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Genetic diversity of *Mycobacterium avium* complex strains isolated in Argentina by MIRU-VNTR

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SUMMARY

Mycobacterium avium sp. *avium* (MAA), *M. avium* sp. *hominissuis* (MAH), and *M. avium* sp. *paratuberculosis* (MAP) are the main members of the *M. avium* complex (MAC) causing diseases in several hosts. The aim of this study was to describe the genetic diversity of MAC isolated from different hosts. Twenty-six MAH and 61 MAP isolates were recovered from humans and cattle, respectively. GenoType CM® and IS*1311*-PCR were used to identify *Mycobacterium* species. The IS*901*-PCR was used to differentiate between MAH and MAA, while IS*900*-PCR was used to identify MAP. Genotyping was performed using a mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) scheme (loci: 292, X3, 25, 47, 3, 7, 10, 32) and patterns (INMV) were assigned according to the MAC-INMV database (http://mac-inmv.tours.inra.fr/). Twenty-two (22/26, 84·6%) MAH isolates were genotyped and 16 were grouped into the following, INMV 92, INMV 121, INMV 97, INMV 103, INMV 50, and INMV 40. The loci X3 and 25 showed the largest diversity (*D*: 0·5844), and the global discriminatory index (Hunter and Gaston discriminatory index, HGDI) was 0·9300. MAP (100%) isolates were grouped into INMV 1, INMV 2, INMV 11, INMV 8, and INMV 5. The HGDI was 0·6984 and loci 292 and 7 had the largest *D* (0·6980 and 0·5050). MAH presented a higher *D* when compared with MAP. The MIRU-VNTR was a useful tool to describe the genetic diversity of both MAH and MAP as well as to identify six new MAH patterns that were conveniently reported to the MAC-INMV database. It was also demonstrated that, in the geographical region studied, human MAC cases were produced by MAH as there was no MAA found among the human clinical samples.

Key words: Mycobacterium avium complex, Diversity, MIRU-VNTR

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are opportunistic pathogens causing mycobacteriosis in humans and animals. The members of *Mycobacterium avium* complex (MAC) are the most frequent etiological

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agents of mycobacteriosis and differ in the type of disease they cause and their pathogenicity [1–3].

M. avium subsp. *paratuberculosis* (MAP) causes paratuberculosis in ruminant animals [4], and it has been postulated that MAP could also have a potential zoonotic role as it has been isolated from patients with Crohn's disease [5, 6]. The other MAC members have no primary host animal and they can be found in the environment as free-living organisms [7, 8].

M. avium, the main MAC mycobacteria, is a heterogeneous group of four subspecies. Identification is based on molecular typing by different genomic targets, including the presence and distribution of several insertion sequences (IS) [9, 10]. The four subspecies of *M. avium* are: (a) MAP that contains IS*900* [7]; (b) *M. avium* subsp. *avium* (MAA), which contains IS*901* and could affect humans [11–14]; (c) MAS (*M. avium* subsp. *silvaticum*) that also has IS*901* [15]; and (d) *M. avium* subsp. *hominissuis* (MAH), the leading member of the MAC causing disseminated or pulmonary disease in humans.

MAH can also produce mycobacterioses in pigs [16] and could be a source of *M. avium* transmission to humans [13, 14, 17, 18]. In fact, there were isolated MAH strains from humans and pigs sharing identical or closely similar genotypes and, while this does not confirm the potential zoonotic role of this subspecies, it cannot be ruled out either [9, 19, 20].

Remarkably the IS*1311* is present in the four *M. avium* subspecies [21] and contains polymorphisms (point mutations) useful to distinguish it from the other subspecies [22].

Molecular techniques based on the polymorphisms present in the length of fragments obtained by restriction enzymes [Restriction fragment length polymorphism (RFLP)] of specific IS (IS*900*, IS*1245*, and IS*1311*) have been used to differentiate MAC subspecies and even strains belonging to the same subspecies [10, 23]. This method, however, is very laborious and requires large amounts of genomic DNA seldom obtained from MAP [24].

Polymerase chain reaction and restriction endonuclease analysis (PCR–REA) (restriction fragment analysis) of the IS*1311* allowed the differentiation between two major MAP lineages (type S, sheep type and type C, cattle type). The strain types were extended to types I and III (subtypes of type S), and type II (subtype of type C) and strains isolated from Bison species [25].

A PCR-based molecular typing method (based on mycobacteria repetitive elements) called mycobacterial interspersed repetitive-unit-variable-number tandem-repeats (MIRU-VNTRs) has been used for genotyping different mycobacteria species [23, 26–28]. *Thibault et al.* (2007) applied a MIRU-VNTR scheme using eight MIRU-VNTR loci (MIRU 292, MIRU X3, VNTR 25, VNTR 47, VNTR 3, VNTR 7, VNTR 10, and VNTR 32) for genotyping MAP isolates. The high discriminatory index (DI: 0·751) made it possible to apply this technique to other MAC members [23, 29].

In this study, we aimed to describe the genetic diversity of MAC, from human and animal origins, causing disease in different hosts in Argentina.

METHODS

MAC isolates

M. avium *isolates*

M. avium isolates from humans were obtained from the Reference Laboratory of Tuberculosis Control Programme at Dr Antonio A. Cetrangolo Hospital where the patients had received medical attention. The study period was from April 2010 to December 2015. To obtain *M. avium* isolates, pulmonary, and extra-pulmonary specimens were homogenized and decontaminated using a mixture of NaCl/NaOH [30]. The mycobacterial isolates from both pulmonary and extra-pulmonary specimens were obtained in Löwenstein–Jensen, Stonebrink and MGIT960™ (BD, Argentina) with the only exception of blood and bone marrow samples that were inoculated in the Myco-F-Lytyc bottles for their incubation in the Bactec 9050™ system (BD).

MAP isolates

MAP isolates were obtained at the Veterinary Research Center (CEDIVE-National University of La Plata (UNLP)) from stool and/or intestinal mucosa from cattle. Clinical samples were homogenized and decontaminated by the HPC (hexadecylpyridinium chloride) method [31] and loaded into Herrolds, M7H10/OADC and M7H9/OADC media supplemented with mycobactin J (2 mg/l) and incubated at 37°C for at least 4 months.

Geographic distribution

All patients affected by MAC came from the 5th Sanitary Region of Buenos Aires Province, which covers a surface of approximately 30 000 km^2, with 13

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districts and municipalities distributed in either rural or urban areas with 3·131·892 inhabitants. MAP isolates came from different locations of Buenos Aires Province such as Tandil, Chascomús, Bartolomé Bavio, Vieytes, Lomas de Zamora. Figure 1 shows the geographical region of the isolates.

Identification of mycobacterial species

When microbial development was observed in culture media, the presence of acid-fast bacilli was confirmed by Ziehl–Neelsen stain. PCR of specific IS (IS*900*, IS*1311*, IS*901*) was performed for species identification and/or through GenoType CM™ (human isolates) [32, 33].

Characterization of MAC subspecies by IS*1311* PCR–REA

The identified MAC subspecies were characterized using restriction enzymes according to the polymorphism present in position 223 of the IS*1311*. The IS*1311* PCR product was digested by *Hinf*I and *Mse*I [33].

Genotyping of MAC isolates by MIRU-VNTR assay

Genotyping of MAC isolates using this system included the amplification of eight MIRU-VNTR loci (292, X3, 25, 47, 3, 7, 10, 32) previously reported by *Thibault et al*. [29]. Primers used to amplify each locus were as previously reported [29]. The amplification protocol was modified to amplify the eight loci simultaneously using touchdown programme at 95°C for 3 min, nine 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 Mix protocol included 1·5 mM $MgCl_2$, 2 µl DMSO, 1·25 U Taq, 2 mM dNTPs mix, and different amounts of primers (from 10 to 25 pmol according to the locus to be amplified). To determine the molecular weight (MW) of each PCR product and to estimate the number of tandem repeats present in each loci, 10 µl of PCR product were loaded in a 2% agarose gel. MW markers (50 and 100 bp) were included on the gel. To digitalize the gel the *Gel Doc TM* imager (Bio-Rad) was used and 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).

Discriminatory power

The allelic diversity (*D*) of each MIRU-VNTR locus and the global discriminatory power of complete MIRU-VNTR scheme (HGDI) were determined using the Hunter and Gaston discriminatory index [34, 35].

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} xj\,(xj-1)$$

where N is the total number of isolates in the typing scheme, s is the total number of distinct subtypes discriminated by the typing method, and xj is the number of isolates belonging to the xth subtype.

The index (*D* and HGDI) were calculated using the online software http://insilico.ehu.es/mini_tools/discriminatory_power/, University of the Basque Country.

The relation among the different INMV profiles was presented in a dendrogram and probable patterns of evolutionary descent between allelic profiles (clonal relationship) was inferred using the goeBURST algorithm, through the Minimum Spanning Tree (MST). It is assumed that the genetic distance between two INMV patterns, is proportional to the difference in the number of repeats at each locus. These relations were established using *Phyloviz 2* software (http://goeburst.phyloviz.net) [36].

RESULTS

MAC isolates

Human isolates

Of 31 *M. avium* isolates that were obtained from human samples at Dr Antonio A. Cetrangolo Hospital, they were identified at species level, by GenoType CM (Hain Lifescience), as *M. avium I* (*n*: 20), *M. avium II* (*n*: 6), and *M. intracellulare* (*n*: 5). The IS*1311* PCR was positive for the 26 isolates classified as *M. avium I* or *II* by GenoType. The 26 isolates were analyzed by PCR for IS*901* and all were found to be negative, confirming that the isolates belonged to MAH and also confirming the absence of MAA among the studied isolates.

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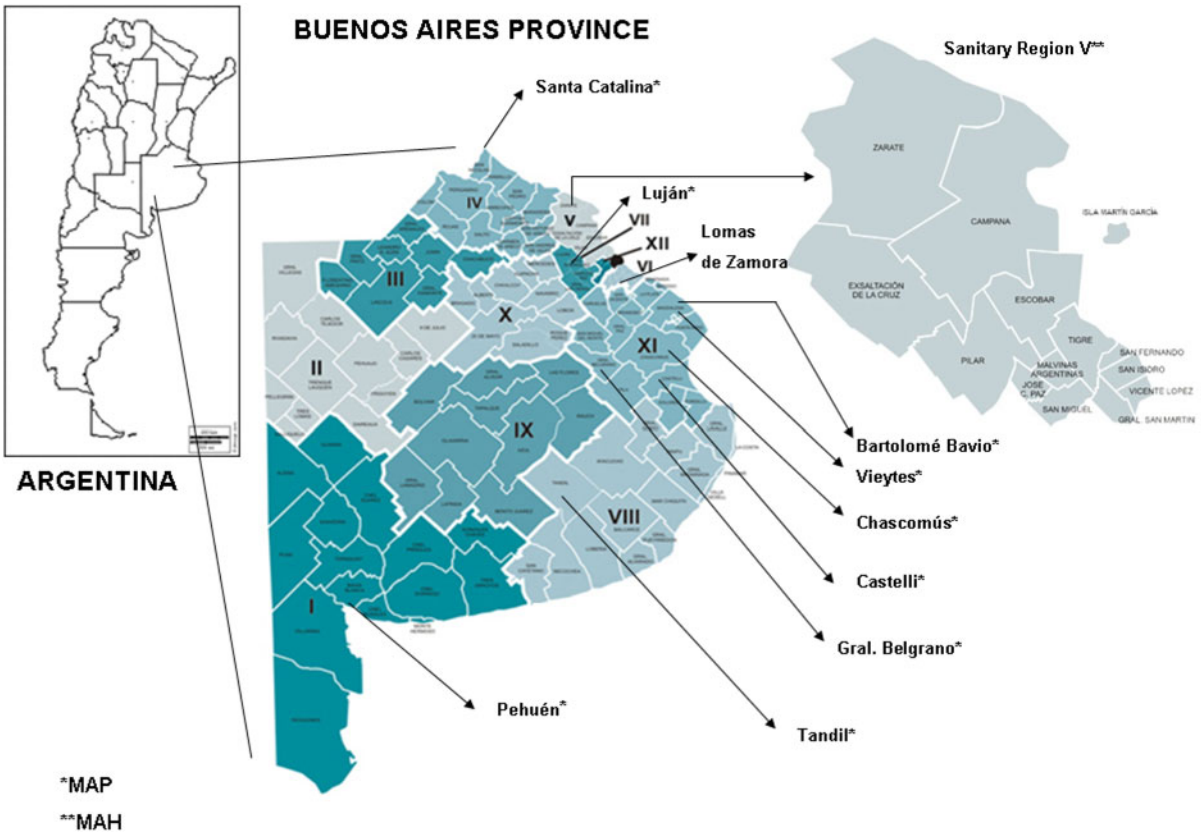

Fig. 1. Geographical region of MAC isolates. MAC, *Mycobacterium avium* complex.

Isolates from cattle

A total of 61 isolates obtained from cattle at CEDIVE, were confirmed as MAC by IS*1311* PCR and as MAP by IS*900* PCR.

Characterization of MAC subspecies by IS*1311* PCR–REA

All MAC isolates were classified according to the polymorphism at the position 223 present in the IS*1311*, by PCR–REA. The restriction pattern obtained after digestion with *Hinf*I (323, 285, 218, and 67 bp) allowed us to identify the most prevalent type of MAP (type II/C or cattle type) among the 61 isolates. As expected, due to the difficulty of culturing type S isolates and their host preference for sheep or goats, no type S/type I/III isolates (with restriction fragments of 285 and 323 bp) were found among the isolates in this study. The 26 MAH isolates showed three restriction bands (134, 189, and 285 bp). Additionally, restriction with *Mse*I allowed discrimination between MAH and MAP (no restriction of MAP).

Genotyping of MAC isolates by MIRU-VNTR assay

A total of 83 out of 87 (95·4%) MAC was successfully genotyped.

MAH isolates

A total of 84·6% (*n*: 22/26) of MAH isolates showed results. Sixteen isolates were grouped among five different previously described INMV patterns (INMV 92, *n*: 4; INMV 121, *n*: 4; INMV 97, *n*: 2; INMV 103, *n*: 2; INMV 50, *n*: 2 and INMV 40, *n*: 2). Six MAH isolates showed novel patterns (NC1, NC2, NC3, NC4, NC5, and NC6) that we reported to National Institute for Agricultural Research and incorporated into the MAC-INMV database. They were assigned to different INMV numbers: 144 (NC1), 145 (NC2), 146 (NC3), 147 (NC4), 148 (NC5), and 149 (NC6). Another four MAH isolates had an incomplete genotyping profile (Table 1). This system showed a high discriminatory power (HGDI: 0·930) for MAH isolates. With regards the discriminatory power (*D*) of each locus, the loci X3 and 25 showed the higher *D* value (*D*: 0·5844), followed by locus 292 (*D*: 0·5714), locus7 (*D*: 0·4848), locus 47

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Table 1. *INMV patterns distribution among MAC isolates*

INMV	TR in locus MIRU-VNTR								Isolates *N* (%)	Geographical area	*M. avium* subsp.
	292	X3	25	47	3	7	10	32			
1	4	2	3	3	2	2	2	8	27 (44·2)	Tandil, Chascomús, Vieytes, Luján, Castelli, Gral. Belgrano, Pehuén, Santa Catalina, Bme Bavio	MAP
2	3	2	3	3	2	2	2	8	17 (27·9)	Tandil, Chascomús, Vieytes, Luján, L. de Zamora, Bme Bavio	
5	4	2	3	3	2	2	1	8	2 (3·3)	Tandil, L. de Zamora	
8	3	2	3	3	2	4	2	8	2 (3·3)	Tandil, Chascomús	
11	3	2	3	3	2	3	2	8	13 (21·3)	Tandil, Luján, L. de Zamora, Chascomús	
92	2	2	2	2	1	1	2	8	4 (15·4)	Escobar	MAH
121	3	2	2	3	1	1	2	8	4 (15·4)	San Isidro, San Fernando, Tigre, San Martín	
97	2	3	2	2	1	1	2	8	2 (7·7)	CABA, Vicente López	
103	2	4	2	2	1	2	2	8	2 (7·7)	San Fernando	
50	3	2	2	3	1	2	1	8	2 (7·7)	Tigre, San Fernando	
40	2	3	2	2	1	2	2	8	2 (7·7)	San Isidro, Vicente López	
144 (NC1)	2	2	2	1	1	1	2	8	1 (3·8)	San Martín	
145 (NC2)	1	3	2	3	1	1	2	8	1 (3·8)	S/D	
146 (NC3)	2	2	4	3	1	1	2	8	1 (3·8)	Zárate	
147 (NC4)	0	4	4	3	1	1	2	8	1 (3·8)	San Fernando	
148 (NC5)	3	3	3	3	1	2	5	8	1 (3·8)	S/D	
149 (NC6)	2	2	4	3	2	2	2	8	1 (3·8)	San Fernando	
IP	–	3	3	0	–	–	–	8	1 (3·8)	Vicente López	
IP	–	3	3	–	–	1	–	8	1 (3·8)	CABA	
IP	3	3	3	3	–	1	–	8	1 (3·8)	San Fernando	
IP	2	2	–	–	1	1	2	8	1 (3·8)	Vicente López	

TR, number of tandem repeats in each locus; MIRU-VNTR, mycobacterial interspersed repetitive-unit-variable-number tandem-repeat; NC, unknown pattern that were recently assigned in the MAC-INMV database with an INMV number; IP, incomplete profile; MAP, *Mycobacterium avium* sp. *paratuberculosis*; MAH, *M. avium* sp. *hominissuis*; CABA, autonomous city of buenos aires; S/D, unknown.

(*D*: 0·3247), locus 10 (*D*: 0·2554). Loci 3 and 32 did not show allelic diversity (*D*: 0·0909 and *D*: 0, respectively).

MAP isolates

The totality of the isolates (100%) were genotyped and grouped into five different INMV previously reported patterns (24, 30). INMV 1 and INMV 2 were the patterns most frequently found (INMV 1, 44·2% and INMV 2, 27·9%), followed by INMV 11 (21·3%), INMV 8 (3·3%), and INMV 5 (3·3%). This technique when applied to MAP showed a lower discriminatory index (HGDI: 0·6984) than that obtained for MAH isolates. Loci 7 and 292 had higher allelic diversities (*D*: 0·6980 and 0·5050, respectively), locus 10 a low *D* (0·0645) while loci X3, 25, 47, 3, and 32 showed no variability.

Relation between isolates

The relation between the different MAC isolates is shown in a dendrogram (Fig. 2) and the clonal relationship between the isolates was calculated using the goeBURST algorithm through the MST (Figs 3 and 4). Figure 3 indicates the INMV 2 of MAP as the original clone from which the others derive. The clonal relationship obtained by goeBURST algorithm including our MAH and MAP isolates, showed two INMV MAH genotypes (NC5: INMV148 and NC6: INMV 149) that were not previously reported and were very closely related to the INMV 2 of MAP that is the original clone from which MAP clones originate. In the case of MAH, clones 92 and 97 are the originals from which all other MAH clones originate. In addition, in Figure 3, it is observed that the unknown patterns of MAH NC5 (INMV 148) and NC6 (INMV 149) originated from INMV 2 of MAP. However, these isolates were negative for IS*900* (data not shown). Interestingly, when our isolates were analyzed in a global context including all the INMV patterns previously reported in the international online MAC-INMV database, it was observed that the

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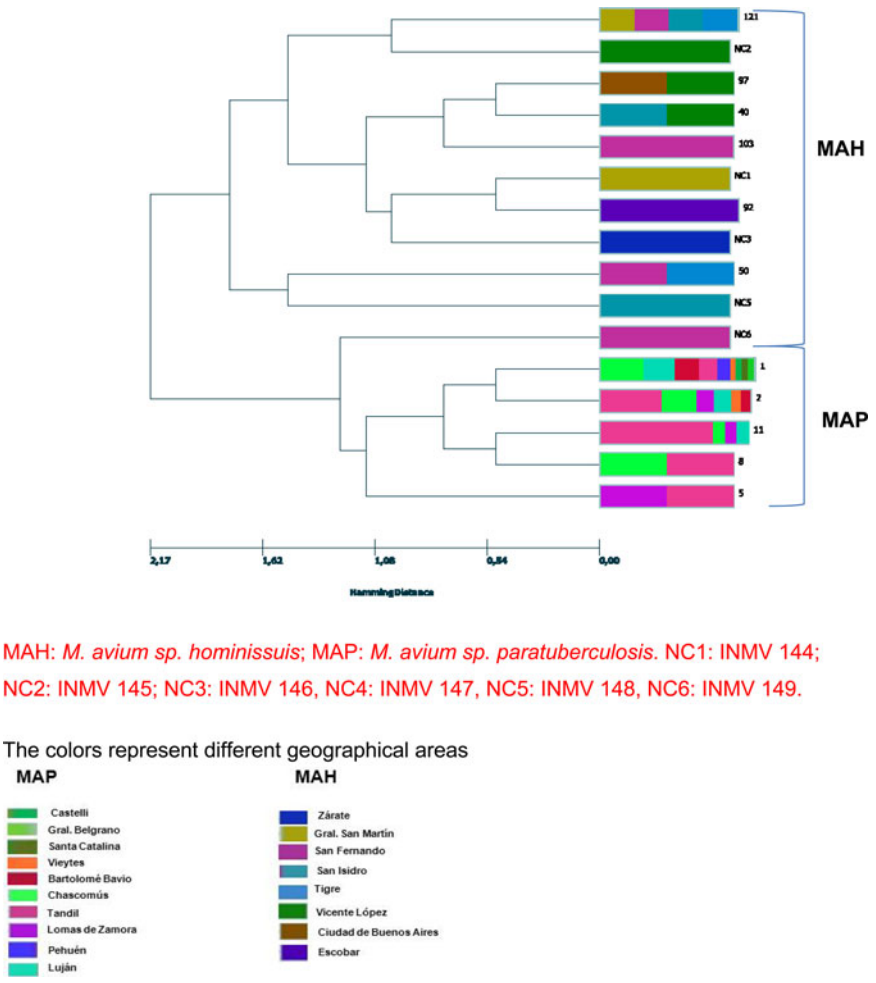

Fig. 2. Dendrogram showing the relation among isolates from cattle and human from Buenos Aires Province. MAH, *Mycobacterium avium* sp. *hominissuis*; MAP, *M. avium* sp. *paratuberculosis*. NC1: INMV 144, NC2: INMV 145, NC3: INMV 146, NC4: INMV 147, NC5: INMV 148, NC6: INMV 149.

patterns NC5 (INMV 148) and NC6 (INMV 149) were grouped with the MAH isolates.

Geographic distribution

While, both MAP and MAC isolates were obtained from Buenos Aires Province, the geographical region of MAH isolates was smaller when compared with the MAP region; however, MAH isolates showed greater genetic variability when compared with the MAP isolates. Table 1 summarizes the different INMV patterns found distributed by geographical area for both MAH and MAP.

DISCUSSION

PCR-based methods have simplified genotyping of microorganisms and in particular the MIRU-VNTR was easy to perform and required a low quantity of DNA. This technique was useful to describe the genetic diversity present among the MAH and MAP isolates studied. MAH showed a higher genetic diversity than MAP, indicating that using only MIRU-VNTR was sufficient for genotyping MAH isolates and to describe the genetic diversity of their subspecies. On the other hand, an acceptable HGDI was obtained for MAP, and was in agreement with those reported by other authors [23, 37].

Since the INMV patterns obtained from the study could be compared with those reported in a free MAC-INMV database (http://mac-inmv.tours.inra.fr/), the eight MIRU-VNTR loci scheme was therefore performed. Six patterns were described for the first time in this study and, following reporting, were subsequently included in the database.

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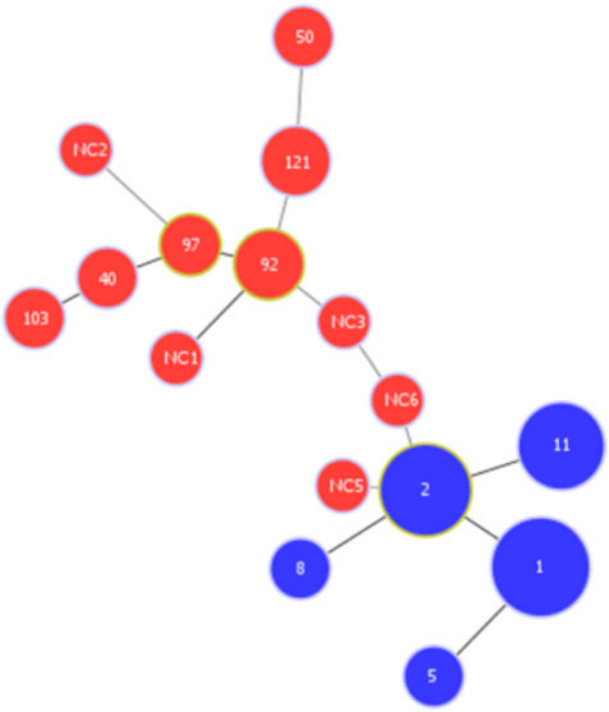

Fig. 3. goeBURST clustering of INMV patterns belonged to *Mycobacterium avium* sp. *paratuberculosis* (MAP) and *M. avium* sp. *hominissuis* (MAH). Red: MAH; blue: MAP. The numbers represents the different INMV patterns found. NC, patterns not previously reported. NC1: INMV 144, NC2: INMV 145, NC3: INMV 146, NC4: INMV 147, NC5: INMV 148, NC6: INMV 149. The size of the pie is related to the number of samples.

In addition, we identified two unknown MAC-INMV patterns (NC5 and NC6) that were grouped within other MAH isolates by MST when all the MAH strains previously reported in the database were included in the analysis (Fig. 4).

In accordance with the *D* value obtained for each locus of the loci and the global HGDI, a shortened scheme could be used for genotyping MAP isolates. This scheme could include only the loci 7, 292, 10, and 25. Additionally, it has been reported that the use of MIRU-VNTR combined with a second method, such as the polymorphisms present in multilocus short sequence repeats (MLSSR) improve the genotyping approach for high-resolution typing of MAP due to their additive discriminatory power [38, 39]. The addition of another genotyping technique using different discriminatory genetic markers could improve the classification of isolates. An example includes where studies assessed the genetic relationship of MAP isolates using whole genome sequencing (WGS) and VNTR typing. The studies reported that VNTR typing may lead to an incorrect assessment of diversity and origins of strains. A weakness of this study was that using VNTR genotyping techniques you may either overestimate or underestimate the relationship between strains due to the instability of some repetitive elements in the genome and the occurrence of homoplasy [40]. Homoplasy is the occurrence of genotypes that are identical by state but not by descent and can be originated by horizontal gene transfer and by convergent and reverse evolution [25, 41].

The main patterns found among the studied MAP isolates were INMV 1 and 2. These findings were in agreement with those previously reported as the most prevalent in different parts of the world [29, 37]. The prevalence reported by the MAC-INMV database for the most common patterns were INMV 1: 27·92% and INMV 2: 23·71% (http://mac-inmv.tours.inra.fr/index.php?p=fa_db).

With regards MAH isolates, INMV 92 and 121 were the most prevalent patterns among our isolates, but they were not reported as the most frequent genotypes of MAH on the database. Interestingly, INMV 92 was found only in one district (Escobar) of the Sanitary Region V of Buenos Aires Province (Fig. 1).

A limitation of this study was that not all MAC subspecies were represented among the isolates studied, although MAH and MAP are the main subspecies of the complex causing disease in humans and animals, respectively.

In this study it was demonstrated that mycobacterioses caused by MAC in humans were produced mainly by MAH, as no MAA clinical isolates were found among the human clinical samples. On the other hand, previous studies carried out also in Buenos Aires Province, but not in the exact region of this study, reported mycobacterioses in pigs caused by MAC. In that study, most of the cases were caused by MAA (n: 30) instead of MAH (n: 6) [14]. However, although it has been postulated the possible zoonotic role of MAH, the INMV patterns found for MAH isolates from pigs in Buenos Aires Province, were different from those found in the present study [14].

The strength of this study was mainly represented by the simplicity and usefulness of the MIRU-VNTR technique and the facilitated analysis on the diversity of strains. In addition, while previous work performed in Argentina showed the distribution of MAP patterns in different host and regions from Buenos Aires Province [37], to the best of our knowledge no previous reports exist to date about the

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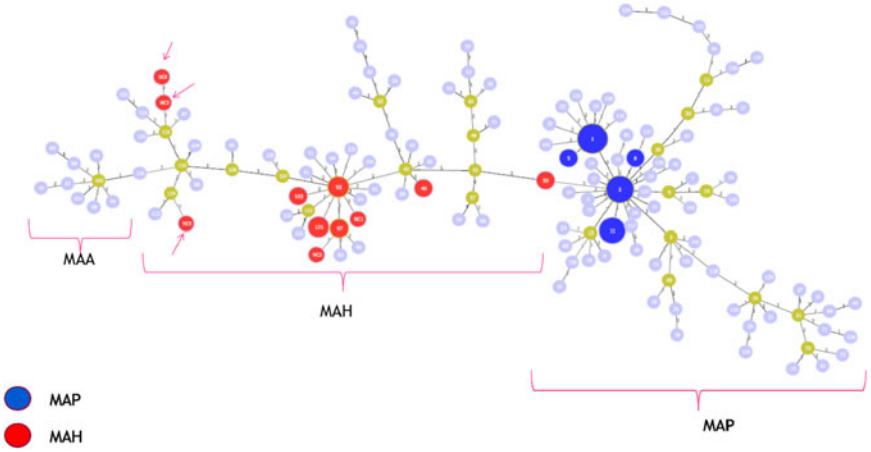

Fig. 4. goeBURST clustering of INMV patterns found and all the INMV patterns reported on the MAC-INMV database. Minimum Spanning Tree-like structure created containing information about the clonal complexes. MAP, *Mycobacterium avium* sp. *paratuberculosis*; MAH, *M. avium* sp. *hominissuis*; NC, patterns not previously reported. NC1: INMV 144, NC2: INMV 145, NC3: INMV 146, NC4: INMV 147, NC5: INMV 148, NC6: INMV 149.

genetic diversity of other MAH members in this country by MIRU-VNTR typing. However, further studies, including more clinical and cattle samples, could contribute to increasing the knowledge of the genotypic diversity of these organisms in addition to exclude or not the MAA as a human pathogen.

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DECLARATION OF INTEREST

None.

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