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Original

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

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ABSTRACT

Objective. To determine *Mycobacterium avium* subsp. *paratuberculosis* (MAP) molecular diversity in environmental samples from Colombian dairy herds. **Materials and methods.** Environmental samples from 25 IS*900*-qPCR MAP-positive dairy herds were cultured by duplicate in Herrold´s egg yolk medium with mycobactin J to obtain isolates. Suspicious colonies were confirmed by MAP-IS*900*-qPCR. Positive DNA was sub-typed using mycobacterial interspersed repetitive units-variable number of tandem repeat (MIRU-VNTR) and multilocus short sequence repeats (MLSSR) techniques to analyze the genetic differences between the isolates. **Results.** Sub-typing revealed two different genotypes by MIRU-VNTR (INMV 2 and INMV 36). MLSSR technique was carried out to increase the discriminatory power from what was obtained by MIRU-VNTR, but no differences were observed among the recovered isolates. **Conclusions.** The present study represents an important approach to the knowledge on MAP epidemiological status in the study population.

Keywords: Genetics; genotyping; Johne's disease; MLSSR; MIRU-VNTR (*Source*: *National Agricultural Library Thesaurus*).

RESUMEN

Objetivo. Determinar la diversidad molecular de *Mycobacterium avium* subsp. *paratuberculosis* (MAP) en muestras ambientales de hatos lecheros colombianos. **Materiales y métodos.** Las muestras ambientales de 25 hatos lecheros positivos a MAP por IS*900*-qPCR se cultivaron por duplicado en medio de yema de huevo de Herrold con micobactina J para obtener aislamientos. Las colonias sospechosas fueron confirmadas para MAP por IS*900*-qPCR. El ADN positivo se subtipó utilizando técnicas de unidades micobacterialess repetitivas intercaladas - número variable de repeticiones en tándem (MIRU-VNTR) y técnicas de repeticiones de multilocus de secuencia corta (MLSSR) para analizar las diferencias genéticas entre los aislamientos. **Resultados.** El subtipado reveló dos genotipos diferentes por MIRU-VNTR (INMV 2 e INMV 36). La técnica de MLSSR se realizó para aumentar el poder discriminatorio de lo obtenido por MIRU-VNTR, pero no se observaron diferencias entre los

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aislamientos recuperados. **Conclusiones.** El presente estudio representa un enfoque importante para el conocimiento del estatus epidemiológico de MAP en la población de estudio.

Palabras clave: Genética; genotipado; enfermedad de Johne; MLSSR; MIRU-VNTR (*Fuente*: *National Agricultural Library Thesaurus*).

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a pathogen that affects several animal species and, under *in vitro* conditions, is dependent on mycobactin, and extremely slow growing. In susceptible animals, MAP infection leads to a chronic granulomatous enteritis, known as paratuberculosis (PTB) or Johne's disease (1). Clinical signs of PTB include diarrhea, weight loss, decreased milk and meat production, and mortality, leading to important economic losses (2). A major concern about MAP infection is the ease with which the bacterium spreads, since subclinical or clinically infected animals shed MAP in feces and milk, enabling dissemination to susceptible young animals, the environment, to food for human consumption (3). MAP-containing milk and meat are of particular concern because the bacterium has been suggested as possibly associated with Crohn's disease in humans (4).

Molecular discrimination of MAP from field isolates become a crucial tool to complement the general knowledge on MAP, its distribution, behavior, and characteristics (5,6). The use of molecular sub-typing methods for MAP has increased in the last two decades (7,8). MAP sub-typing is a useful tool in epidemiological research, offering a better understanding of MAP-infection origin, dynamics, associated risk factors, transmission profiles, pathogenesis, among other related features, allowing a rational design of adequate control measures, diagnosis improvement, and vaccine development (7,9). Nevertheless, sub-typing of MAP strains represents a challenge since they are genetically monomorphic and traditional molecular techniques have limited discriminatory power. The advances and availability of whole-genome sequencing have extended possibilities for the characterization of MAP, providing a phylogenetic context to enhance global epidemiology studies (10).

Sub-typing techniques include mycobacterial interspersed repetitive units-variable number of tandem repeat (MIRU-VNTR), which are based on the polymorphism of repetitive elements, and multilocus short sequence repeats (MLSSR), consisting on the detection of simpler tracts of 2-5 bp tandem repeats (11,12). The combination of methods targeting different genomic MAP-structures (i.e. MIRU-VNTR, MLSSR) has been reported to increase the discriminatory ability of combined methods compared to the ability of each method used separately (5,13,14).

Some knowledge about PTB has been achieved over time in Colombia, however, information about the impact and distribution of the disease, as well as its molecular epidemiology in the country is still limited, even being a mandatory reporting disease in the since 2015 (15,16). Therefore, this study aimed to determine MAP molecular diversity in isolates obtained from environmental samples of dairy herds located in the Northern region of the Province of Antioquia (Colombia) by MIRU-VNTR and MLSSR techniques.

MATERIALS AND METHODS

Study herds. The present study was carried out in 25 dairy herds previously detected as MAP-infected by IS*900*-qPCR on environmental samples. The infected herds were detected from a population of 386 dairy herds located in 62 districts of six different municipalities in the Northern region of the Province of Antioquia (Colombia), sampled during July and October, 2016. The 25 infected herds of study were located in six different districts in three different municipalities. Each participating herd was visited once during the study period to collect two composite environmental samples. The first one contained material from at least six different sites (sub-samples) of concentration of adult cattle and/or high traffic areas (e.g. paddocks, areas surrounding waterers and feeders, alleyways, gutters, milking parlor-holding areas). The second one contained manure from the milking parlor collected in the manure storage lagoon, after mixing its content for at least 5 min. The six sub-samples from the second place were obtained from different places of the perimeter of the lagoon by submerging the sampling container up to 10 cm beneath the surface. The final composite samples were approximately of 20 g, kept under refrigeration at 4°C during

transport back to the laboratory and, in once there, homogenized and then frozen at -20°C until DNA extraction. The DNA extraction was carried out using a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, CA, USA). The protocol included a bead-beating *prior* step (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA). The extracted DNA was analyzed using a commercial duplex IS*900*-qPCR kit (Bactotype MAP PCR Kit®, Qiagen, Leipzig, Germany).

Culture. Environmental samples from the IS*900*-qPCR-positive herds were decontaminated with 0.75% (w/v) hexadecylpyridinium chloride solution (HPC) for 24 h, according to standard procedures (15). The inoculums were cultured in Herrold´s egg yolk medium (HEYM) with mycobactin J by duplicate. All culture media were incubated at 37°C for 24 weeks and were checked weekly for mycobacterial growth or contamination with undesirable germs. MAP growth was visually monitored for typical slow growth rate and colony morphology according to previous descriptions (colonies developing after ≥ 3 weeks of incubation, initially round, smooth and white, tending to heap up slightly and becoming dull light yellow with wrinkling of the surface). Colonies were sub-cultured in HEYM and those showing MAP compatible growth were considered as suspicious. All isolates were subjected to confirmatory IS*900*-qPCR (Bactotype MAP PCR Kit®, Qiagen, Leipzig, Germany) to confirm the identity of the isolates. Decontamination, as well as culture procedures, were carried out in the diagnostic unit at the Facultad de Ciencias Agrarias, Universidad de Antioquia, in Medellín (Colombia).

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

MIRU-VNTR. The procedure was carried out by amplifying eight MIRU-VNTR loci 3, 7, 10, 25, 32, 47, 292, and X3 (alias 1658) using the PCR conditions previously reported (12), with slight modifications in the PCR conditions. The PCR mixture was composed of 2 µL from tenfold-diluted DNA solution added to a final volume of 50 µL containing 0.25 µL of GoTaq® DNA polymerase (Promega; 5 U/µL), 2 µL of betaine (Sigma), dTTP (Qiagen, Leipzig, Germany), 5 µL of PCR buffer (supplied by the manufacturer), 1 µM of primers, and 2 mM of $MgCl_2$. The reactions were carried out using a SimpliAmp thermal cycler (Applied Biosystems, Darmstadt, Germany). The PCR conditions included 1 cycle of 3 min at 95°C, 1 cycle of 1 min at 95°C, 30 cycles of 1 min at 58°C, 1 cycle of 1 min at 72°C, and 10 min at 72°C. To determine the molecular weight of each PCR product and to estimate the number of tandem repeats present in each loci, 10 µl of PCR product was loaded in a 2% agarose gel and a 50 bp ladder. To digitalize the gel, the Gel Doc TM imager (BioRad) was used. The results were expressed by an octal code and the genotype pattern (INMV) was determined using the international free access on-line database (http://mac-inmv.tours.inra.fr/). MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus. MAP K10 strain was used as the reference control.

MLSSR. The procedure was carried out by amplification of four short sequence repeats (SSR) loci, locus 1 (g-repeats), locus 2 (g-repeats), locus 8 (ggt-repeats), and locus 9 (tgc-repeats) using PCR conditions as previously reported (11), with slight modifications in the PCR conditions. The selection was carried out by selecting the loci with the highest discriminatory index (11). The PCR mixture was composed of 2 µL from tenfold-diluted DNA solution added to a final volume of 50 µL containing 0.5 µL of GoTaq® DNA polymerase (Promega; 5 U/µL), dTTP (Qiagen, Leipzig, Germany), 10 µL of PCR buffer (supplied by the manufacturer), 1 µM of primers, and 2 mM of MgCl2. The reactions were carried out using a SimpliAmp thermal cycler (Applied Biosystems, Darmstadt, Germany). The PCR conditions included 1 cycle of 3 min at 95°C, 1 cycle of 1 min at 95°C, 35 cycles of 1 min s at 60°C, 1 cycle of 2 min at 72°C, and 7 min at 72°C. PCR products were analyzed by electrophoresis using 1.5% agarose gels (agarose electrophoresis grade; TransGen Biotech). All amplicons of every locus were purified using the MinElute® PCR Purification Kit (Qiagen, Leipzig, Germany) and sequenced independently (Genomics unit, Biotechnology Laboratory, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina). The quality of sequencing and the number of short repeat units to identify the alleles were analyzed using the Sequencing Analysis Software v5.4 (Applied Biosystems). MLSSR genotypes were expressed as the combination of the number of repeats found in the four loci amplified by PCR. If the number of g-repeats at locus 2 was higher than 11, g-repeats for such locus were denoted as >11g as previously suggested (13). Sub-typing procedures were carried out at the

Institute of Agrobiotechnology and Molecular Biology (IABIMO) INTA-CONICET in Buenos Aires, Argentina.

RESULTS

Of the 25 MAP-IS*900*-qPCR positive environmental samples, 13 showed MAP-compatible growth in one or both HEYM slants, six of which were confirmed as MAP by IS*900*-qPCR. All isolates grew within 8-15 weeks of incubation. In total, three MAP isolates recovered from environmental samples collected from the wastewater lagoon from three different herds were suitable for MAP sub-typing. The three isolates revealed two different genotypes by MIRU-VNTR [INMV 2 (numerical code: 32332228) and INMV 36 (numerical code: 32342228)]. MLSSR was carried out to increase the discriminatory power, but no differences were observed, as it can be seen in Table 1. Nevertheless, MLSSR profile obtained from herd 3 may correspond to MLSSR 50 (numerical code: 711555455455) according to previous reports (13).

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

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

DISCUSSION

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

Thirteen of the cultures showed MAP compatible growth, six of them were confirmed as MAP by IS*900*-qPCR, and of these, three different isolates were suitable for MAP sub-typing. The low recovery rate of the bacteria could be due to the fact that the samples were frozen at -20°C for a maximum of 56 days, which could affect the sensitivity of the detection (17). In accordance with the characteristics reported in the literature regarding color, appearance, and differential growth rate of MAP (17), compatible isolates were considered, although they were not subsequently confirmed from the molecular test. This can occur due to unfavorable conditions related to DNA quality, mainly as a consequence of transport and preservation processes of the samples, as well as the DNA extraction processes, considering that the typing tests used require a defined quality of the source DNA. (11). Additionally, no genetic poly-diversity was found in any of the samples from the techniques used. This may, hypothetically, be due to chance at the time of washing the media, which would correspond to the analysis matrix.

Although comparisons with other studies are very difficult because of the use of different loci for analysis, MIRU-VNTR profiles 1 (INMV 1) and 2 (INMV 2) has been previously reported as the most common genotypes found in isolates from other Latin-American countries (12,18,19,20), as well as from European isolates (5,21,22,23). Nonetheless, our findings cannot be directly compared with those previously reported (18), since we considered eight MIRU-VNTR loci and

four SSRs and they used eleven loci [1, 3, 7, 4, 10, 25, 32, 47, 259, 292, and X3 —alias 1658] and three SSRs [1, 2, and 8], respectively.

On the other hand, some reports using the same loci/SSRs we used have been previously published. The INMV 2 profile was reported in an isolate from cattle feces (24). Seven different INMV profiles, including INMV 2 in cattle and goats was reported from Argentinian and Mexican isolates (19). The INMV 2 was reported as the second most common profile found in stool and/or intestinal mucosa from cattle and humans in Argentina, also concluding that INMV 2 was the original clone from which the others derive (20). According to MLSSR profiles reported herein, the sub-types isolated in our study are commonly found in cattle and other species in different countries (13,18,25). Interestingly, a bovine isolate from Colombia's neighbor country Venezuela has shown a different genotype by SSR (11g-10g-5ggt-5ggt), suggesting strain diversity in the Northern part of South America (13).

At a national scale, a molecular characterization by MIRU-VNTR and MLSSR of eight MAP isolates (obtained from feces, tissue, and wastewater lagoons) from five dairy herds in the Northern region of Antioquia was reported, already defined as MAP-infected according to direct and indirect testing (18). Authors revealed two different combined-strain profiles (1A and 2B), being the first molecular characterization of MAP in Colombia to that date. The findings presented herein cannot be compared with those previously reported in the country (18), since we considered eight MIRU-VNTR loci and they used twelve [1, 2, 3, 4, 7, 10, 25, 32, 47, 259, 292, and X3 —alias 1658]. Nevertheless, authors considered the same four SSRs we did, reporting the MLSSR-genotype B [7-g, 10-g, 5-ggt, 4-tgc], obtained from tissue and wastewater lagoon samples.

As a conclusion, and based on the sub-typing patterns of the MAP isolates obtained herein, from 386 dairy herds located in the Northern dairy region of Antioquia (Colombia), we found different MAP genotypes circulating in the study region: INMV 2 and INMV 36 according to MIRU-VNTR analysis. The isolates with identical INMVs, from two different municipalities, could not be discriminated by MLSSR. The findings of this study lead to important epidemiological implications concerning control and prevention of PTB in Colombia. It is suggested that newer typing methods, such as single nucleotide polymorphism (SNP), which is capable of detecting differences among major types may be used to obtain further discernment into the epidemiological features of MAP in the country, including the influence of culture media, the role played by the local wildlife, the diversity of agro-ecosystems, and the crossbreeding of imported and indigenous animals to be taken into account in the analysis as possible sources of genomic diversity of MAP.

Conflict of interest

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

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