antioquia, Colombia were tested. According to

the collected data, the average population in the flocks was 155 animals and mostly had an area of less than 2 Has, 67,5% of the flocks had other ruminants in their facilities, mainly bovines, and in 40,3% of the cases these species shared paddocks. A 61,1% of the flocks shared roads with other farms and 52,6% used manure as fertilizer for pastures. A 47,3% of the flocks actively purchase animals and in 79% of the cases they never had cases compatible with paratuberculosis.

Fecal culture

The fecal culture using the BACTEC™ MGIT™ para TB System detected 64 (71,1%) positive pools out of 90 fecal pools tested. MAP positive pools (one or more) were detected in 83,3% (IC :67,3-99,3) of the flocks. The prevalence values among the regions were 100, 67 and 72,7% in the Metropolitan Area region, Northern region and Eastern region, respectively (Table 1). Fecal pools from animals belonging to flocks 12,19,21,24 did not produce positive results by fecal culture (table 2).

qPCR

The qPCR detected 25 (27,7%) positive pools out of 90 fecal pools tested, as indicated by the ROCHE LightCycler System. MAP positive pools (one or more), were detected in 45,8% (IC : 24,3-67,3) of the flocks. The apparent prevalence among regions were 40, 100 and 36,4% in the Metropolitan Area region, Northern region and Eastern region, respectively (table 1). All PCR positive results were produced in fecal pools from animals belonging to flocks 2,6,10,14,15,17,18,19,20,21,22. Fecal pools from animals of remaining flocks did not produce positive results by PCR (Table 2).

Table 1. Prevalence of MAP infection in sheep by fecal culture and qPCR in three regions of Antioquia, Colombia

Region	Flocks	Fecal culture		qPCR	
		Positive flocks[a]	Prevalence	Positive flocks[a]	Prevalence
Metropolitan Area region	10	10	100	4	40
Northern region	3	2	67	3	100
Eastern region	11	8	72,7	4	36,4
All regions	24	20	83,3	11	45,8

[a]At least one positive pool to MAP.

Table 2. Prevalence of MAP infection by qPCR and Fecal culture in 16 municipalities in three regions of Antioquia, Colombia.

Region	*Municipality*	*Flock*	*Number of pools*	*Fecal culture*		*qPCR*	
				Positive pools	*Prevalence*	*Positive pools*	*Prevalence*
	Barbosa	1	1	1	100	1	100

Metropolitan Area Region		22	4	4	100	3	75
	Envigado	2	5	5	100	2	40
	Bello	4	1	1	100	0	0
	Girardota	5	3	3	100	0	0
	Copacabana	6	1	1	100	1	100
		10	14	10	71,4	4	28,5
	Caldas	7	6	6	100	0	0
		8	1	1	100	0	0
	Medellín	9	2	1	50	0	0
Northern region	San Pedro De Los Milagros	14	8	4	50	3	37,5
		15	2	1	50	1	50
	Santa Rosa	19	3	0	0	1	33,3
Eastern region	San Vicente	3	2	2	100	0	0
		21	1	0	0	1	100
	Marinilla	11	1	1	100	0	0
		20	3	1	33,3	1	33,3

Guarne	12	1	0	0	0	0
Santuario	13	2	2	100	0	0
La Ceja	16	10	8	80	0	0
	17	2	2	100	2	100
	18	12	9	75	6	50
El Peñol	23	1	1	100	0	0
Rionegro	24	4	0	0	0	0

Discussion

We report the results of a cross-sectional study carried out using a combination of direct diagnostic methods to detect MAP infection in sheep in the Metropolitan Area region, the Northern region, and the Eastern region of the province of Antioquia, Colombia. We consider that a combination of PCR and fecal culture was necessary to obtain an accurate detection of MAP infection in the regions of study. To our knowledge, this is the first epidemiological report on MAP infection in the country, performed on small ruminants aiming the determination of the MAP prevalence using direct diagnostic methods.

Although few randomized studies have been carried out in sheep in the regions of study and there is no prevalence estimates to make comparisons, the observed apparent flock-level prevalence of MAP infection in sheep flocks (45,8% by qPCR and 83,3% by fecal culture) could be considered as high. A recent study from Canada, which used direct diagnostic methods (ELISA, bacterial

culture and PCR) and a 3-test latent class Bayesian model found a sheep flock-level prevalence of 66,8% (IC: 41,6-91,4%)(Bauman, C., Jones-Bitton, A., Menzies, P., Toft, N., Jansen, J., Kelton, 2016).

The lower prevalence obtained by qPCR compared to BACTEC™ MGIT™ para TB System was expected based on previous reports. Alinovi *et al.*, (2009) compared the qPCR to culture for the detection of MAP using a Bayesian methodology finding a sensitivity and a specificity of the qPCR test of 0.72 and 0.96, respectively. In another study, the diagnostic accuracy of qPCR was compared to liquid radiometric fecal culture showing that only 84% of the ovine-culture positive samples were positive in the qPCR test (High-through-Jhones, Plain *et al.*, 2014).

It is possible that the matrix used had an effect on the results of the qPCR. Feces are considered a difficult sample for the molecular detection of microbial pathogens due to the presence of PCR inhibitors. Ruminant feces are expected to include high levels of PCR inhibitors (Monteiro et al., 1997; Thornton & Passen, 2004) which are difficult to remove (Harris and Barletta, 2001). One strategy used to decrease inhibitors in pool samples is to dilute them to decrease the concentration of these inhibitors, which leads to a dilution effect in the sample, causing decreased sensitivity (Mita, A., Mori, Y., Nakagawa, T., Tasaki, T., Utiyama, K., Mori, 2016). Considerable variation in the consistency (e.g. water content and presence of mucus) and composition (e.g., fiber vs. grain) of sheep feces in the current study was observed, which may have an impact on the efficacy of inhibitor removal. We used the amount of feces recommended by the protocol. However, little is known about the spatial distribution of bacteria in feces, and if the small amount of feces sampled may provide an erroneous measure of MAP prevalence. It may be possible to increase the amount

of feces sampled, but increased sample amount may overload the purification step and increase the amount of inhibitory compounds during DNA extraction (Inglis & Kalischuk, 2003). Complex polysaccharides are commonly encountered inhibitors of PCR found in feces. Furthermore, polyphenolic compounds of plant origin can be very inhibitory to PCR (Monteiro et al., 1997), and the high plant content of sheep feces would be expected to contribute large quantities of both complex polysaccharides and polyphenolic compounds, a factor which may limit the efficiency of the protocol used. Although no clinical sheep were found in the study sample, in some cases is highly probable that feces from animals with clinical paratuberculosis may contain heme (a complex of iron with protoporphyrin IX) and epithelial cells, being these components reported to be inhibitory to PCR (Correa-Valencia, N., Ramírezn, N., Bülte, M., Fernández-Silva, 2017).

Nevertheless, our results in the qPCR protocol applied could be better explained by the already reported physiopathology of the disease rather than to qPCR misclassification. According to Whitlock *et al.* (2000), the disadvantages of some detection test are due mainly because of the intermittent shedding of microorganisms. This means that the sensitivity of direct tests to detect symptomatic animals is high, but low for detection of infected/subclinical animals.

In this study, no positive pools were identified in the flocks 12 and 24 belonging to the municipalities of Guarne and Rionegro, respectively. According to the information provided by the flock owners or flock managers during data collection of the present study, is probable that the contact between neighboring flocks and livestock be less frequent in these flocks, since the paddocks are distant to each other or the flocks are isolated and does not allow the entry of animals. However, taking into account the wide distribution of MAP in the study regions, the high

prevalence obtained and the presence and confirmed circulation of MAP in cattle of the region (Fernández-Silva *et al*., 2011; Ramírez *et al*., 2011; Correa-Valencia *et al.*, 2016). It is probable that the results for the tests did not necessarily mean that the animals were not infected, because the shedding phase has not started (infected animal in a noninfectious phase) or was absent at the moment of fecal sampling (intermittency, McKenna et al., 2006; Nielsen, 2010). Considering MAP-shedding characteristics as the major limitation in the detection of infected animals, it should be taken into account that the elimination of the bacteria through feces happens at all stages but at different levels and sporadically, which demands repeated testing to detect animals shedding very low number of MAP, which could anyway go undetected (Stevenson, 2010b).

The results of this study confirm that the MAP infection is widespread in sheep in the study regions. The comparison of the tests used was not an objective of this study, however a different behavior was observed among them obtaining a greater number of positive pools with the culture in comparison to the qPCR. It underlines the importance of using a combination of diagnostic test to determine the real status of infection in small ruminants in Colombia. Further studies at a regional and national scale considering follow-up studies and molecular characterization of MAP are necessary to determine the distribution of MAP infection in the Colombian sheep industry.

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Statement of Animal Rights

The authors declare that the study does not contain clinical studies or patient data. Informed consent was obtained from all individual participants included in the study. The study was conducted according to the current law of animal protection in Colombia and was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act 111, May 2017).

Conflict of interest

The authors declare that they have no conflict of interest.

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General conclusion

The objectives of this master's degree were oriented to stablish MAP prevalence using three different diagnostic techniques like ELISA, fecal culture and qPCR, and to explore the association between serological status and potential flock level risk factors in some regions of the Province of Antioquia, Colombia. The hypotheses consider in this work included an expected MAP prevalence of 50% at the flock and individual level and that at least one flock management practice is a risk factor for MAP presence in the study flocks.

As a result of the investigation, we found a high prevalence of MAP in the study population (higher than expected but according to other studies around the world). Additionally, flock population was identified as a risk factor for the presence of antibodies against MAP.

The results of this work confirm the presence of MAP in the sheep located in the regions of study, and the usefulness of three diagnostic techniques (direct and indirect) to improve the overall diagnostic ability of the investigation.

Further microbiological and epidemiological studies at a regional and national scale considering follow-up studies and molecular characterization of MAP are necessary to determine the distribution of MAP infection in the Colombian sheep industry and increase the knowledge about ovine Johne's disease and the risk factors in our agroecological conditions.

Annexes

Annex 1: Authors guidelines

Revista Colombiana de Ciencias Pecuarias (RCCP)

http://www.scielo.org.co/revistas/rccp/iinstruc.htm

Tropical Animal Health and Production

https://link.springer.com/article/10.1023%2FB%3ATROP.0000009625.94908.bb

Annex 2: Approval of Comité de Ética para la experimentación Animal (CEEA)

UNIVERSIDAD DE ANTIOQUIA
Vicerrectoría de Investigación

Comité de Ética para la Experimentación con Animales

Medellín, 19 de mayo de 2017

Investigador
Jorge Fernández Silva
Grupo de investigación "Centauro"
Universidad de Antioquia

Proyecto: "Prevalence and risk factors associated with Mycobacterium avium subsp. paratuberculosis (MAP) infection in flocks of sheep located at some regions in the Department of Antioquia, Colombia" / "Prevalencia y factores de riesgo asociados con la infección por Mycobacterium avium subsp. paratuberculosis (MAP) en apriscos de ovinos ubicados en algunas regiones del Departamento de Antioquia, Colombia".

Resultado de la revisión: Otorgar aval[1]

Cordial saludo.

Después de ser estudiada su solicitud y como constará en el acta No. 110 de la reunión extraordinaria realizada el 17 de mayo a las 2:00 pm, en la sala de reuniones del Bioterio de la Sede de Investigación Universitaria de la Universidad de Antioquia, el **Comité de Ética para la Experimentación con Animales** le expresa que se otorga el aval solicitado.

Con toda atención.

JOSÉ IGNACIO CALLE POSADA
Coordinador
Comité de Ética para la Experimentación con Animales
Universidad de Antioquia

[1] *El aval otorgado hace referencia única y exclusivamente al proyecto y/o a los procedimientos que se mencionan, además será válido solamente por el tiempo que dure (n) este (os).*

Página 1 de 1

Annex 3: Questionnaire for the determination of risk factors for paratuberculosis

Este predio ha sido seleccionado al azar para participar en el proyecto de investigación: "Prevalencia y factores de riesgo asociados con la infección por *Mycobacterium avium* subs *paratuberculosis* en ovejas ubicadas en algunas regiones del departamento de Antioquia, Colombia", de acuerdo a nuestros criterios de inclusión y a su autorización. El investigador responsable de la toma de muestras es José Miguel Hernández, MV, MCV(est) y el investigador principal es Jorge Fernández Silva, MV, MSP, Dr. Med. Vet. Ambos pertenecen al grupo de investigación CENTAURO de la Universidad de Antioquia. Para la ejecución del proyecto se requieren muestras sanguíneas y fecales. Por lo cual, la intervención de los animales es requerida. La recolección de las muestras se realizará bajo métodos estandarizados con el objetivo de evitar estrés y dolor innecesario en los animales. Las muestras recolectadas podrán ser usadas para posteriores análisis y estudios relacionados con el tema. Este cuestionario es un complemento del muestreo y se necesitan aproximadamente 10 minutos para resolver las preguntas incluidas. El mismo está orientado a la determinación de la prevalencia y de factores de riesgo a nivel de granja para la enfermedad de interés. Le pedimos dar respuestas claras y reales a las siguientes preguntas, así mismo, garantizamos la confidencialidad tanto de la información proveída como de los resultados.

Como propietario/administrador de esta granja, acepto voluntariamente participar en esta investigación

Nombre completo del propietario/administrador _____

Firma del propietario/administrador _____

Nombre del hato _____

Vereda y municipio _____

Número telefónico _____

Correo electrónico _____

Firma del Investigador/Auxiliar que toma el consentimiento

_____

Fecha _____ (día/mes/año)

Información general de la granja				**[código]**
1. Número del cuestionario (consecutivo)				1.[ ]
Fecha y hora de toma de las muestras		Fecha y hora de congelación de las muestras		
Nombre del predio				
Nombre del propietario/administrador				
Teléfono(s) de contacto				
2. Municipio				2.[ ]
3. Zona		[1] Área Metropolitana [2] Oriente [3] Norte		3. [ ]
4. Vereda				4.[ ]
5. ¿Cuál es el área destinada a la producción de ovinos? (N° de Has)		[1] < 2 Has	[2] ≥ 3 - ≤ 5 Has [3] > 6 Has	5.[ ]
6. ¿Cuál es la población de ovejas?		Animales < 1 año _____ Machos adultos _____	Hembras adultas _____ Total Población _____	6.[ ]
Prácticas de manejo de la granja				
7. ¿Posee otro tipo de rumiantes en sus instalaciones? (bovinos, cabras, búfalos)		[1] Sí [0] No		7.[ ]

	Por favor especifique _____ _____	
8. ¿Se encuentran los diferentes rumiantes en los mismos potreros?	[1] No [2] Sí [3] N/A	8.[ ]
9. ¿Los corderos se quedan con las madres después del parto?	[0] No [1] Sí Especifique días _____	9.[ ]
10. ¿Comparte carreteras con granjas vecinas?	[0] No [1] Sí	10.[ ]
11. ¿Utiliza el estiércol o compostaje como fertilizante en sus pastos?	[0] No [1] Sí	11.[ ]
12. ¿Cada cuánto desparasita los ovinos?	[1] No desparasita [2] Una vez al año [3] 2 veces al año [4] 3 o más veces al año	12.[ ]
13. ¿Moviliza usted animales de diferentes especies entre granjas?	[0] No [1] Sí	13.[ ]
14. ¿Compra ovejas? (hato abierto)	[0] No [1] Sí	14.[ ]
Conocimiento sobre la enfermedad		
15. ¿Cuánto sabe sobre paratuberculosis?	[1] Amplio conocimiento [3] Reconozco el nombre	15.[ ]

	[2] Alguna información [4] Nunca había oído hablar de la enfermedad	
16. ¿Ha tenido casos de animales con diarrea y pérdida progresiva de peso, refractarios al tratamiento?	[1] Actualmente [2] En los últimos 2 años, pero no en el presente [3] Nunca	16.[ ]
17. ¿Cuál es la principal causa de descarte de los animales?		