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Research Article

Diagnosis and Molecular Characterization of *Mycobacterium avium* subsp. *paratuberculosis* from Dairy Cows in Colombia

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The objective of this study was the serological, bacteriological and molecular diagnosis, as well as the molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in adult cows of five Colombian dairy herds. Serum samples were tested by an indirect absorbed enzyme–linked immunosorbent assay (ELISA-C). All fecal samples were tested by pooled culture. After that, fecal samples of Map positive pools were tested individually by culture and polymerase chain reaction (PCR). In one herd, slurry and tissue samples from one animal were also taken and tested by PCR and culture. Map isolates were analyzed by the Multilocus Short Sequence Repeat (MLSSR) and the Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) methods. ELISA produced positive results in 1.8% (6/329) of the animals and 40% (2/5) of the herds. Four fecal, two tissue, and two slurry samples from a herd were Map positive by culture and PCR. MLSSR and MIRU-VNTR revealed two different strain profiles among eight Map isolates recovered. This study reports the first molecular characterization of Map in one dairy herd in Colombia, the limitations for individual diagnosis of subclinical Map infections in cattle, and the usefulness of pooled fecal samples and environmental sampling for Map diagnosis.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) is a slow growing, mycobactin-dependent acid fast bacterium that causes paratuberculosis or Johne's disease in cattle [1]. Paratuberculosis produces a considerable economic impact on the cattle industry, especially on milk production and body condition. Map has also been related to the chronic human enteritis known as Crohn's disease, but this relationship still remains controversial [2].

Enzyme-linked immunosorbent assay (ELISA), bacteriological cultivation of fecal samples, and polymerase chain reaction (PCR) are test widely used for the antemortem diagnosis of paratuberculosis in cattle herds [3–5]. On the other hand, bacteriological culture of pooled fecal samples and environmental sampling are cost-effective methods to classify herds as Map infected [6, 7]. In addition, sampling all adult cattle in every herd, environmental sampling, serial testing, and the use of two to three diagnostic tests have been recommended for herd screening and to increase the accuracy of Map diagnosis [5, 8].

Strain differentiation of Map is very useful to understand the origin of the infections and the disease transmission dynamics, to design more adequate control measures, and to improve diagnosis rates and the development of vaccines [9]. Although paratuberculosis is a notifiable disease in Colombia, the lack of an official national control program and some limitations of the diagnostic tests have contributed to the reduced local information on the disease.

The objective of this study was the serological, bacteriological, and molecular diagnosis of Map in adult cows of five Colombian dairy herds, as well as the molecular characterization of Map isolates and the comparison of results with similar studies.

Table 1: Information on herd management of five dairy herds examined for Map in Colombia.

Herd[a]	District	Number of cattle purchased in the last two years		Raising of own replacement heifers	Proportion of ELISA-A positive results 2007[b,d]
		2007[b]	2009		
1	Monterredondo	0	0	Yes	15% (3/20)
2	El Yuyal	10	5	Yes	20% (4/20)
3	El Yuyal	0	0	Yes	27.2% (6/22)
4	Santo Domingo	0	0	Yes	21.7% (5/23)
5	Santa Bárbara	N.A.[c]	4	Yes	NA

[a] Herd 2 and herd 4 belong to the same farmer and cattle exchange between both herds occurs frequently.
[b] According to Fernández-Silva et al. 2011 [10].
[c] NA: not applicable. This herd was not sampled in 2007.
[d] Refers to the proportion of positive animals by ELISA-A to the number of animals sampled in the herd.

2. Materials and Methods

2.1. Selection of Herds. Between November and December of 2009, five dairy herds were selected to be examined for Map (Table 1 and Figure 1). Of these five herds, four herds (herds 1, 2, 3, and 4) tested ELISA and PCR positive but culture negative for Map in a previous cross-sectional study in 2007 [10]. In this study, 13 herds without previous history of paratuberculosis and one herd (herd 1 of the present study) with previous diagnosis of Johne's disease [11] were examined. In every herd, only a sample (between 19 and 29 randomly selected adult cows, depending on the herd population) was tested. Serological testing was initially carried out to all sampled animals using a commercial lipoarabinomannan- (LAM-) based indirect ELISA test without preabsorption (Svanovir Para-TB Ab ELISA Kit, Uppsala, Sweden) (ELISA-A). For confirmation of positives samples by ELISA-A, a commercial indirect ELISA test based on detection of antibodies to protoplasmic Map antigens, including a pre-absorption step with *Mycobacterium phlei* (*M. phlei*) (ELISA paratuberculosis antibody verification, Institut Pourquier, Montpellier, France) (ELISA-B) was used. For the PCR diagnosis, two methods targeted to F57 and IS*Mav2*, and to IS*900*, respectively, were applied [10].

The four herds previously tested in 2007 and selected in the present study (herds 1, 2, 3, and 4) have never followed any structured or consistent control program for prevention or control of paratuberculosis before the first study in 2007, or in the period between both studies. However, culling of animals with nonresponsive diseases (including animals with compatible signs of Johne's disease in herd 1) or low productive or reproductive performance was done permanently. The remaining herd (herd 5) had a cow with weight loss and nonresponsive diarrhea compatible with paratuberculosis, but did not have a history of Johne's disease or a previous diagnosis of Map.

From the herds tested, only herd 2 has purchased animals before the first study in 2007 and between both studies. Herd 5 has purchased animals in the last two years before the sampling of the present study. Between herd 3 and herd 4, which belong to the same farmer, cattle exchange occurs usually. All herds raise their own replacement heifers (Table 1).

2.2. Collection of Samples. Serum and fecal samples were taken from all adult dairy cows (≥2 years) in every herd. In herd 2, 110 cows were sampled for feces, but only 53 of them were sampled for serum due to reluctance of farmer to sample all animals. In one herd (herd 1) that had slurry pit collecting liquid manure and wastewater from the herd's milking parlor, slurry samples were additionally taken from three different places of the pit. From one animal of the same herd (herd 1), a section of thick intestine (colon) and a mesenteric lymph node were obtained after euthanasia and necropsy due to advanced clinical symptoms compatible with Johne's disease. Information about age was collected from all animals with exception of six animals, from which farmers did not have available data at the moment of sampling.

2.3. ELISA. Serum samples ($n = 329$) were tested with an ELISA test based on detection of antibodies to Map extract (ID Screen Paratuberculosis Indirect, IDVET, Montpellier, France) (ELISA-C). This test included a pre-absorption step with *M. phlei*. ELISA-C was not carried out in duplicate due to economical reasons. A herd was considered positive if at least one animal tested positive by ELISA-C.

2.4. Bacteriological Culture. Fecal samples ($n = 386$) were examined on the basis of a strategic pooling procedure. Fecal samples were sorted on the basis of birth order of the animals, and 2 g of feces from each cow was mixed at the laboratory into pooled fecal samples of 8–12 cows per pool. After this, 3 g of the pooled fecal sample was decontaminated with 0.75% (w/v) Hexadecylpyridinium Chloride solution (0.75% HPC) for 24 h, according to standard procedures [12]. Briefly, 3 g of feces were added to a 50 mL sterile tube (Sarstedt, Nümbrecht, Germany) containing 30 mL of a 0.75% HPC. This suspension was manually mixed by shaking and vortexting and let in vertical position for 5 min at room temperature to allow the precipitation and sedimentation of big particles. Approximately 20 mL of the upper portion of the supernatant was transfer to another 50 mL sterile tube in which the whole suspension was agitated for 30 min by 200 U/min. Tubes were placed in vertical position in the dark for 24 h at room temperature. Decontaminated pooled fecal samples were centrifuged at 900 ×g during

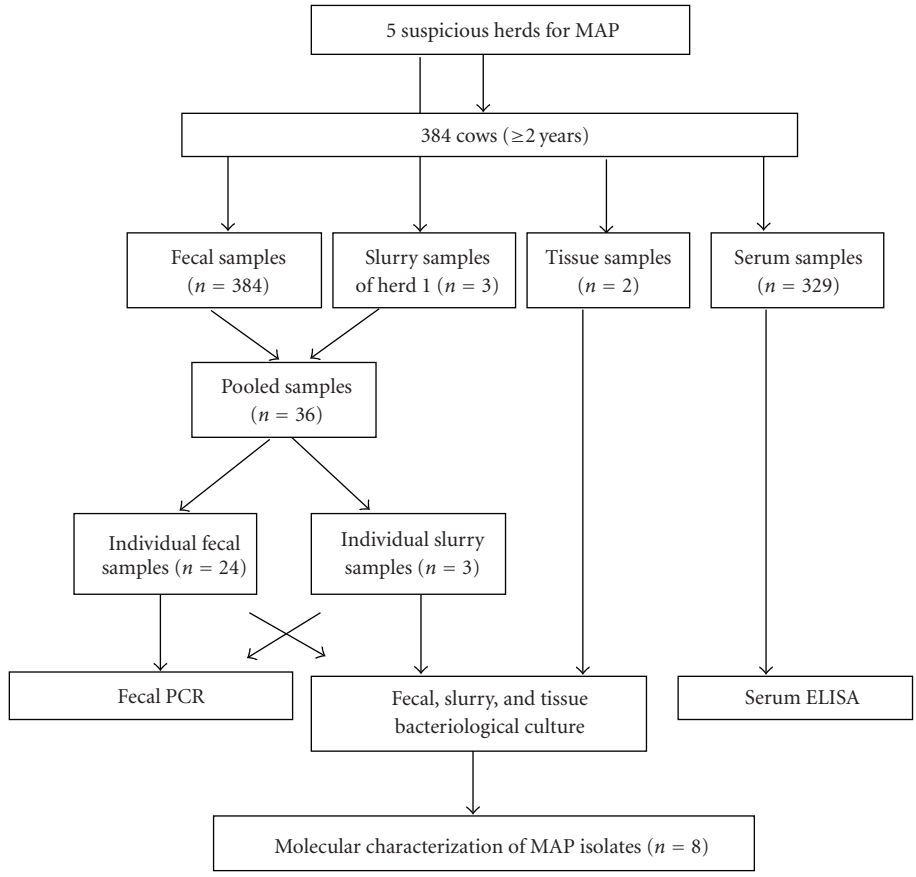

Figure 1: Overview of the study design.

30 min, supernatant was discarded, and two Herrold's Egg Yolk Agar medium (HEYM) slants, supplemented with mycobactin J (Prepared Culture Media, Becton Dickinson, Heidelberg, Germany) were inoculated with 300 μL of the decontaminated pellet [12]. The slants were incubated at 37°C for a maximum of 20 weeks and checked at 1-2-week intervals.

Slurry samples were also pooled, decontaminated, and inoculated as described above for fecal samples. If HEYM slants inoculated with pooled fecal or slurry samples showed mycobacterial growth, single fecal and slurry samples were cultured individually. The individual samples from a negative pool were assumed negative and not tested individually, except for the fecal samples from ELISA-C-positive animals of herd 2, which were cultured individually regardless of their culture results.

Tissue samples (colon and mesenteric lymph node) were prepared, decontaminated, and inoculated in duplicate onto HEYM slants [12]. Briefly, the colon tissue was cut open, and the mesenteric lymph node was released from adipose tissue. Both samples were cut up separately, and approximately 1 g of the respective tissue material was put in a stomacher bag with 7 mL of 0.9% (w/v) Hexadecylpyridinium Chloride solution (0.9% HPC) and was homogenized for 6 min in the stomacher. The homogenized tissue was put in a 50 mL sterile tube (Sarstedt, Nümbrecht, Germany) and shaken at room temperature, 200 U/min for 5–10 min. After that, tubes were place in vertical position in the dark for 24 h at room temperature. After decontamination, the tubes were centrifuged at 1880 ×g, at 20°C for 20 min. The supernatant was discarded, and the sediment was resuspended in PBS-Buffer pH 7.2 and vortexted. Finally, two HEYM slants, supplemented with mycobactin J (Prepared Culture Media, Becton Dickinson, Heidelberg, Germany) were inoculated with 300 μL of the decontaminated pellet. As done with fecal samples, slants were incubated at 37°C for maximum 20 weeks and checked at 1-2-week intervals for mycobacterial growth or contamination. Contamination rate was estimated in 8.3% (3/36) for the fecal and the slurry pooled samples, and 3.7% (1/27) for the individual fecal samples (including fecal cultures from ELISA-C-positive animals of herd 2) and the tissue samples. In all contaminated samples, only one slant of the duplicate was affected. In case of mycobacterial growth, Map was confirmed by the real-time PCR method described as follows.

2.5. Polymerase Chain Reaction (PCR). PCR was carried out only on individual fecal and slurry samples that were part of positive fecal and slurry pooled samples by culture, and to fecal samples of four positive ELISA-C animals of herd 2 ($n = 27$) (Figure 1). DNA isolation from fecal and slurry samples was carried out using a commercial DNA

preparation kit (High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany). Briefly, 1.5 g of bovine feces was put in a 15 mL sterile, nonpyrogenic centrifuge tube (Sarstedt, Nümbrecht, Germany). Five mL of a buffer for stabilization (Stool Transport and Recovery-S.T.A.R. buffer, Roche, Mannheim, Germany) was added to fecal sample and homogenized. This suspension was subsequently centrifuged for 1 min by 1000 ×g and 1 mL of the supernatant was put in a 2 mL conical sample tubes (Biozym Scientific, Hess. Oldendorf, Germany) containing ceramic beads, size range 1.4–1.6 mm, Genotype ZY (Zirkonoxid-Beads, Yttrium stabilized) (Sigmund Lidner, Warmensteinach, Germany). A mechanical cell disruption step was carried out in an automated biological sample lyser (Precellys 24, Bertin technologies, Montigny-le-Bretonneux, France) to achieve efficient cell lysis. The mixture was subsequently incubated at 95°C for 10 m and centrifuged 5 min by 5000 ×g. Two hundred microliters of the supernatant was added to a 1.5 mL reaction tube containing 5 μL of lysozyme (Merck, Darmstadt, Germany) solution. Further processing was done according to kit's protocol for isolation of nucleic acids from bacteria and yeast. DNA isolation was always carried out in duplicate.

DNA isolation from bacteria for Map confirmation and molecular characterization was carried out using a commercial preparation kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany). This preparation included overnight lysis buffer incubation at 37°C, proteinase K/AL-buffer incubation for 90 min at 56°C, and final incubation for 15 min at 95°C, as a modification of the protocol of the commercial kit. DNA from fecal samples and from bacterial culture was tested in duplicate for Map with the real-time PCR method targeted to F57 and IS*Mav2* described by Schönenbrücher et al. [13]. Samples were also tested in duplicate with the nested-PCR targeted to IS*900* described by Bull et al. [14]. Additional to the samples, a positive and a negative preparation control, as well as a blank control were included. In the PCR system, a positive Map control (DNA of a positive Map strain), a non-Map negative control (DNA of a non-Map mycobacteria), and a master-mix blank control were also included. The real-time PCR method also included an internal amplification control (IAC) to avoid the misinterpretation of false negative results [13].

2.6. Molecular Characterization. For molecular characterization of the Map strains isolated, a combination of two different genotyping methods based on PCR amplification of repetitive elements of Map genome was applied. The Multilocus Short Sequence Repeat (MLSSR) analysis was carried out by amplification of the short sequence repeats (SSRs) found in locus 1, 2, 8, and 9 according to primers and PCR conditions reported by Amonsin et al. [15]. The final PCR reaction volume (30 μL) contained GeneAmp 10x PCR Puffer (Applied Biosystems, Darmstadt, Germany), dNTP-Mix (10 μM each) (Roche, Mannheim, Germany), 0.2 μM of each primer (Eurofins MWG, Martinsried, Germany), 10% Dimethil Sulfoxide (DMSO) (Roth, Karlsruhe, Germany), 1 U of AmpliTaq Gold Polymerase 5 U/μL (Applied Biosystems, Langen, Germany), and 3 μL of DNA. A master mixture blank (without DNA) was included as control in every PCR reaction. Seven microliters of every PCR product were mixed with 2 μL of loading buffer, and electrophoresed in a 1.5% agarose gel. All amplicons in every SSR locus were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced by an independent laboratory (Sequence Laboratories, Göttingen, Germany). MLSSR genotypes were expressed as the combination of the number of repeats found in the four loci amplified by PCR.

The Mycobacterial Interspersed Repetitive Units (MIRU) loci MIRU-1, MIRU-2, MIRU-3, and MIRU-4 were selected to analyze the Map isolates according to Bull et al. [16]. The Variable Number of Tandem Repeats (VNTRs) loci VNTR-292, VNTR-1658 (alias X3), VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32, and VNTR-259 were selected to analyze the Map isolates according to Overduin et al. [17], Thibault et al. [18], and Castellanos et al. [19]. For all loci, primers used were those suggested by the authors mentioned above. Except for the PCR conditions of MIRU-1, VNTR-7, and VNTR-10 carried out according to Möbius et al. [20], and of VNTR-25 and VNTR-47 according to Castellanos et al. [19]. The final reaction volume of PCR (30 μL) for MIRU-VNTR was the same as described for MLSSR. However, for the PCR amplification of VNTR-32, 5 μL of Betain (Sigma-Aldrich, Schenelldorf, Germany) was additionally added to the mix as suggested by Thibault et al. [18].

Calculation of the number of repeats per locus for MIRU-VNTR was initially performed according to the size of the amplicon determined by electrophoresis in 1.5% agarose gel. Additionally, the amplicons of representative alleles in every locus were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced by an independent laboratory (Sequence Laboratories, Göttingen, Germany). MIRU and VNTR genotypes were confirmed by the number of repeat units and the number of tandem repeats in selected sequences. MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus in the order MIRU-1, MIRU-2, MIRU-3, MIRU-4, VNTR-292, VNTR-1658 (alias X3), VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32, and VNTR-259. The INRA Nouzilly MIRU-VNTR (INMV) nomenclature as defined by Thibault et al. [18] was taken into account for ease comparison with previous studies. For this purpose only the results of loci VNTR-292, VNTR-1658, VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, and VNTR-32 were considered.

2.7. Data Analysis. The descriptive analysis of age, the estimation of standard deviation (SD), and the determination of confidence intervals 95% (95% CI) were carried out using the program packages BMPD release 8.1 (Berkeley, USA) and BIAS release 8.2 (Hochheim-Darmstadt, Germany). The estimation of the testing agreement between ELISA and culture (Cohen's kappa (κ) coefficient) was done with the program Win-Episcope 1.0 (Zaragoza, Spain). True prevalence was estimated based on the apparent prevalence obtained by ELISA-C using sensitivity (42%), and specificity (99%) values determined previously on asymptomatic infected animals [21]. The relation age versus ELISA results

Table 2: Enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and fecal culture positive results of five dairy herds examined for Map in Colombia.

Herd	District	Herd cattle population	Number of samples[a]	Serum ELISA-C	Fecal culture[c]	Fecal PCR[e]
1	Monterredondo	125	75	2	4[d]	2
2	El Yuyal	174	53[b]	4	0	0
3	El Yuyal	144	84	0	0	ND[f]
4	Santo Domingo	172	94	0	0	ND
5	Santa Bárbara	38	23	0	0	ND
Total		653	329	6	4	2

[a] Only cows over 2 years of age were sampled.
[b] In this herd, 110 animals were sampled for feces, but only 53 of them were sampled for serum.
[c] Refers to fecal samples cultured individually.
[d] Positive results of one lymph node, one colon tissue, and two slurry samples are not included.
[e] Refers to individual fecal samples part of pooled fecal samples positive by culture, and to fecal samples of positive ELISA-C animals tested individually.
[f] ND not done.

was analyzed descriptively, according to age classes defined arbitrarily.

3. Results

3.1. ELISA. ELISA-C produced positive results in 1.8% (6/329) (95% C.I.; 0.7–3.9%), negative results in 97.5% (321/329), and doubtful results in 0.6% (2/329) of the serum samples examined, as well as positive results in 40% (2/5) of the herds. Of the six positive ELISA-C samples detected, two were detected in herd 1 and four were detected in herd 2 (Tables 2 and 3). The true Map-prevalence based on ELISA-C-apparent prevalence, sensitivity (42%) and specificity (99%) was 2.2%.

The age of the animals sampled ranged between 2.2 and 14 years (mean 5.9, SD 2.8). Analysis of age of animals versus ELISA-C result (positive, negative, doubtful) revealed that the group of >11 years of age, was the group in which the highest proportion (6.3%, 1 out of 16) of ELISA-positive samples were produced (Table 4). However, it was in the group of 5.1–8 years of age in which the highest absolute number of ELISA-positive animals ($n = 3$) of the whole study was found. In the group of the youngest cows (>2.2–2.9 years of age), no positive result by ELISA was produced. In the group of 3–5, 5.1–8, and 8.1–10.9 years, 0.8%, 2.9%, and 1.9% of the samples produced positive results, respectively (Table 4).

3.2. Bacteriological Culture and Polymerase Chain Reaction. The strategic pooling procedure for fecal samples from the five herds resulted in 36 pools, including the slurry pool prepared from herd 1, which had a slurry pit collecting liquid manure and wastewater from the herd's milking parlor. Two pools from herd 1 out of 36 pools analyzed produced positive results by culture after 5-6 weeks of incubation with >50 Colony Forming Units (CFU)/tube. The slurry pool produced positive results by culture after 17 weeks of incubation with <10 CFU/tube. Isolates obtained from pooled samples were confirmed as Map by the real-time PCR method described above. Remaining pools of herds 2, 3, 4, and 5 did not show mycobacterial growth by culture in 20 weeks of incubation.

Table 3: Enzyme-linked immunosorbent assay (ELISA-C), polymerase chain reaction (PCR), and culture results of animals and individual slurry samples from positive pooled samples or ELISA-C-positive animals of herd 1 and herd 2.

Herd	Pool	Source	ELISA-C	PCR	Culture
1	1	C1	−	−	+
		C2	−	−	+
		C3	+	+	+
		C4	+	−	−
		C5	−	−	−
		C6	−	−	−
		C7	−	−	−
		C8	−	−	−
		C9	−	−	−
	2	C1	−	−	−
		C2	−	−	−
		C3	−	−	−
		C4	−	+	+
		C5	−	−	−
		C6	−	−	−
		C7	−	−	−
		C8	−	−	−
		C9	−	−	−
		B	−	−	−
	3	S1	N.A.	−	−
		S2	N.A.	−	−
		S3	N.A.	−	−
2	1	C1	+	−	−
	2	C2	+	−	−
	3	C3	+	−	−
	4	C4	+	−	−

C: cow, B: bull, S: slurry pit, +: positive result, −: negative result, NA not applicable.

Fecal samples that were a part of the two positive pools of herd 1 ($n = 19$) produced four positive results by individual culture (Table 3). All isolates showed no pigmentation and were confirmed as Map by real-time PCR. Two cows

Table 4: ELISA results according to group of age of 323 animals from five dairy herds.

Group of age	ELISA result			Total (%)
	Positive (%)	Negative (%)	Doubtful (%)	
2.2–2.9	0 (0.0)	32 (100)	0 (0.0)	32 (9.9)
3–5	1 (0.8)	116 (98.3)	1 (0.8)	118 (36.5)
5.1–8	3 (2.9)	101 (97.1)	0 (0.0)	104 (32.2)
8.1–10.9	1 (1.9)	51 (96.2)	1 (1.9)	53 (16.4)
>11	1 (6.3)	15 (93.8)	0 (0.0)	16 (5)

Table 5: Isolates of *Mycobacterium avium* subsp. *paratuberculosis* recovered in a dairy herd.

Isolate number	Source	Isolated from	Molecular genotype	
			MLSSR-Type[a]	MIRU-VNTR-Type[b]
(1)	Cow 1	Feces	A	1
(2)	Cow 2	Feces	A	1
(3)	Cow 3	Feces	A	1
(4)	Cow 3	Mesenteric lymph node	B	2
(5)	Cow 3	Colon tissue	A	1
(6)	Cow 4	Feces	A	1
(7)	Slurry pit	Slurry	A	1
(8)	Slurry pit	Slurry	B	2

[a] MLSSR-genotype A: 7g-10 g-4ggt-5tgc and MLSSR-genotype B: 7g-10g-5ggt-4tgc.
[b] MIRU-VNTR genotype 1: 3951-42332228-2 (INMV 1) and MIRU-VNTR genotype 2: 3751-32332228-2 (INMV 2).

(both ELISA-C-negative, PCR-negative, asymptomatic, 7.1 years old) were low shedders (<10 CFU/tube), one cow (ELISA-positive, PCR-positive, symptomatic, 6 years old) was a heavy shedder, and one cow (ELISA-negative, PCR-positive, asymptomatic, 9.5 years old) was a heavy shedder (>50 CFU/tube) (Table 3). The cow 3 from pool 1 (herd 1) also produced positive results by mesenteric lymph node and colon tissue culture (Tables 3 and 5). On HEYM-slants inoculated with mesenteric lymph node tissues, visible colonies grew before 16 weeks of incubation, while in those inoculated with colon tissues no visible Map colonies grew in this period of time. Four fecal samples from positive ELISA-C animals of herd 2 produced negative results by culture and PCR; these samples were all from different pooled fecal samples (Table 3). Surprisingly, although the pooled slurry sample produced positive results by culture and PCR, their individual samples ($n = 3$) were negative by PCR, and by culture after 20 weeks of incubation (Table 3).

ELISA-C results were confirmed by culture in only one symptomatic animal (cow 3) of herd 1. Thus, ELISA-C, culture, and PCR only agreed on one single animal out of four animals that delivered positive culture results. According to these results, calculated agreement between ELISA-C and culture was poor ($\kappa = 0.19$, 95% C.I. 0.09–0.29). ELISA-C did not detect Map antibodies in three serum samples from asymptomatic animals of herd 1 (Cow 1 and Cow 2 from pool 1, and Cow 4 from pool 2) that produced positive results by fecal culture. In fecal samples from two of these three animals, Map was also not detected by PCR. In one case, a cow (cow 4 of pool 1 from herd 1) produced negative results by ELISA-C, but positive results by PCR and culture (Table 3).

3.3. Comparison of Results 2007–2009. Based on results of a previous study [10], it was determined that some animals ($n = 11$) tested in 2007 were tested again by ELISA, PCR and culture in this study (2009). None of these animals have presented symptoms of paratuberculosis before 2007 or between the two studies. Results can be classified in five different categories, in which only positive findings are described, meaning that other tests produced negative results. In the category 1, one single animal produced positive results by ELISA-A and real-time PCR (molecular target F57) in 2007 and ELISA-C-positive results in 2009. In the category 2, one single animal produced positive results in ELISA A, PCR and real-time PCR (molecular target IS*Mav2*) in 2007. In the category 3, two animals produced ELISA-A and ELISA-B positive results in 2007. In the category 4, one single animal produced positive results by ELISA-A and PCR in 2007. Finally, in the category 5, six animals produced positive results by ELISA-A (Table 6).

3.4. Molecular Characterization. In total eight Map isolates were recovered. Four isolates were from fecal samples, one from mesenteric lymph node, one from colon tissue sample, and two from pooled slurry samples (Table 5). All isolates were confirmed as Map by the real-time PCR method described above. All isolates were obtained from samples from herd 1. All isolates grew within 6–16 weeks of incubation, except from samples of slurry and colon tissue, which grew after the 16th week.

The four isolates obtained from fecal samples, the isolate obtained from colon tissue, and one of the two isolates obtained from slurry sample were of the MLSSR genotype

Table 6: Comparison of diagnostic test results of single animals ($n = 11$) tested for Map in 2007 and in 2009

	2007[a]					2009			
Category	ELISA-A[b]	ELISA-B[c]	PCR[d]	Real-time PCR[d]	Culture	ELISA-C[e]	Culture[f]	PCR[g]	Real-time PCR[g]
1	+	−	−	+ (F)	−	+	−	−	−
2	+	−	+	+ (I)	−	−	−	N.D	N.D
3	+	+	−	−	−	−	−	N.D	N.D
4	+	−	+	−	−	−	−	N.D	N.D
5	+	−	−	−	−	−	−	N.D	N.D

[a] According to Fernández-Silva et al. 2010 [10].
[b] ELISA-A (Svanovir Para-TB Ab ELISA Kit, Svanova Biotech AB).
[c] ELISA-B (ELISA paratuberculosis antibody verification, Institute Pourquier) performed only to positive and doubtful ELISA-A samples.
[d] PCR and real-time PCR performed only to fecal samples from positive animals by ELISA-A.
[e] ELISA-C (ID Screen Paratuberculosis Indiret, IDVET) performed to herds in the present study (2009).
[f] Culture performed initially from pooled fecal samples, and then individually if pooled sample was Map positive.
[g] PCR and real-time PCR performed only to individual fecal samples from culture-positive pooled samples, and to fecal samples from ELISA-C-positive animals regardless of culture result.
+: positive, −: negative, (F): positive real-time-PCR in marker F57, (I): positive real-time-PCR in marker IS*Mav2*, ND: not done.

7g-10g-4ggt-5tgc (hereafter MLSSR-genotype A). The isolates obtained from mesenteric lymph node and the remaining isolate obtained from slurry sample were of the MLSSR genotype 7g-10g-5ggt-4tgc (hereafter MLSSR-genotype B). Similarly, the combination of MIRU-VNTR showed two different MIRU-VNTR genotypes, genotype 3951-42332228-2 (hereafter MIRU-VNTR genotype 1) and 3751-32332228-2 (hereafter MIRU-VNTR genotype 2). Interestingly, strain types A-1 and B-2 were both identified in cow 4, representing a case of double strain infection.

4. Discussion

To our knowledge, this is the first report of isolation and molecular characterization of Colombian Map-strains from dairy herds. For achievement of this goal, herds with history of Johne's disease (report of cases and/or positive diagnosis) were selected to increase the likelihood of detection and isolation of Map. Despite the importance of the cattle production in Colombia, paratuberculosis has remained relatively uninvestigated, and very limited epidemiological information and data on molecular characterization of Map were available. For about 60 years, some studies have tried to widen the clinical and epidemiological information of paratuberculosis in the country through research on diagnosis, treatment, epidemiology, and molecular biology. Now, a consistent study including a significant cattle population, using different diagnostic tests, and including the molecular characterization of the circulating causal agent is presented. As previously suggested in some studies, ELISA, PCR, and culture were used to increase sensitivity of Map detection, in order to confirm whether herds with history of Johne's disease or Map diagnosis were truly infected [22].

The lower proportion of the current ELISA-C positive results (1.8%) compared to the previous ELISA-A positive results (10.1%) in four of the five herds examined was surprising at first sight, but it is explained by the characteristics of the ELISA tests used in both studies. ELISA-C is an absorbed test using purified Map extract, IgG-conjugate and preincubation with *M. phlei*, which are characteristics that have been considered of critical influence on the increment of specificity for the serological diagnosis of paratuberculosis [23–26]. Therefore, the use of an absorbed test (ELISA-C) has produced negative or a lower proportion of positive results in herds with previous Map diagnosis (ELISA-A and PCR), or even with previous history of clinical cases of paratuberculosis (e.g., herd 1), compared to the previous study of 2007. In this previous study, herds of a dairy region mostly without previous diagnosis of paratuberculosis were tested using an unabsorbed test that used LAM as Map antigen (ELISA-A), which produced a higher proportion of seropositives confirmed only in two animals by an absorbed ELISA (ELISA-B). Interestingly, the results of both absorbed tests, ELISA-B in 2007 and ELISA-C in 2009, produced closer results (5.1% versus 1.8%) than those obtained with the unabsorbed ELISA-A (10.1%) in 2007. This suggests that the characteristics of the tests used were determinant in the different proportions of seropositives obtained in both studies.

Furthermore, the absence of reliable preliminary epidemiological information on the disease makes also plausible that dairy herds in the region of study were of a very low prevalence or even negative for Map, or at least undetectable with the current diagnostic tests, if only cross-sectional studies instead of a longitudinal study or serial testing is carried out. In any case, these studies are the first step of the systematic epidemiological study of paratuberculosis in Colombia, and therefore further studies have to be conducted to elucidate the situation of the disease in the country.

The case of herd number 2, in which clinical paratuberculosis has never been reported, but some animals were positive by ELISA-A and PCR in 2007, and again in 2009 (four positive ELISA-C, but negative results by culture) is striking and difficult to explain. In this case, it could be possible that other mycobacteria could influence the positive results of the unabsorbed ELISA-A in 2007 and of the ELISA-C positive in 2009, making the proportion of positive animals higher than it really is [27]. In the study of 2007, atypical mycobacteria (*Mycobacterium engbaekii*) were isolated [10].

However, the lack of testing of the half of the adult cattle population of this herd for ELISA-C limits any definitive conclusion about the current negative PCR and culture results obtained from seropositive animals, taking into account the high quality of the tests employed. Additionally, the four fecal samples from the positive ELISA-C animals were cultured individually, which could have been reduced the chances of a probable concentration of low quantities of Map, if these animals were really shedders. Likewise, it is possible that the positive ELISA results, not only in herd 2, but all herds of both studies have been produced due to the interference with tuberculin from intradermal tests (caudal fold tuberculin test) [28] applied occasionally in order to be certified as free herd from bovine tuberculosis in frame of the national program for eradication of tuberculosis. In conclusion, some of these results of ELISA-A and even ELISA-C could have been simply false positives in 2007 and in 2009, respectively.

The low apparent prevalence and the true prevalence obtained (1.8 versus 2.2%) was probably related to the high specificity of test used (99%), as previously reported for studies with these characteristics [29]. Although our study was biased for prevalence determination, due to analysis of herds with previous history or diagnosis of paratuberculosis, animal level apparent prevalence calculated appeared to be lower compared to prevalences obtained in European countries [30]. However, no similar studies were found aiming at the determination of prevalence of Map infection by using the ELISA-C, which made impossible a better comparison of results.

In this study, only 0.8% of ELISA-C-positive cows were detected in the group of 3–5 years and the only symptomatic animal found was a 6- year- old cow. This results slightly disagree with a report of a higher probability of testing positive by ELISA between 2.5 and 5.5 years in infected animals [31] and with the knowledge that most clinical cases of paratuberculosis occur between 3–5 years [32]. Cattle in Colombia tend to be kept longer in production and to be culled later compared to North American or European countries. In this manner, cows can live long enough to be tested and detected by ELISA or fecal culture, or to show symptoms of paratuberculosis out of the age limits reported for other countries.

As reported before, culture of pooled fecal samples of 8–12 animals per pool permitted the examination of a high number of fecal samples by culture at low cost and with acceptable sensitivity [6, 33, 34]. The option of pooling 5 fecal samples instead of 10 was not considered due to economic reasons. Furthermore, precise information about Map within-herd prevalence was absence to take a better decision of the best pool size according to a previous modeling study [35]. This option was also discarded because of the reported insignificant difference in sensitivity between pooling 5 or pooling 10 cows in a previous study in a comparable South American cattle production system, in which it was concluded that the sensitivity of the pool is related more to the prevalence of the herd and to the infection status of the cows as with the size of the pool [6]. In the same way, a study in the United States reported acceptable sensitivity with 10 samples per pool (35%), compared to pooled samples of 5 animals (44%), leading to the conclusion that in herds with at least one high fecal shedder, pools of more than five samples might also detect Map [36]. Although some studies have concluded a better sensitivity of pooling five animals instead of 10 or more, these studies have been based on the detection of Map using radiometric fecal cultures to reliably detect low-shedders, which could be not comparable to classical bacteriological methods [37]. Other studies refer more to a theoretical calculation than to a sensitivity estimation, difficult to extrapolate to South American field conditions [35], or reported the use of five samples per pool focusing on the determination of the sensitivity of culture of pooled fecal samples compared with culture of individual fecal samples, with special attention to the number of pooled fecal samples per herd, rather than to the number of animals to be included in the pooled fecal samples [36].

The detection of a positive pooled slurry sample by culture from a positive ELISA-C herd (herd 1) agrees with the knowledge of the correlation of this finding with seropositive results, and with the higher probability of isolation from lagoon samples compared to other environmental samples [7]. The result of the single slurry samples producing negative results by individual culture and PCR has been reported and has been attributed to uneven distribution of Map in the fecal sample [6], to the lack of homogeneity in the fecal sample or to different sensitivities of individual fecal culture procedure between laboratories [34], to the presence of Map in the feces of at least one animal within the pooled fecal sample, although this animal was not detected by bacteriological culture of individual fecal samples [38], or to unclear reasons [36]. In any case, Kalis et al. properly concluded that there is an element of chance apart from the element of dilution related to the detection of Map in feces, particularly when samples contain low numbers of the organism, and the bacteria are not uniformly distributed in the fecal samples [33]. Thus, although a complete homogenization of the pooled slurry sample was achieved and the PCR systems used are very reliable, it is possible that the 3 g or 1.5 g of slurry samples taken to test by individual culture and by PCR, respectively, lacked enough Map cells to be detected by bacteriological culture in a 20-week period of incubation, and in two PCR systems (F57-Is*Mav2*-real-time and IS*900*-nested-PCR) carried out in duplicate. The PCR systems used, specially the real-time PCR, are strict tested for specificity, included an IAC and use multiple reaction controls, which avoid the misinterpretations of results due to disturbed contamination or very improbable false positive results [13].

Close analysis of the individual results obtained in herd 1 revealed that one single symptomatic animal producing positive results by ELISA-C and PCR, confirmed that regardless of the ELISA or PCR type used sensitivity is higher for detection of symptomatic animals and fecal high shedders [21, 39]. The results of three asymptomatic ELISA-C-negative cows that produced positive results by culture could be: two cases (Map low-shedders and PCR-negative) of the known "passing through" phenomenon previously described [40], and one case (Map high-shedder and PCR-positive) of a positive animal with undetectable antibodies.

On the other hand, the results of one ELISA-C positive-animal of herd 1, that produced negative results by culture, does not necessarily mean that the animal was not really infected, but that the shedding phase has probably not yet started (infected animal in a noninfectious phase) or was absent at the moment of fecal sampling (intermittency). Another possibility is that in this animal Map-antibodies have been detected prior to the start of bacterial shedding, which could begin later and could be then detected by PCR or culture [41]. Map is shed in feces of infected animals 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 [5].

One ELISA-C-negative animal was positive by PCR, real-time PCR, and culture. On the contrary, one ELISA-C-positive animal in the same herd showed negative results by PCR, real-time PCR, and culture. In herd 2 four ELISA-C-positive animals produced negative results by fecal PCR and fecal real-time PCR, as well as negative results by individual fecal culture. Muskens et al. found a low percentage of ELISA-positive cattle testing fecal culture-positive for all age groups included. Among their arguments, they stated a possible limited sensitivity of the fecal culture and/or false-positive ELISA test results and a nonhomogeneous distribution of Map in feces especially for low shedders [42]. In general, explanations for the poor concordance of diagnostic tests could be attributed to false-positive ELISA results, to nonhomogeneous distribution of Map in feces (especially for low shedders), to relatively low prevalence of Map infection, and very low positive predictive value of ELISAs applied. In addition, it has to be taken into account that not only the combination of different tests, but repeated sampling is necessary to achieve the identification of individual animals [5].

Although many animals sampled in a previous study (2007) were no longer in the herds at the time of the second sampling (2009), it was an interesting finding to compare the diagnostic results of animals sampled in 2007 with those results obtained from the same animals in 2009, simulating a longitudinal study or repeating testing for these animals. Changes in our diagnostic test results between 2007 and 2009 agree with studies that report fluctuations of serum ELISA, PCR, and culture results overtime [43–45]. Many test factors (sensitivity, specificity, within-herd prevalence of herd) in every diagnostic procedure influence the variability of results, when the same animals are tested more than once overtime. Particularly for ELISA, fluctuations in test results have been attributable to false-positive results on the first or on the second test, fluctuation in antibody production by the cow, application of tests to low prevalence herds, in which the positive predictive value of tests is lower, or to analytic error. Analytic error occurs when samples were not tested in duplicate as suggested by manufacturers, and repeat analysis gives negative results [43]. Nevertheless multiple testing over time increases the chance of detection of an infected animal, this would also increase the chances of a false-positive result [43]. Therefore, ELISA results have to be analyzed carefully when this test is applied for individual animal diagnosis [21, 46]. However, this is not an uniform process because, as it has been reported, cows with negative results are less likely to change ELISA status than cows with positive results, regardless of within-herd prevalence [44].

Phenotypic characteristics of fast growth, mycobactin dependency, and no pigmentation of Colombian Map isolates coincide with the description of type II (or cattle type) strains described in previous studies [47]. The combination of MIRU-VNTR and MLSSR, as done previously [48] made possible the reliable differentiation for the first time of two Map genotypes among eight different Map isolates of one herd in Colombia. These methods were applied combined to increase the minimum discriminatory ability needed and not reached if one single method had been used, as reported before by Stevenson et al. 2009 [49]. According to MLSSR, the types isolated in our study are commonly found in cattle and other species in different countries [48, 50–52]. Interestingly, a bovine isolate from Colombia's neighbor country Venezuela has shown a different genotype (11g-10g-5ggt-5ggt), suggesting strain diversity in the northern part of the subcontinent [48].

Although comparison with other studies is very difficult because of the use of different loci for analysis, genotype 1 (INMV1) and genotype 2 (INMV 2) were previously reported as the most common genotypes found in isolates from Argentina and Venezuela [18], and in European isolates [49, 53]. Cases of double strain infection has been also reported at herd level in the United States [54], Germany [20], and the Netherlands [55], while cases of double strain infection at animal level have been reported in Germany [51].

The finding of two strain types among eight isolates recovered from herd 1, including isolates from four cows all born in the herd, but unrelated each other, and isolates of slurry samples of the slurry pit collecting liquid manure and wastewater from the herd's milking parlor, suggests the circulation of Map from and to the environment, and among different animals in the herd. In the same way, the isolation of two different types in one single animal, types that were also isolated in slurry samples, supports the idea of a highly Map contaminated environment, which leads to the infection with more than one different strain genotype in the herd. In herd 1, animal feces are used as fertilizer on the pastures and no paratuberculosis control program is carried out. It has been presenting sporadic cases of animals with symptoms of paratuberculosis confirmed by histopathology (unpublished data), and Map has been detected by PCR and serology [10, 11]. Shedding cows were relatively old cows ($\geq$6 years) at the time of sampling suggesting that these animals have been contaminating the environment with Map until they are removed from the herd, contributing to the perpetuation of Map and the presentation of new infections, if no control program is established.

Regarding technical considerations of the genotyping methods, we agree that MLSSR could be less accessible and more expensive than MIRU-VNTR due to the sequencing step required [48]. This aspect could represent a limitation in some developing countries (e.g., Colombia) in which sometimes sequencing has to be carried out abroad incrementing even more the costs of application of MLSSR method. However, we agree that MLSSR analysis is an excellent Map

molecular characterization method in terms of in vitro stability and discriminatory index [54], which could justify the cost of the sequencing step needed.

The results of this study confirm the presence and suggest the circulation and transmission of different Map strains types between individuals of the infected herd. In addition, the study confirmed the limitations of current tests for individual diagnosis of subclinical Map infections in cattle, and the usefulness of pooled fecal samples and environmental sampling to screen herds for Map.

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