Original

Mycobacterium avium subsp. paratuberculosis (MAP) in a dairy buffalo herd in Colombia

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ABSTRACT

Objective. To determine the presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) using serological, microbiological, and molecular methods, to genotype MAP isolates, and to explore factors associated to MAP-seropositive status in a buffalo herd of the province of Antioquia (Colombia). **Materials and methods.** The study was carried out to test fecal and serum samples from 21 asymptomatic adult buffaloes using culture and ELISA tests. Suspicious culture isolates were confirmed by MAP-IS*900*-PCR (IS*900*-PCR). The positive DNA was retested using MAP-IS*900*-quantative PCR (IS*900*-qPCR), and then, sub-typed using techniques based on the detection of mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) and multilocus short sequence repeat (MLSSR). **Results.** At sampling, the average age of animals was 4.7 years, 90.5% (19/21) were females and 84.2% (16/19) were pregnant. The average body condition score was 4.2 (in a 1-to-5 scale). The average milk yield was 3.5 L/day/animal. No MAP-seropositive animals were detected. One dairy buffalo cow out of the 21 buffaloes was positive by fecal culture at week 6 of incubation and then confirmed by IS*900*-PCR. Sub-typing analysis revealed that the MAP isolate corresponded to the INMV 3 genotype by MIRU-VNTR. **Conclusions.** To the best of the author's knowledge, this is the first report of MAP diagnosis and isolation in Colombian buffaloes and the first time an INMV 3 MAP-profile is found in this species the country.

Keywords: Buffaloes; Colombia; culture; PCR; paratuberculosis (*Fuente: MeSH*).

RESUMEN

Objetivo. Determinar la presencia de *Mycobacterium avium* subsp. *paratuberculosis* (MAP) mediante métodos serológicos, microbiológicos y moleculares, para genotipar los aislamientos de MAP y explorar factores asociados a la seropositividad a MAP en un hato de búfalos del departamento de Antioquia (Colombia). **Materiales y métodos.** Se realizó un estudio transversal para analizar muestras fecales y de suero de 21 búfalas adultas asintomáticas mediante cultivo y ELISA. Los cultivos con aislamientos sospechosos se confirmaron mediante PCR de MAP-IS*900* (IS*900*-PCR). El

ADN positivo se volvió a analizar mediante PCR cuantitativo de MAP-IS*900* (IS*900*-qPCR), y luego, se subtipificó utilizando las técnicas basadas en la detección de unidades repetitivas intercaladas de micobacterias-número variable de repeticiones en tándem (MIRU-VNTR) y repetición de secuencia corta de múltiples locus (MLSSR). **Resultados.** Al muestreo, la edad promedio de los animales era de 4.7 años, el 90.5% (19/21) eran hembras y el 84.2% (16/19) estaban preñadas. El puntaje promedio de condición corporal fue 4.2 (en una escala de 1 a 5). La producción de leche promedio fue de 3.5 L/día/animal. No se detectaron animales seropositivos a MAP. Una hembra de los 21 búfalos fue positiva mediante cultivo fecal en la semana 6 de incubación y luego se confirmó mediante IS*900*-PCR. El análisis de subtipado reveló que el aislado de MAP correspondía al genotipo INMV 3 por MIRU-VNTR. **Conclusiones.** Según el conocimiento de los autores, este es el primer diagnóstico y aislamiento de MAP en búfalos en Colombia y la primera vez que se encuentra un perfil INMV 3 de MAP en esta especie en el país.

Palabras clave: Búfalos; Colombia; cultivo; PCR; Paratuberculosis (*Fuente: MeSH*).

INTRODUCTION

Paratuberculosis (PTB) or Johne's disease (JD) is a granulomatous enterocolitis that affects both domestic and wild ruminants. The etiological agent of PTB is *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (1).

Diagnosis of PTB in ruminants include several types of tests. Sensitivity (Se) and specificity (Sp) of diagnostic tests vary depending on clinical stage of PTB infection (2).

Due to the rusticity of buffalo, PTB is likely to occur in an asymptomatic way, leading to a silent dissemination of the bacterium, not only inside the herd, but to other ruminant populations (3,4) Data about the distribution of MAP in buffalo populations worldwide is available. Reports from India, Brazil, and Italy have been published delivering diverse frequency and diagnostic tests used (3,5,6,7,8). The PTB is a notifiable disease in buffalo in Colombia, Brazil, Italy, Japan, and Switzerland (9).

In 2019, the buffalo population of Colombia was reported to be around 356,908 heads according to official records. The province of Antioquia reported 58,891 buffalos, being the second in the country —out of 32 provinces, with the largest number of animals of this species (15.94%) (10). Despite these data, the PTB affecting buffaloes has not been investigated in Colombia and no control measures or control program for the disease have been established so far (11).

Therefore, the aim of this study was to determine the presence of MAP using serological, microbiological, and molecular methods, to genotype MAP isolates, and to explore factors associated to MAP-seropositive status in a buffalo herd in one municipality of Antioquia (Colombia).

MATERIALS AND METHODS

Ethical considerations. This study was accepted by the Committee of ethics for animal experimentation of the Universidad de Antioquia (Act # 91, September 25th, 2014).

Type of study, population and location. A cross-sectional study was carried out in May 2015 in an adult buffalo herd (>2 years old; n=21) in a farm located in the municipality of Gómez Plata, province of Antioquia (Colombia), at 1,080 m.a.s.l. The farm has an area of 84 hectares and is located in a highly-humid mountain forest zone, with an annual average temperature of 25°C, a relative humidity of 80%, and annual rainfall of 1,800 mm (12). In addition to buffaloes, the herd also breeds seedstock cattle of the Colombian creole breed Colombian Black-Ear White cattle (Blanco OrejiNegro-BON) and its crossbreed with Holstein. In a previous study, it was determined that 4% (2/50) of the adult Colombian Black-Ear White cattle population of this farm was MAP-seropositive by ELISA.

Samples collection. Blood samples were taken from 20 of the 21 adult buffaloes of study (since one of the buffaloes was not blood-tested because of difficult handling). The blood sample was taken from the coccygeal or jugular vein, previously disinfected with antiseptic alcohol, and then collected using Vacuette® tubes (Kremsmunster, Kremsmünster. Austria). The blood samples were left at room temperature to ease the clot formation. Afterwards, the blood

serum was separated from the cell components using centrifugation at 2.000 r.p.m during 5 min. The serum was stored at 4ºC until analysis (12-24 h later). A 20 g fecal sample was taken from each of the 21 adult buffaloes. The sample was taken directly from the rectum using an individual new latex glove, collected in a sterile plastic container.

ELISA test. For the serological analysis, a commercial pre-absorbed ELISA kit (ID Screen® paratuberculosis Indirect, ID-Vet, Grabels, France) was used.

Fecal culture. The fecal samples were stored at 4ºC until processing (12-24 h later). Fecal samples were then decontaminated according to a procedure previously published (13). Briefly, 30 ml of a 0.75% hexadecylpyridinium chloride solution (HPC) were added to 3 g of feces in a sterile 50 ml Falcon tube, then mixed by agitation and left in a vertical position for 5 min to allow precipitation and sedimentation of large particles. Later, 20 ml of supernatant was taken from the solution, which was transferred to a new 50 ml sterile Falcon tube, and left in a vertical position during 24 hours at room temperature and in the dark. After 24 hours, the tubes were centrifuged at 900 × g for 30 min. The supernatant was discarded and 300 µl of the decontaminated pellet was inoculated in duplicate in slants of the Herrold's egg yolk medium (HEYM) with mycobactin J (Becton Dickinson, Sparks, New Jersey, USA). The inoculated tubes were incubated at 37ºC during 20 weeks and checked weekly to verify bacterial growth.

IS*900*-PCR. The DNA from MAP-compatible colonies was extracted using a commercial kit (InnuPREP Bacteria DNA Kit®, Analytic Jena, Jena, Germany). Molecular confirmation was carried out using a conventional PCR for the detection of IS*900* (IS*900*-PCR), using the primers TJ1 (GCT GAT CGC CTT GCT CAT) and TJ2 (CGG GAG TTT GGT AGC CAG TA), according to Bull et al (14) with some modifications on temperature and time conditions of the thermal cycler: 1 cycle of 95°C for 10 min and then 35 cycles of 94°C for 30 s, 60°C for 130 s and 72°C for 30 s, followed by 1 cycle of 72°C for 5 min. For the visualization of the 355 bp product of the IS*900*-PCR, a 1.5% agarose gel was used.

IS*900*-qPCR. Prior to sub-typing, the positive DNA was retested using a duplex MAP-IS*900*-quantative PCR (IS*900*-qPCR) (Bactotype MAP

PCR Kit®, Qiagen, Leipzig, Germany), including an internal amplification control and a MAP-positive and negative controls. The total reaction volume was 25.2 µl (17 µl of master mix, 8 µl of sample, and 0.2 µl of the IAC, diluted at 1:5 in ultrapure DNase- and RNase-free distilled water).

MAP subtyping. For the molecular characterization of MAP isolates, the DNA extracted from the HEYM culture and defined as MAP-positive by IS*900*-PCR and IS*900*-qPCR was used. A combination of two different sub-typing methods, both based on PCR-amplification of repetitive elements of MAP genome, was applied.

Mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR). As the first genotyping method, it was carried out by amplifying eight MIRU-VNTR loci 3, 7, 10, 25, 32, 47, 292, and X3 (alias 1658), as well as, PCR conditions as previously reported by Thibault et al (18). The PCR conditions included 1 cycle of 3 min at 95°C, 1 cycle of 1 min at 95°C, 30 cycles of 1 min s at 58°C, 1 cycle of 1 min at 72°C, and 10 min at 72°C. Molecular weight (MW) was determined of each PCR product and to estimate the number of tandem repeats present in each locus, 10 µl of PCR product was loaded in a 2% agarose gel. In addition, 50 bp ladder was used to determine the MW. Gel Doc TM imager (BioRad) was used. The results were expressed by an octal code and the genotype pattern (INMV) was determined using the international free access on-line database (http://mac-inmv. tours.inra.fr/). MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus. MAP K10 strain was used as the reference control.

Multilocus short sequence repeat (MLSSR). As the second genotyping method, it was carried out by amplification of four short sequence repeats (SSR) in locus 1 (g-repeats), locus 2 (g-repeats), locus 8 (ggt-repeats), and locus 9 (tgc-repeats), as well as, PCR conditions as previously reported by Amonsin et al (19)di-, and trinucleotide repeat sequences dispersed throughout the M. paratuberculosis genome, of which 78 were perfect repeats. Comparative nucleotide sequencing of the 78 loci of six M. paratuberculosis isolates from different host species and geographic locations identified a subset of 11 polymorphic short sequence repeats (SSRs. The PCR conditions included 1 cycle of 3 min at 95°C, 1 cycle of 1 min at 95°C, 35 cycles of 1 min s at 60°C, 1 cycle of 2 min at

72°C, and 7 min at 72°C. PCR products were analyzed by electrophoresis using 1.5% agarose gels (agarose electrophoresis grade; TransGen Biotech). All amplicons of every locus were purified using the MinElute® PCR Purification Kit (Qiagen, Leipzig, Germany) and sequenced independently (Genomics unit, Biotechnology Laboratory, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina). The quality of sequencing and the number of short repeat units to identify the alleles were analyzed using the Sequencing Analysis Software v5.4 (Applied Biosystems). MLSSR genotypes were expressed as the combination of the number of repeats found in the four loci amplified by PCR. If the number of g-repeats at locus 2 was higher than 11, g-repeats for such locus were denoted as >11g as previously suggested (15). Sub-typing procedures were carried out at the Institute of Agrobiotechnology and Molecular Biology (IABIMO)-CONICET, INTA in Buenos Aires, Argentina.

Information collection. A questionnaire at the moment of blood and fecal sampling was administered to the herd manager in a face-to-face interview, to collect information on animal-level factors [i.e. sex, age, body condition score —BCS, by direct observation according to Alapati et al (16), daily milk yield, origin —born in or outside the herd, reproductive status —pregnant or empty, months after calving]. Information collected was entered into an Excel worksheet (Microsoft Corp., Redmond, WA, USA) and then exported to Stata 14.0 (StataCorp, 2017, Texas, USA) for analysis. Descriptive statistics were reported using percentages. Chi-square test was applied to explore associations between the animal-level associated factors and the serological result.

RESULTS

At the time of sampling, the age of the buffaloes was 4.7 years in average. A 90.5% (19/21) were females and 84.2% (16/19) were pregnant, and 66.7% of the buffaloes (14/21) were born outside the herd (external origin). The average BCS was 4.2 (in a 1-to-5 scale). The average time since the last calving was 7.4 months, and the average daily milk yield was 3.5 L (Table 1).

The ELISA did not detect MAP-positive or suspicious results in the 20 serum samples of analysis. Therefore, the animal-level factors were not able to be explored for associations.

One out of 21 fecal samples (4.8%) showed MAP-compatible colonies (i.e. white, circular, shiny, raised and convex) on HEYM at week 6, and was confirmed as MAP by IS*900*-conventional PCR (Figure 1), and subsequently, by IS*900*-qPCR as previously mentioned in the methodology. The buffalo from which the positive fecal sample was obtained was pregnant and apparently healthy at the time of sampling, and it corresponded to the animal from which it was not possible to collect the blood sample.
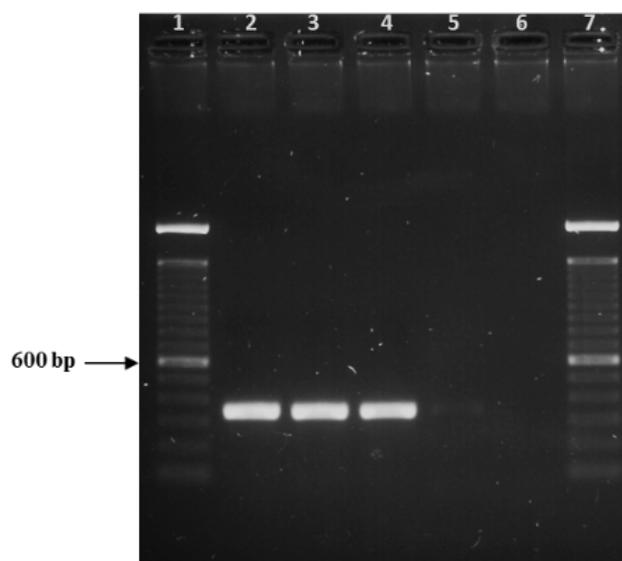

Figure 1. PCR results for the detection of the *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-specific IS*900* insertion sequence (14). Lane 1 and 7: Molecular marker (100 bp ladder), Lane 2: Suspicious fecal sample from the dairy buffalo cow #1, Lane 3: ATCC reference strain, Lane 4: Cow confirmed as MAP-positive from a previous study (17), Lane 5: Negative DNA-extraction control, Lane 6: Negative PCR control.

To the sub-typing, the MAP isolate was found to be INMV 3 by MIRU-VNTR (numerical code: 32332218; Table 2). The MLSSR result did not show any differential profile.

Table 1. Animal-level factors and *Mycobacterium avium* susp. *paratuberculosis* (MAP) diagnostic results of 21 buffalos of a herd in Colombia (2015).

Sex	#	Age (years)	BCS	Daily milk yield (L)	Origin	Reproductive status	Months after calving	Results Culture, IS900-PCR	ELISA
Female	1	6.8	4.0	4.5	E	Pregnant	8.8	Positive	Not performed
	2	6.6	4.3	2.0	E	Pregnant	8.0	Negative	Negative
	3	6.3	4.3	4.0	E	Pregnant	7.8	Negative	Negative
	4	5.9	3.8	4.5	E	Pregnant	7.0	Negative	Negative
	5	5.7	4.0	5.5	E	Pregnant	7.0	Negative	Negative
	6	5.7	4.0	4.0	E	Pregnant	10.0	Negative	Negative
	7	5.7	4.0	8.5	E	Empty	19.0	Negative	Negative
	8	5.7	4.0	0.0	E	Pregnant	7.0	Negative	Negative
	9	5.5	4.3	4.5	E	Pregnant	0.0	Negative	Negative
	10	5.4	4.0	3.5	E	Pregnant	8.0	Negative	Negative
	11	5.2	4.0	7.5	E	Empty	17.0	Negative	Negative
	12	5.2	3.8	7.5	E	Pregnant	7.0	Negative	Negative
	13	5.1	4.3	6.0	E	Pregnant	6.0	Negative	Negative
	14	5.0	4.3	0.0	E	Pregnant	6.8	Negative	Negative
	15	3.2	4.5	0.0	I	Pregnant	0.0	Negative	Negative
	16	3.0	4.3	4.0	I	Empty	0.0	Negative	Negative
	17	2.8	4.3	0.0	I	Pregnant	0.0	Negative	Negative
	18	2.8	4.5	0.0	I	Pregnant	0.0	Negative	Negative
	19	2.7	4.5	0.0	I	Pregnant	0.0	Negative	Negative
Male	20	2.7	4.5	NA[2]	I	NA	NA	Negative	Negative
	21	2.7	4.0	NA	I	NA	NA	Negative	Negative

BCS: Body condition score obtained by direct observation according to Alapati et al (16); E: External; I: Internal; HEYM: Herrold's egg yolk medium; PCR: Polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; NA: not applicable.

Table 2. MIRU-VNTR and MLSSR profiles of a *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolate obtained from a buffalo cow of a herd in Colombia (2015).

Number of copies of MIRU-VNTR								INMV profile	Number of copies of SSR Loci			
3	7	10	25	32	47	292	X3 (1658)		1 (g)	2 (g)	8 (ggt)	9 (tgc)
2	2	1	3	8	3	3	2	3	7	9	5	5

INMV profile performed according to Thibault et al (18); MLSSR profiles performed according to Amonsin et al (19).

DISCUSSION

In the present study, MAP was detected in a buffalo herd located in one municipality of Antioquia (Colombia), using serological, microbiological, and molecular approaches.

Concerning the animal-level factors considered in the present study, most of the sampled animals were born outside the herd, including the only buffalo cow found to be MAP-positive by fecal culture. Hence, it might be hypothesized that they were not infected at the herd. However, the presence of infected bovines in the same herd might indicate that MAP could be circulating between both populations.

In the present study, serum-ELISA did not detect anti-MAP antibodies in the buffalo population of interest. Therefore, none of the animal-level factors of interest were able to be explored for association with MAP-serostatus (i.e. sex, age, BCS, daily milk yield, origin, reproductive status, months after calving). A previous study found an association between MAP-seropositive status to a buffalo-herd management factor, such as the absence of a calving area or the presence of cow-calf systems (8). Future studies in the national buffalo population could indicate whether this or other herd-management or animal-level factors may be considered as risk factors for MAP-infection in Colombia.

The negative results obtained by the ELISA test contrast with previous studies performed in buffaloes from Italy, Brazil, and India, in which a seroprevalence of 4, 10.3, and 28.6% was found, respectively (5,6,8). Such differences can be due to factors such as sample size —lower for the present study, reducing the probability of detecting seropositive animals, and the low Se reported for the ELISA test in asymptomatic animals. Serum and milk ELISA Se in bovine are approximately 25.6-45.3% and its Sp are higher (97.6-98.9%) (20). This is the main reason to obtain false-negative animals (21).

MAP was isolated from one fecal culture and was then confirmed by IS*900*-PCR and IS*900*-qPCR. The absence of ELISA analysis of this only buffalo cow did not allow to fully confirm its immunological status. This animal could represent the case of the *pass-through* shedding phenomenon, in which animals that were not infected at early stages of life can ingest significant amounts of MAP from the environment and then eliminate them through feces (22) The source of MAP in the farm could be the buffalo population or the infected bovine cattle which coexist and graze on the same pastures.

Other studies on MAP in buffaloes using serological and molecular methods have been reported in Philippines and Brazil, showing variations between their estimations and the present study (23,24). These variations can be due to the sample inherent characteristics and to the collection method, as well as other population-related factors. It must be kept in mind that, in the subclinical cases of the infection, the elimination of MAP is intermittent and it is possible that the bacterial charge was reduced or absent at the moment of the sample collection, underestimating the serological and culture outcomes (3). Thus, there are reports of subclinical PTB presentation in buffaloes, as mentioned by de Moraes et al (4), who found 30% (31/115) samples of intestinal tissues with histopathological lesions compatible with PTB of slaughtered buffaloes without obvious clinical signs. Also, MAP was isolated in 4.3% (5/115) by fecal culture and 13% (15/115) by IS*900*-qPCR.

The MIRU-VNTR profile found in the present study (INMV 3) has been previously reported in cattle in the world (15,18,25,26,27,28). However, no report has been done in buffalo and conclusions regarding molecular epidemiology of MAP based on subtyping results are difficult to make because of the use of different technical approaches and different loci for analysis.

In conclusion, MAP was isolated from a fecal sample of a dairy buffalo cow of a herd of 21 MAP-ELISA negative adult buffaloes. Sub-typing analysis revealed that the MAP isolate corresponded to the INMV 3 genotype by MIRU-VNTR. For the authors, this is the first report of MAP diagnosis and isolation in Colombian buffaloes and the first time an INMV 3 MAP-profile is found in this species the country.

Conflict of interests

The authors declare that there is no conflict of interest for the publication of this article.

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