[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]

Original Article

# Genetic Diversity of *Mycobacterium avium* sp. Paratuberculosis by Mycobacterial Interspersed Repetitive Unit–Variable Number Tandem Repeat and Multi-Locus Short-Sequence Repeat One-Sentence Summary: Genetic Diversity of *Mycobacterium avium* sp. Paratuberculosis Isolates

**Roberto Damián Moyano[1], Belén Rocío Imperiale[2], Magalí Romero[3], María de la Paz Santangelo[1], María Fiorella Alvarado Pinedo[3], Gabriel Travería[3], María Isabel Romano[1]**

[1]Institute of Agrobiotechnology and Molecular Biology (IABIMO)-CONICET, INTA De Los Reseros y Las Cabañas S/N, Hurlingham, [2]Institute of Experimental Medicine (IMEX)-CONICET, National Academy of Medicine. Pacheco de Melo, [3]Faculty of Veterinary Sciences, National University of La Plata, CEDIVE, Buenos Aires, Argentina

Dr. Santangelo and Dr. Imperiale contributed equally to the manuscript.

## Abstract

**Background:** Paratuberculosis is an enteric disease caused by Mycobacterium avium sp. paratuberculosis (MAP) that affects mainly ruminant producing losses to the livestock industry. Many molecular epidemiological methods have been used to discriminate MAP isolates. **Method:** The aim of this study was to describe the genetic diversity of the Argentinean MAP isolates using a combination of two molecular systems, the mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) ("automated and "non-automated") and the multi-locus short-sequence repeat (MLSSR) system. **Results:** Thirty-two isolates were identified as MAP of C type by IS900 polymerase chain reaction (PCA) and IS1311 PCA-restriction enzyme analysis. The main patterns found by both MIRU-VNTR systems were INMV1 (54.5%), INMV2 (24.2%) and INMV11 (9.1%). The INMV5, INMV8 and INMV16 were represented with one isolate each (3.0%). Only 4 MIRU-VNTR loci were polymorphic. **Conclusion:** Those isolates sharing the same INMV patterns were analyzed by MLSSR, being locus 2 the most polymorphic one showing isolates with 9, 10, 11, and more than 11 "G" repeats. Besides, the global discriminatory power among isolates could be increased using both techniques. Based on these results, a short version of the "automated" MIRU-VNTR could be used as a screening tool to group isolates genetically related and subsequently perform the SSR using locus 2 on those isolates sharing the same INMV pattern.

**Keywords:** Automated mycobacterial interspersed repetitive unit–variable number tandem repeat typing, genetic diversity, multi-locus short-sequence repeat typing, *Mycobacterium avium* sp. paratuberculosis

**Submitted:** 03-Dec-2020 **Accepted:** 13-Jan-2021 **Published:** 28-Feb-2021

## Introduction

Johne's disease or paratuberculosis (PTB) is an enteric disease caused by *Mycobacterium avium* sp. *Paratuberculosis* (MAP) that affects mainly ruminants. PTB occurs worldwide, is highly prevalent in many countries and produces significant economic losses associated to the livestock industry.[1] The main clinical signs of PTB in cattle are diarrhea, weight loss, and edema because of hypoproteinemia caused by protein-losing enteropathy.[2] Besides, MAP may have a potential role in human Crohn disease but this postulation remains controversial.[3-6]

Access this article online

Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_229_20

**Address for correspondence:** Dr. Roberto Damián Moyano, Institute of Agrobiotechnology and Molecular Biology (IABIMO)-CONICET, INTA. De Los Reseros y Las Cabañas S/N, Hurlingham, Buenos Aires, Argentina. E-mail: moyanord@gmail.com

**ORCID:** Roberto Damián Moyano: 0000-0002-2133-6457

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

**For reprints contact:** WKHLRPMedknow_reprints@wolterskluwer.com

**How to cite this article:** Moyano RD, Imperiale BR, Romero M, Santangelo MP, Pinedo MF, Travería G, *et al.* Genetic diversity of *Mycobacterium avium* sp. paratuberculosis by mycobacterial interspersed repetitive unit–variable number tandem repeat and multi-locus short-sequence repeat one-sentence summary: Genetic diversity of *Mycobacterium avium* sp. paratuberculosis isolates. Int J Mycobacteriol 2021;10:51-9.

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



MAP isolates can be discriminated in different lineages depending on the animal from which it was isolated: S type (Sheep-type); C type (Cattle-type); and B type (Bison-type).[6,7]

The development of a rapid and simple molecular epidemiological method is needed to describe PTB outbreaks and to determine the epidemiological situation from a particular geographical region or country including disease surveillance investigation. Epidemiological studies have included different molecular techniques to discriminate among MAP isolates, establish evolutionary relationships among them and determine the population structure.

Genetic diversity of MAP isolates has been performed through several molecular tools using different genetic markers such as insertion elements, repetitive sequences, and single nucleotide polymorphisms. Restriction fragment length polymorphism of Insertion Sequences (IS900-RFLP, IS1245-RFLP), pulsed-field gel electrophoresis, mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) analysis, and multi-locus short-sequence repeat (MLSSR) analysis have been extensively used to study the diversity of MAP.[8-10] All these techniques have different discriminatory power among MAP isolates and the combination of them may increase discrimination.[11]

Although IS*900*-RFLP analysis was a very extensively used method for typing MAP isolates, it is not easy to perform because MAP is a very slowly growing microorganism and is difficult to obtained the high amounts of DNA required for this technique.[12]

The MLSSR system is based on the search of short sequence repeats or microsatellites in multiple loci and could be used to discriminate MAP isolates.[13-15] These loci, could be used to analyze the sequence of simple homopolymeric tracts of single, di- or trinucleotides.[16-18] Several studies analyzed some locus markers to determine the genetic diversity of MAP. These locus were: 1 (Locus: 1793091), 2 (Locus: 2719085), 3 (Locus: 607784;), 4 (Locus: 3406364), 5 (Locus: 3735342), 6 (Locus: 4286068), 7 (Locus: 4310932), 8 (Locus: 1028129), 9 (Locus: 2955362), 10 (Locus: 3558075); and 11 (Locus: 1536798). The position of these loci in the genome is the coordinate of the SSR locus in the *M. paratuberculosis* strain K10 genome (GenBank accession number AE016958). However, it was demonstrated in previous studies that loci 1 (position in the genome of the SSR: 1793091-1793109), 2 (position in the genome of the SSR: 2719085-2719094), 8 (position in the genome of the SSR: 1028129-1028145), and 9 (position in the genome of the SSR: 2955362-2955378) were the most polymorphic loci having a high discriminatory power.[16,18]

The genotyping using MLSSR consisted in a polymerase chain reaction (PCR) of the specific loci and subsequent sequencing. It yields results that are easy to interpret and highly reproducible. MLSSR typing is expressed as numerical genotypes, reflecting the repeat copy numbers in the respective loci.[12,14] MLSSR has also been reported as fragment analysis because some authors analyze several loci simultaneously.[19] Besides, the stability of the SSR loci is inferred by the repeatability of the strain genotype. While L1, L8, and L9 were reported as the most stables, L2 was reported as not sufficiently stable by some authors. Nevertheless, L2 was included to be tested in our study for being a highly polymorphic locus[16,19,20] and also, there are reports that could differentiate two MAP strains (one virulent and the other one an attenuated strain) with the same MIRU pattern (INMV2) using SSR L2.[21]

Although as it was suggested previously,[18] reading of repetitive sequence requires some expertise to avoid mistakes. Besides, in order to avoid interpretation errors, alleles with more than 11 repeats should be assigned as alleles as >11, despite some loss of information to be a reliable interpretation.

Multiple-locus variable-number tandem repeat analysis is a fast, PCR-based method previously described for typing bacteria. A specific typing method based on mycobacteria repetitive elements, called mycobacterial interspersed repetitive-unit-variable-number tandem repeats (MIRU-VNTRs) have been used for genotyping different mycobacteria species. This system also expressed the different genotypes as a numerical code.[22-26]

We have previously reported a MIRU-VNTR system, to genotype MAP isolates, based on eight different MIRU-VNTR loci described by Thibault *et al*. 2007. This system allowed us to discriminate among Argentinean MAP isolates with a relative acceptable discriminatory power (Hunter and Gaston discriminatory index [HGDI]: 0.7).[10,24] Although it is a PCR based method, each one of the PCR should be performed individually and the detection system used is through an agarose gel using a molecular weight (MW) market to determine the MW of each PCR product.

An alternative of this MIRU-VNTR system, using primers labeled with different fluorochromes would allow to perform multiplex-allele-specific-PCR (MAS-PCR) of each MIRU-VNTR loci and to detect the MW of products by capillary electrophoresis using an automated sequencer (GA 3500XL, Applied Biosystems). This system allows the detection of the fragments in an automated way. Authors will call from now on: "automated" MIRU-VNTR assay and "non-automated" MIRU-VNTR assay to differentiate both systems.

At the same time, as it was previously reported, the combination of two molecular techniques such as MLSSR plus MIRU-VNTR system increase the discrimination among isolates.[16,18]

For that reason, the aim of this study was to describe the genetic diversity of the Argentinean MAP isolates using a combination of two molecular systems, the MIRU-VNTR ("automated and "non-automated") and the MLSSR system.

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



## Method

### *Mycobacterium avium* sp. paratuberculosis isolates

MAP isolates were obtained at the Veterinary Research Center (CEDIVE-National University of La Plata, UNLP) from cattle stool. The samples were homogenized, decontaminated by the hexadecylpyridinium chloride method, then loaded into Herrold medium supplemented with mycobactin J (2 mg/L) and finally incubated at 37ºC at least for 4 months.

Isolated MAPs were collected from different locations of Buenos Aires Province: Castelli, Gral Belgrano, Tandil, Chascomús, Bartolomé Bavio, Vieytes, Pehuén, Luján and Lomas de Zamora.

### DNA extraction from *Mycobacterium avium* sp. paratuberculosis

A loopful of colonies of each MAP isolate was added into different sterile water containing tubes and then the isolates were heated at 95°C for 30 min in a thermal block. After that, five freeze-thaw cycles of 1 min each were performed on liquid $N_2$. The supernatants were used for PCR.

### Identification of mycobacterial isolates

Positive mycobacterium cultures were confirmed by Ziehl–Neelsen stain and MAP was identified by IS*900* PCR. IS*1311* PCA-restriction enzyme analysis (PCR-REA) was used to discriminate MAP isolates in different lineages; S type (sheep-type), C type (cattle-type), and B type (Bisson-type).[6,24-26] MAP K10 strain was used as a reference control.

### Genotyping of *Mycobacterium avium* sp. paratuberculosis isolates

#### *"Non-automated" mycobacterial interspersed repetitive unit–variable number tandem repeat assay*

This system included the amplification of eight MIRU-VNTR loci (292, X3, 25, 47, 3, 7, 10, 32). Primers used to amplify each locus were previously reported.[10] The PCR protocol was modified in order to amplify the eight loci simultaneously using a touchdown program. Amplification cycle was: 95°C for 3 min; 9 cycles of 95°C for 30 s, 62°C (−0.5°/cycle) for 30 s, and 72°C for 30 s; followed by 30 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 7 min. The PCR protocol included 1.5 mM $MgCl_2$, 2 µl DMSO, 1.25 U Taq, 2 mM dNTPs mix, and different amounts of primers according to the locus to be amplified (25 pmoles for loci 292, 25, 10, 79, and 10 pmoles for loci 3, 47, X3, 32). MW of each PCR product and the number of tandem repeats present in each locus were determined or estimated by loading 10 µl of PCR product in a 2% agarose gel. MW markers (50 and 100 bp) were included in the gel. The Gel Doc TM imager (Bio-Rad) was used to digitalize the gel. The results were expressed by an octal code and the genotype pattern (INMV) was determined using the international online MAC-INMV database (http://mac-inmv.tours.inra.fr/index.php?p = nomenclature). MAP K10 strain was used as reference control.

#### *"Automated" mycobacterial interspersed repetitive unit–variable number tandem repeat assay*

This system included the amplification of the same eight MIRU-VNTR loci used in the "non-automated" system. The "automated" MIRU-VNTR system used two different MAS-PCR to amplify the eight loci. Primers used in the MAS-PCR were the same as those used in the "non-automated" MIRU-VNTR assay[10] but in this case, the primers were labeled with fluorochromes. Each MAS-PCR mix contains four different pairs of fluorochromes labeled primers (FAM, VIC, NED, PET) that emit fluorescence of different color, Table 1.

Both MAS-PCR were performed simultaneously in the same run using a touchdown program described above for the "non-automated" system. The MAS-PCR master mix 1 included 5 µl of buffer ClK 10X, 3 mM of $MgCl_2$, 1.25 U Taq, 10 mM dNTPmix, 2 µl of DMSO, and 25 pmoles of each pair of primers (loci 292, 25, 10, 7). While for mix 2, 10 pmoles of each one of the primers were used (loci 3, 47, X3, 32).

MAS-PCR products were diluted 1/20 in water and then run by capillary electrophoresis in an automated sequencer GA3500XL (Applied Biosystems, Buenos Aires, Argentina) using a MW marker labeled with a fluorochrome (GS-500 LIZ, Applied Biosystems, Buenos Aires, Argentina) that was not included in the MAS-PCR system. These runs were performed in IABIMO-CONICET, INTA. Results were analyzed using the GeneMapper 4.1 software (Applied Biosystems, Buenos Aires, Argentina) and then expressed by an octal code to determine the genotype pattern (INMV) through the international online MAC-SSR-PLUS database (http://mac-ssr.tours.inra.fr/). MAP K10 strain was used as reference control.[27,28]

### Multi-locus short-sequence repeat assay. This assay was carried out performing polymerase chain reaction amplification of different loci and then sequencing the polymerase chain reaction products

Those MAP isolates with the same INMV pattern were studied analyzing the shorts sequences repeats in multiple-loci, to determine if the MAP isolates that were previously grouped by MIRU-VNTR were really identical or not. The SSR loci, 1, 2, 8, and 9, were selected for being the most polymorphic ones with a high discriminatory power to discriminate among type C MAP isolates according to previous reports[16,18] and the INRA MAC-SSR-PLUS *Database* (http://mac-ssr.tours.inra.fr/).

There is no previous data about genotyping Argentinean MAP isolates by MLSSR, but previous reports indicated that all MAP isolates from Buenos Aires Province belonged to C type according to IS*1311*-RFLP.[24,29]

Table 2 shows the primers used to amplify each locus and the sequence of repeats in each one. These primers were previously reported.[16]

The PCR protocol included 0.6 µM of each primer, 200 µM of dNTP mix and 0.5 U of Taq, with and initial denaturation step of 3 min at 94°C, followed by 35 cycles at 95°C for

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



**Table 1: Primers used in the "automated" mycobacterial interspersed repetitive unit-variable number tandem repeat system**

| Locus | Primers (5'-3')-labeled | mT (°C) | MAS-PCR |
|---|---|---|---|
| 292 | F: CTTGAGCAGCTCGTAAAGCGT-(FAM)<br>R: GCTGTATGAGGAAGTCTATTCAT | 58 | 1 |
| 25 | F: GTCAAGGGATCGGCGAGG-(VIC)<br>R: TGGACTTGAGCACGGTCAT | 58 | |
| 10 | F: GACGAGCAGCTGTCCGAG-(NED)<br>R: GAGAGCGTGGCCAGAG | 60 | |
| 7 | F: GACAACGAAACCTACCTCGTC-(PET)<br>R: GTGAGCTGGCGGCAAC | 60 | |
| 3 | F: CATATCTGGCATGGCTCCAG-(FAM)<br>R: ATCGTGTTGACCCCAAAGAAAT | 60 | 2 |
| 47 | F: GACAACGAAACCTACCTCGTC-(VIC)<br>R: GTGAGCTGGCGGCAAC | 60 | |
| X3 | F: AACGAGAGGAAGAACTAAGCCG-(NED)<br>R: TTACGGAGCAGGAAGGCCAGCG | 58 | |
| 32 | F: CCACAGGGTTTTTGGTGAAG-(PET)<br>R: GGAAATCCAACAGCAAGGAC | 55 | |

Primers were previously described by Thibault *et al*., 2007. MAS-PCR: Multiplex-allele specific polymerase chain reaction, mT: Melting temperature, F: Forward, R: Reverse

**Table 2: Primers used in multi-locus short-sequence repeat system**

| Locus | Primers | | SSR |
|---|---|---|---|
| | Forward | Reverse | |
| 1 (1793091) | 5'-TCAGACTGTGCGGTATGGAA-3' | 5'-GTGTTCGGCAAAGTCGTTGT-3' | GGGGGGGGGGGGGGGGGGG |
| 2 (2719085) | 5'-GTGACCAGTGTTTCCGTGTG-3' | 5'-TGCACTTGCACGACTCTAGG-3' | GGGGGGGGGG |
| 8 (1028129) | 5'-AGATGTCGACCATCCTGACC-3' | 5'-AAGTAGGCGTAACCCCGTTC-3' | GGT GGT GGT GGT GGT GG |
| 9 (2955362) | 5'-GACAAGTTCGGGTTGACCAC-3' | 5'-AGTTCCTCGACCCAGTCGT-3' | TGC TGC TGC TGC TGC TG |

Primers were previously described by Amonsin *et al*., 2014. SSR: Short sequence repeat

30 s and an annealing at 60°C for 1 min. The extension step was 2 min at 72°C with a final extension of 7 min at 72°C. Then, PCR products were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). Both DNA strands were sequenced, through the Sanger's sequencing, using the same PCR primers and by a DNA sequencer GA3500XL (Applied Biosystems, Buenos Aires, Argentina) in IABIMO-CONICET, INTA.

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, Buenos Aires, Argentina).

Besides, the Basic local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the flanking sequences of the SSRs during the validation of the process. Each obtained sequence was compared with the DNA sequence of the MAP reference strain K-10.

The results could be also compared with those published on the MAC-SSR-PLUS database (http://mac-ssr.tours.inra.fr/).

### Discriminatory power

The allelic diversity (D) of each MIRU-VNTR locus and of each locus included in MLSSR system was determined by the formula previously described.[30] Besides, the global discriminatory power of complete MIRU-VNTR scheme and the MLSSRs were determined by the HGDI[31,32] using the online software http://insilico.ehu.es/mini_tools/discriminatory_power/, University of the Basque Country. The discriminatory power of the combination of both techniques together was also established.

### Clonal relationship

The clonal relationship among the obtained patterns was established by creating a minimum spanning tree (MST) through the goeBURST algorithm using the Phyloviz 2 software (http://goeburst.phyloviz.net).[33]

## Results

### Genotyping of *Mycobacterium avium* sp. paratuberculosis isolates

A total of 32 MAP isolates were grew on Herrold medium at CEDIVE. These isolates were identified as MAP according to IS*900* PCR and all of them belonged to the C type by IS*1311* PCR-REA.

### "Non-automated" and "Automated" mycobacterial interspersed repetitive unit–variable number tandem repeat assay

All MAP isolates (n: 32) could be genotyped by both "automated and non-automated" systems. Fully agreement

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



was obtained between them, as the same INMV patterns for all isolates were obtained.

MAP isolates were grouped into six different INMV patterns. INMV1 was the most frequent pattern found (n: 18, 54.5%), followed by INMV2 (n: 8, 24.2%) and INMV11 (n: 3, 9.1%). INMV5, INMV8 and INMV16 were represented with one isolate each (3.0%).

Loci 7 and 292 had the highest allelic diversity (D: 0.6980 and 0.5050, respectively). Locus 10 showed a low D (0.0645) whereas loci X3, 25, 47, 3, and 32 showed no variability.

The global discriminatory power (HGDI) using the MIRU-VNTR system to genotype MAP isolates was 0.6290.

Regarding the "automated" system, all MAP isolates could be amplified using MAS-PCR. Besides, detection of labeled PCR products was easily detected by capillary electrophoresis using the GA3500xl (Applied Biosystems, Buenos Aires, Argentina). Figure 1 shows a scheme of the amplification region [Figure 1a] and an example of the detection of PCR products by the GeneMapper software [Figure 1b]. Besides, Table 3 contains the possible fragments (bp) to be obtained according to the number repeat (0–12) presented in each one of the studied loci.

## Multi-locus short-sequence repeat assay

MAP isolates showing the same INMV pattern were studied using the MLSSR system to increase the discrimination among them.

MLSSR revealed L2 as the most polymorphic one. We found isolates with 9, 10, 11 and more than 11 "G" repeats in this locus. However, studying L1 we found that all isolates had 7 "G" repeats in this locus, but the reference strain K10 that showed 10 "G" repeats. Besides, all isolates showed 4 "G" repeats in SSR L8 and L9, while K10 had 5 repeats.

Using SSR L2 we could differentiate MAP isolates sharing the same MIRU-VNTR pattern (INMV). Fourteen out of 18 isolates with INMV1 showed valid SSR L2 results and they

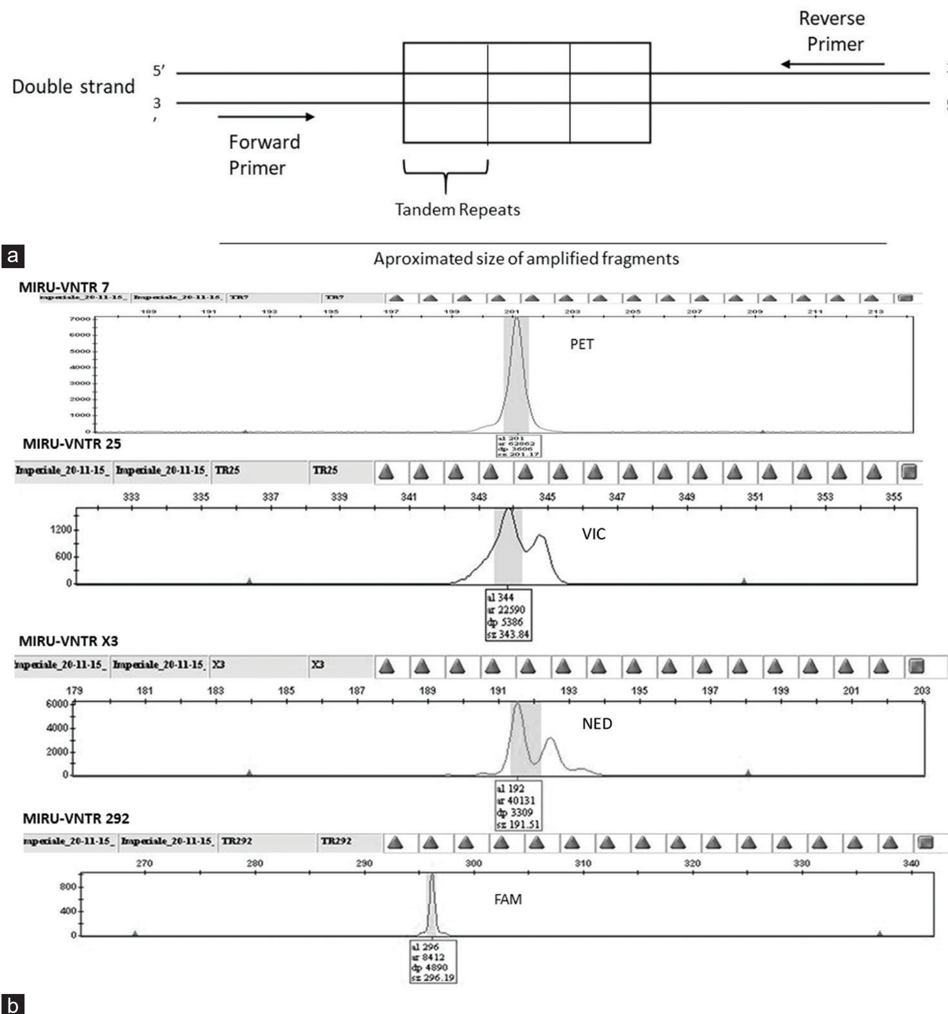


**Figure 1:** (a) Scheme of the amplification region. (b) Chromatograms from capillary electrophoresis (ABI 3130 × l) of the runs of the "automated" mycobacterial interspersed repetitive unit–variable number tandem repeat system to determine the molecular weight. The molecular weights given by the GeneMapper software are indicated below each peak. The polymerase chain reaction products are labeled with different fluorophores: PET, NED, VIC, FAM

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



**Table 3: Fragments to be obtained according to the number of repeats (0-12) contained in each one of the studied loci**

| Locus | Fluorophore | Flanking region to TR (bp) | TR (bp) | Possible size of amplified fragments (bp) for number repeats (0-12) |
|---|---|---|---|---|
| 292 | FAM | 141 | 53 | 141, 194, 247, 300, 353, 406, 459, 512, 565, 618, 6711, 724, 777 |
| X3 | NED | 94 | 53 | 94, 147, 200, 253, 306, 359, 412, 465, 518, 571, 624, 677, 730 |
| 25 | VIC | 176 | 58 | 176, 234, 350, 408, 466, 524, 582, 640, 698, 756, 814, 872 |
| 47 | VIC | 112 | 35 | 112, 147, 182, 217, 252, 287, 322, 357, 392, 427, 462, 497, 532 |
| 3 | FAM | 154 | 27 | 154, 181, 208, 235, 262, 289, 316, 343, 370, 397, 424, 451, 478 |
| 7 | PET | 159 | 22 | 159, 181, 203, 225, 247, 269, 291, 313, 335, 357, 379, 401, 423 |
| 10 | NED | 193 | 55 | 193, 248, 303, 358, 413, 468, 523, 578, 633, 688, 743, 798, 853 |
| 32 | PET | 154 | 18 | 154, 172, 190, 208, 226, 244, 262, 280, 298, 316, 334, 352, 370 |

TR: Tandem repeat, bp: Base pair

were separated in four MAP patterns, each one with different number of "G" repeats. One MAP isolate belonging to INMV1 had 9 "G" repeats, 3 isolates showed 10 "G" repeats, 4 isolates 11 "G" repeats, and 6 isolates presented more than 11 "G" repeats.

The discriminatory power (D) for SSR L2, to discriminate among INMV1 MAP isolates, was 0.7363.

Nevertheless, SSR L1, L8 and L9 failed to discriminate among INMV1 isolates, since all isolates had the same quantity of "G" repeats: 7 in L1, and 4 in L8 and L9.

MAP isolates belonging to INMV2 (n: 8) were separated in 4 different MAP patterns according the "G" repeats in the SSR L2. One isolate showed 9 "G" repeats, another one had 10 "G" repeats and other had 11 "G" repeats. More than 11 "G" repeats were found in four isolates, while one isolate did not amplify by PCR. Besides, K10 strain showed 10 "G" repeats.

The D value for SSR L2 was 0.7143 to discriminate among INMV2 MAP isolates.

On the other hand, two out of three isolates belonging to INMV11 pattern showed valid results, with 10 and 11 "G" repeats in SSR L2.

SSR L1, L8, and L9 did not allow discrimination among INMV11 isolates, since 7 "G" repeats were found with SSR L1 and 4 "G" repeats with SSR L8 and L9 in both isolates.

Using the combination of both molecular techniques (first MIRU-VNTR and then SSR L2) to discriminate among INMV1 and INMV2 MAP isolates, an increase in the HGDI (0.9077) was observed.

## Clonal relationship

Figure 2 shows the MST with the clonal relationship among MAP patterns obtained by MIRU-VNTR and SSR L2 systems. It was assumed that the genetic distance between two INMV patterns is proportional to the difference in the number of repeats at each locus. And the size of the circles indicates the population size of each pattern.

Figure 3 shows a dendrogram with the relationship among MAP isolates that were grouped according to the genetic distance obtained among them.

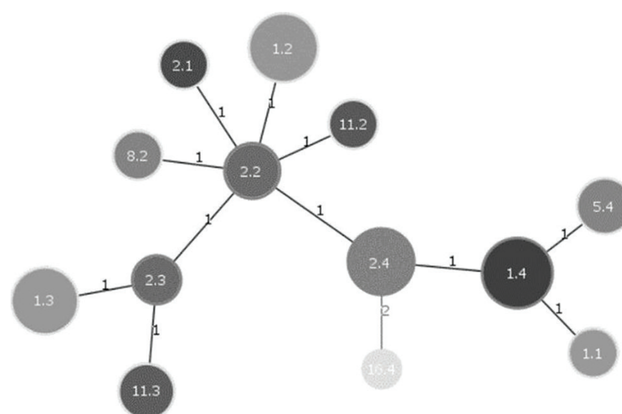


**Figure 2:** goeBURST clustering of INMV patterns belonging to MAP goeBURST clustering of MAP patterns obtained by mycobacterial interspersed repetitive unit–variable number tandem repeat and SSR L2 systems. The size of the pie is related to the number of samples. The numbers indicate the genetic distance between two INMV patterns

| REF | INMV | SSRL2 (number of repeats) |
|---|---|---|
| 1.1 | 1 | 9 G |
| 1.2 | 1 | 10 G |
| 1.3 | 1 | 11 G |
| 1.4 | 1 | >11 G |
| 2.1 | 2 | 9 G |
| 2.2 | 2 | 10 G |
| 2.3 | 2 | 11 G |
| 2.4 | 2 | >11 G |
| 5.4 | 5 | >11 G |
| 8.2 | 8 | 10 G |
| 11.2 | 11 | 10 G |
| 11.3 | 11 | 11 G |
| 16.4 | 16 | >11 G |

## Discussion

MAP isolates could be genotyped using both, the "nonautomated" and the "automated" MIRU-VNTR systems, and a fully concordance within both techniques was obtained.

Nevertheless, the fact of being able to apply the MAS-PCRs for the "automated" version, allowed simplifying the procedures of the system. Using only two separated tubes we were able to amplify

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



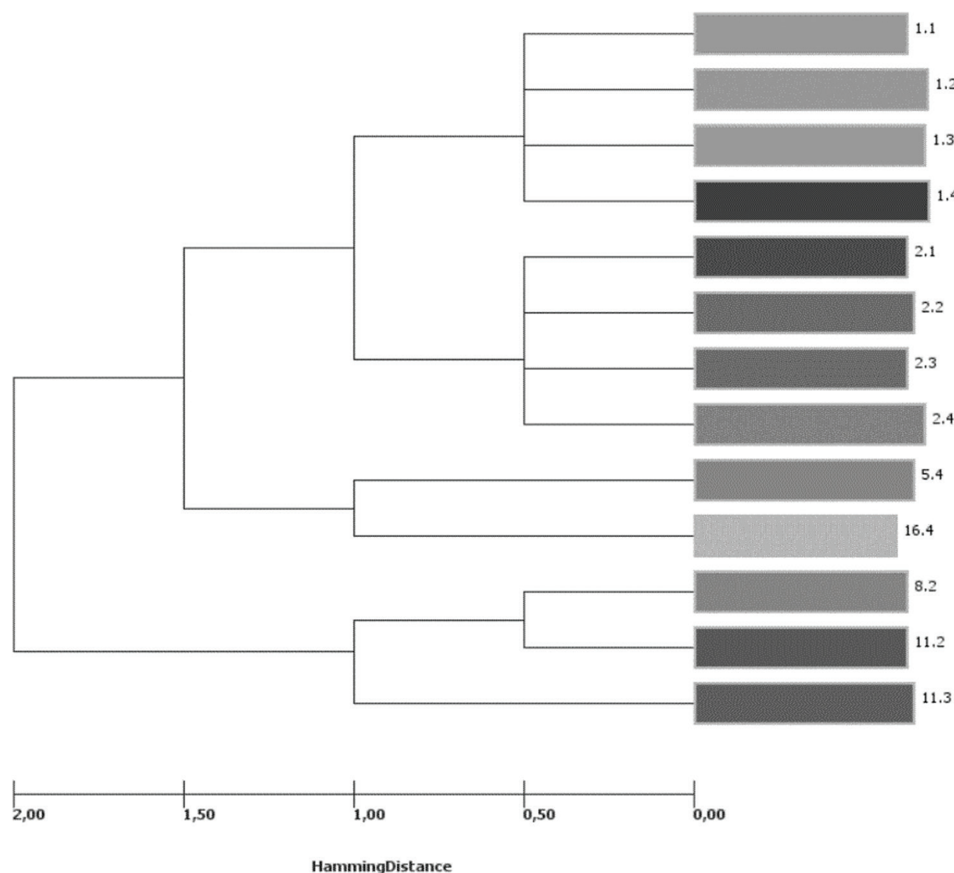


**Figure 3:** Dendrogram showing the relation among MAP isolates according with mycobacterial interspersed repetitive unit–variable number tandem repeat and SSR L2

| REF | INMV | SSRL2 (number of repeats) |
|---|---|---|
| 1.1 | 1 | 9 G |
| 1.2 | 1 | 10 G |
| 1.3 | 1 | 11 G |
| 1.4 | 1 | >11 G |
| 2.1 | 2 | 9 G |
| 2.2 | 2 | 10 G |
| 2.3 | 2 | 11 G |
| 2.4 | 2 | >11 G |
| 5.4 | 5 | >11 G |
| 8.2 | 8 | 10 G |
| 11.2 | 11 | 10 G |
| 11.3 | 11 | 11 G |
| 16.4 | 16 | >11 G |

the 8 MIRU-VNTR loci, instead of using eight PCRs individually. Besides, using capillary electrophoresis to run amplified products and then the GeneMapper software to determine MW, it was possible to save time and make the system less cumbersome, more reproducible and easier to perform. This system allows running several isolates simultaneously. In contrast, the detection of MW by agarose gel is always operator dependent, and must sometimes be repeated to get the correct results.

In addition, taking into account the D values obtained for each one of the analyzed loci by MIRU-VNTR system, a shortened scheme using only the one mixture mix 1 of MAS-PCR amplifying the MIRU 292, VNTR 7, VNTR 10, and VNTR 25 loci could be proposed as a first approach to genotype MAP isolates.

Besides, using MLSSR analysis on MAP isolates previously grouped in different INMV patterns by MIRU-VNTR analysis, the global discriminatory power among isolates could be increased by the combination of both systems (HGDI: 0.9077).

Nevertheless, only SSR L2 was able to discriminate MAP isolates belonging to INMV1 and INMV2. This is in

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



concordance with previous studies that reported that L2 had the highest D (0.707) compared to the other studied loci that showed very low D values.[34]

## Conclusions

Based on the obtained results, MAP isolates from Argentina could be discriminate using a short version of the "automated" MIRU-VNTR scheme as a screening tool to group isolates genetically related and subsequently perform the SSR using Locus 2 on those isolates sharing the same INMV pattern.

The strength of this study was mainly represented by the improve of the MIRU-VNTR system that was obtained using the "automated" version and the addition of another genotyping method using different discriminatory genetic markers, such as MLSSR that allowed to improve the discrimination of MAP isolates.

### Ethical clearance

The Institutional Animal Care and Use Committee (CICUAE) of CICVyA-INTA, whose regulations are in agreement with the European Union Laws for protection of experimental animals, authorized this study. Necropsies were performed in slaughterhouses authorized by the National Service of Agricultural and Food Health and Quality (SENASA).

### Acknowledgments

BRI, MPS, MIR, RDM are fellows from CONICET. Authors would like to acknowledge to Dr. Julia Sabio y García for reviewed the English of the entire manuscript.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

## References

1. Sweeney RW. Transmission of paratuberculosis. Vet Clin N Am Food Anim Pract 1996;12:305-12.
2. Coussens PM. Mycobacterium paratuberculosis and the bovine immune system. Anim Heal Res Rev 2001;2:141-61.
3. Sechi LA, Manuela M, Francesco T, Amelia L, Antonello S, Giovanni F, *et al*. Identification of *Mycobacterium avium* subsp. paratuberculosis in biopsy specimens from patients with Crohn's disease identified by *in situ* hybridization. J Clin Microbiol 2001;39:4514-7.
4. El-Zaatari FA, Osato MS, Graham DY. Etiology of Crohn's disease: The role of *Mycobacterium avium* paratuberculosis. Trends Mol Med 2001;7:247-52.
5. Echeverr G, Escobar H, Changoluisa D, Ron L, Proanõ A, Proanõ-Pérez F, *et al*. Prevalence of paratuberculosis in dairy cattle in ecuador. Int J Mycobacteriol 2020;9:1-6.
6. Sonawane GG, Narnaware SD, Tripathi BN. Molecular epidemiology of *Mycobacterium avium* subspecies paratuberculosis in ruminants in different parts of India. Int J Mycobacteriol 2016;5:59-65.
7. Hemalatha S, Roy P, Purushothaman V, Iyue M. Paratuberculosis in different breeds of sheep: A retrospective study of cases. Int J Mycobacteriol 2013;2:166-70.
8. Pavlik I, Horvathova A, Dvorska L, Bartl J, Svastova P, du Maine R, *et al*. Standardisation of restriction fragment length polymorphism analysis for *Mycobacterium avium* subspecies paratuberculosis. J Microbiol Methods 1999;38:155-67.
9. Sevilla I, Li L, Amonsin A, Garrido JM, Geijo MV, Kapur V, *et al*. Comparative analysis of *Mycobacterium avium* subsp. paratuberculosis isolates from cattle, sheep and goats by short sequence repeat and pulsed-field gel electrophoresis typing. BMC Microbiol 2008;8:204.
10. Gutierrez MC, Supply P, Biet F. New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. paratuberculosis and *M. avium* strains: Comparison with IS900 and IS1245 restriction fragment length polymorphism typing. J Clin Microbiol 2007;45:2404-10.
11. Orti G, Pearse DE, Avise JC. Phylogenetic assessment of length variation at a microsatellite locus. in: Mol. Ecol. Evol. Org. Side Sel. Writings from Avis. Lab., 2010: in: Mol. Ecol. Evol. Org. Side Sel. Writings from Avis. Lab., 2010: pp. 520–524. https://doi.org/10.1142/9789814317764_0008.
12. Biet LA, Thibault F, Grayon V, Supply M, Guilloteau P. Development of VNTR typing of *M. avium* sp. paratuberculosis: Comparison of results with those obtained by IS900 RFLP. In: Manning EJ, Nielsen SS, editors. Proceedings of the 8th International Colloquium on Paratuberculosis. Madison, WI: International Association for Paratuberculosis; 2006. p. 366-74.
13. Sreenu VB, Kumar P, Nagaraju J, Nagarajaram HA. Simple sequence repeats in mycobacterial genomes. J Biosci 2007;32:3-15.
14. Motiwala AS, Amonsin A, Strother M, Manning EJ, Kapur V, Sreevatsan S, *et al*. Molecular epidemiology of *Mycobacterium avium* subsp. paratuberculosis isolates recovered from wild animal species. J Clin Microbiol 2004;42:1703-12.
15. Ghadiali AH, Strother M, Naser SA, Manning EJ, Sreevatsan S. *Mycobacterium avium* subsp. paratuberculosis strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. J Clin Microbiol 2004;42:5345-8.
16. Amonsin A, Li LL, Zhang Q, Bannantine JP, Motiwala AS, Sreevatsan S, *et al*. Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. paratuberculosis strains. J Clin Microbiol 2004;42:1694-702.
17. Harris NB, Payeur JB, Kapur V, Sreevatsan S. Short-sequence-repeat analysis of *Mycobacterium avium* subsp. paratuberculosis and *Mycobacterium avium* subsp. avium isolates collected from animals throughout the United States reveals both stability of loci and extensive diversity. J Clin Microbiol 2006;44:2970-3.
18. Thibault VC, Grayon M, Boschiroli ML, Willery E, Allix-Beguec C, Stevenson K, *et al*. Combined multilocus short-sequence-repeat and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing of *Mycobacterium avium* subsp. paratuberculosis isolates. J Clin Microbiol 2008;46:4091-4.
19. Oakey J, Gavey L, Singh SV, Platell J, Waltisbuhl D. Variable-number tandem repeats genotyping used to aid and inform management strategies for a bovine Johne's disease incursion in tropical and subtropical Australia. J Vet Diagn Invest 2014;26:651-7.
20. Kasnitz N, Köhler H, Weigoldt M, Gerlach GF, Möbius P. Stability of genotyping target sequences of *Mycobacterium avium* subsp. paratuberculosis upon cultivation on different media, *in vitro*- and *in vivo* passage, and natural infection. Vet Microbiol 2013;167:573-83.
21. Colombatti Olivieri MA, Moyano RD, Traveria GE, Alvarado Pinedo MF, Mon ML, Gravisaco MJ, *et al*. Protection efficacy of Argentinian isolates of *Mycobacterium avium* subsp. paratuberculosis with different genotypes and virulence in a murine model. Res Vet Sci 2018;121:4-11.
22. Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P, *et al*. Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: Association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol 2003;3:125-33.
23. Supply P, Lesjean S, Savine E, Kremer K, Van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J Clin Microbiol 2001;39:3563-71.
24. Imperiale BR, Moyano RD, DI Giulio AB, Romero MA, Alvarado Pinedo MF, Santangelo MP, *et al*. Genetic diversity of *Mycobacterium avium* complex strains isolated in Argentina by MIRU-VNTR. Epidemiol Infect 2017;145:1-10.
25. Zhang L, Budiawan T, Matsuoka M. Diversity of potential short tandem repeats in *Mycobacterium leprae* and application for molecular typing.

[Downloaded free from http://www.ijmyco.org on Saturday, March 6, 2021, IP: 190.188.46.244]



J Clin Microbiol 2005;43:5221-9.

26. Iakhiaeva E, McNulty S, Brown Elliott BA, Falkinham JO, Williams MD, Vasireddy R, *et al*. Mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) genotyping of mycobacterium intracellulare for strain comparison with establishment of a PCR-based database. J Clin Microbiol 2013;51:409-16.
27. Bryant K, Thibault JM, Smith VC, Mc Luckie DG, Heron J, Sevilla I, *et al*. Phylogenomic exploration of the relationships between strains of *Mycobacterium avium* subspecies paratuberculosis. BMC Genomics 2016;17:79.
28. Whittington RJ, Marsh IB, Whitlock RH. Typing of IS 1311 polymorphisms confirms that bison (Bison bison) with paratuberculosis in Montana are infected with a strain of *Mycobacterium avium* subsp. paratuberculosis distinct from that occurring in cattle and other domesticated livestock. Mol Cell Probes 2001;15:139-45.
29. Gioffré A, Muñoz MC, Alvarado Pinedo MF, Vaca R, Morsella C, Fiorentino MA, *et al*. Molecular typing of Argentinian *Mycobacterium avium* subsp. paratuberculosis isolates by multiple-locus variable number-tandem repeat analysis. Braz J Microbiol 2015;46:557-64.
30. Nei M. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci U S A 1973;70:3321-3.
31. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988;26:2465-6.
32. Hunter PR. Reproducibility and indices of discriminatory power of microbial typing methods. J Clin Microbiol 1990;28:1903-5.
33. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA. PHYLOViZ: Phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics 2012;13:87.
34. Park HT, Park HE, Bin Park W, Kim S, Hur TY, Jung YH, *et al*. Genetic diversity of bovine *Mycobacterium avium* subsp. paratuberculosis discriminated by IS1311 PCR-REA, MIRU-VNTR, and MLSSR genotyping. J Vet Sci 2018;19:627-34.