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Tuberculosis in swine co-infected with *Mycobacterium avium* subsp. *hominissuis* and *Mycobacterium bovis* in a cluster from Argentina

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SUMMARY

In Argentina little is known about the epidemiology of tuberculosis (TB) infection in swine. We characterized the epidemiological dynamics of *Mycobacterium avium* complex (MAC) infection in a swine population of Argentina using molecular tools and spatial analysis techniques. Isolates ($n = 196$) obtained from TB-like lesions ($n = 200$) were characterized by polymerase chain reaction. The isolates were positive to either *M. bovis* (IS*6110*) ($n = 160$) or *M. avium* (IS*1245*) ($n = 16$) while the remaining 20 (10·2%) isolates were positive to both *M. bovis* and *M. avium*. The detection of both bacteria together suggests co-infection at the animal level. In addition, MAC-positive isolates ($n = 36$) were classified as *M. avium* subsp. *avium* (MAA) ($n = 30$) and *M. avium* subsp. *hominissuis* (MAH) ($n = 6$), which resulted in five genotypes when they were typed using mycobacterial interspersed repetitive unit, variable number of tandem repeats (MIRU-VNTR). One significant ($P = 0·017$) spatial clustering of genotypes was detected, in which the proportion of MAH isolates was larger than expected under the null hypothesis of even distribution of genotypes. These results show that in Argentina the proportion of TB cases in pigs caused by *M. avium* is larger than that reported in earlier studies. The proportion of *M. bovis*–MAC co-infections was also higher than in previous reports. These results provide valuable information on the epidemiology of MAC infection in swine in Argentina.

Key words: Co-infection, MIRU-VNTR, *M. bovis*, *M. avium*, PCR.

INTRODUCTION

Tuberculosis (TB) is an infectious and chronic disease affecting a wide range of mammalian species. Within those species, swine are susceptible to infection with a variety of *Mycobacteria*, including *Mycobacterium bovis, M. tuberculosis,* and *M. avium* complex (MAC) [1–3]. TB infection affects animal production because of its economic impact and its potential risk for zoonotic transmission [4, 5]. Because mycobacterial infection in pigs normally does not produce a clinical manifestation of disease, TB is usually only detected by the presence of macroscopic lesions during slaughterhouse inspection. MAC infection typically causes localized, proliferative lesions and usually affects the submandibular and mesenteric lymph nodes [1, 6, 7]. On the other hand, *M. bovis* infection

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typically produces caseous and necrotic lesions in the lungs, spleen and lymph nodes in the thoracic and/or abdominal cavity [7]. In countries in which bovine TB (bTB) is yet to be eradicated, disease prevalence in pigs is related to that found in cattle and is usually due to *M. bovis* [8–10]. Conversely, in regions free from bTB, the disease in swine is more frequently associated with MAC [11, 12].

MAC includes *M. intracellulare, M. avium* subsp. *paratuberculosis* (MAP), *M. avium* subsp. *silvaticum* (MAS), *M. avium* subsp. *avium* (MAA) and *M. avium* subsp. *hominissuis* (MAH). In particular, MAA and MAH are the *M. avium* subspecies that are most frequently associated with TB in pigs [11, 12].

Before the use of molecular techniques, MAA was believed to be responsible for most of the MAC cases of infection in pigs. However, MAH infection in pigs and humans is more frequent than originally suspected [1, 5].

The molecular characterization of *Mycobacteria* is conducted using alternative polymerase chain reaction (PCR) target sequences. For instance, *M. bovis* is typically identified using the insertion sequence IS*6110*, followed by spoligotyping for between- and within-species differentiation [13, 14]. Regarding MAC, the insertion sequence IS*1245* is characteristic of *M. avium* and is useful to discriminate between *M. avium* and *M. intracellulare* [15], whereas IS*901* is present in MAA and MAS but is absent in MAH [4, 16]. The molecular typing reference method for epidemiological links of *M. avium* is the restriction fragment length polymorphism (RFLP) with IS*1245*, IS*1311* and IS*900* as probes [6, 17]. However, in recent years, the development of molecular typing methods based on PCR has substantially simplified the studies of the TB infection.

Multilocus variable number tandem repeat analysis (MLVA) is based on the identification of mycobacterial repetitive elements referred to as a mycobacterial interspersed repetitive unit, which results in a variable number of tandem repeats (MIRU-VNTR). Nowadays, MIRU-VNTR analysis is increasingly used for typing *M. avium* isolates [18–22]. A panel of eight MIRU-VNTR markers has been identified as a suitable tool to discriminate isolates and to study the genetic variability of the strains within the MAC.

M. bovis infection is more frequent than MAC infection in the pig population of Argentina [23–25]. This may be the reason why MAC infection has been little characterized in the country.

The aim of this study was to characterize the epidemiology of MAC infection in a swine population of Argentina by means of molecular tools and spatial analysis techniques. Specifically, we (1) studied the frequency of TB cases caused by MAC infection, which was also stratified by type of *Mycobacteria* (i.e. MAA or MAH) and genotyped using MIRU-VNTR; (2) assessed if co-infection of MAC and *M. bovis* occurs in the field; and (3) described the relative frequency and spatial distribution of MAC genotypes. The results herein presented will be useful to characterize and quantify the impact of the disease and, ultimately, to promote disease control measures in the most important porcine production regions of Argentina.

METHODS

Samples

Pig carcasses were bromatologically inspected at slaughterhouses. The selection criteria of samples consisted of presence of macroscopic granulomatous lesions caseo-necrotic and/or proliferative. The head and mesenteric lymph nodes as well as the liver and lungs of the pigs were routinely examined; gross lesions in other locations were also recorded. About 10 g of the lymph nodes with gross pathological lesions were placed in a sterile bag and transported to the laboratory in a refrigerated container. The samples were stored at −20 °C for up 2 months until bacteriological processing.

Isolates

TB-like lesions ($n = 200$) were collected from May 2007 to November 2009 at three slaughterhouses located in Buenos Aires province, Argentina. The studied pigs came from herds located in three provinces (Buenos Aires, Santa Fe, Cordoba) of the Pampa region of Argentina, which accounts for most of the country's swine production. The samples were homogenized and decontaminated using Petroff's method [26] with 4% sodium hydroxide followed by seeding for 8 weeks at 37 °C in Löwenstein–Jensen (LJ) and Stonebrink (ST) media to stimulate the developing of MAC and *M. bovis*, respectively. The cultures were examined weekly. A bacteriological typing of the isolates was performed based on the growing time, culture media, colony morphology, and the presence of acid-fast bacilli (AFB), which was detected through Ziehl-Neelsen staining [26].

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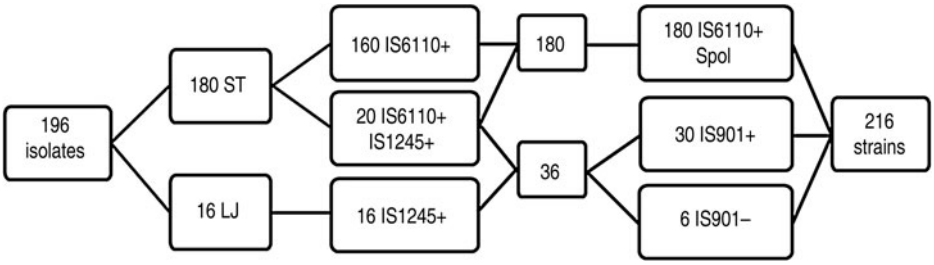

Fig. 1. Scheme of the results obtained by culture, polymerase chain reaction (PCR) and spoligotyping. ST, Stonebrink medium; LJ, Löwestein–Jensen medium; Spol, spoligotyping; IS, insertion sequence. Positive and negative PCR results are denoted by + or –, respectively.

Molecular typing

The AFB-positive isolates were tested by PCR for species-specific identification of *Mycobacteria*. The PCR-targeted sequences were IS*6110* for *M. tuberculosis* complex (CMTB) [13]; IS*1245* for *M. avium* [15], and IS*901* for MAA and MAS, specifically [16]. The IS*6110*-positive samples were subsequently subjected to spoligotyping to identify *M. bovis* (absence of 3, 9, 16 and 39–43 spacers) and for intraspecies differentiation [14].

Detection of co-infection

The positive samples were considered co-infected with *M. bovis* and MAC if the isolates developed in ST and/or LJ culture media and had a PCR-positive result for IS*6110* and IS*1245* along with spoligotypes characteristic of *M. bovis*. Although MAC is not a strict pathogen, which decreases the chances of environmental pollution, this possibility cannot be totally excluded. However, the isolation of bacteria was done from granulomatous lesions; which strengthen the idea that MAC is the infective agent. The criteria used to determine co-infection were confirmation by PCR of two species in suspensions from at least two colonies grown in culture media. We considered infection present when a single species of *Mycobacteria* was confirmed by PCR [1, 5].

Genotyping of *M. avium* strains

The MAC-positive isolates were typed using MIRU-VNTR. The scheme selected was based on eight MIRU-VNTR loci, namely, VNTR-292, MIRU-X3, VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, and VNTR-32 (simplified as 292, X3, 25, 47, 3, 7, 10, and 32, respectively). Primer sequences and PCR conditions were as described elsewhere [18]. Molecular weights were calculated using BioNumerics software v. 3·5 (Applied Maths, Belgium) and the number of tandem repeats in each locus was assigned following a protocol described previously [20]. Numerical profiles were classified according to INMV (INRA, Nouzilly, MIRU-VNTR) combinations [18, 20].

Spatial distribution of MAC genotypes

Because the farm origin was unknown, the MAC-positive isolates were assumed to have originated from the centroid of the department in which the farm of origin was located. The multinomial model of the spatial scan statistic was used to identify if the samples belonging to a given INMV category were spatially clustered [27]. The null hypothesis was that INMVs were randomly distributed in the study area, whereas the alternative hypothesis was that at least one of the INMVs was spatially clustered. The significance of clustering was tested using a simulation process and the multinomial model of the scan statistic was implemented using SaTScan software [28].

RESULTS

A total of 216 mycobacterial strains were identified by PCR, of which 160 (74·07%), 16 (7·40%), and 20 (9·25%) were positive to *M. bovis* (IS*6110*), *M. avium* (IS*1245*), and both *M. bovis* and *M. avium*, respectively (Fig. 1). *Mycobacterium* isolates ($n = 196$) were obtained from most (98%) of the TB-like lesions assessed ($n = 200$). Of the 216 mycobacterial strains, 180 (83·33%) were *M. bovis* (IS*6110*) positive with typical spoligotypes, including 143 isolates described elsewhere [28]; and 36 (16·66%) remaining strains were MAC (IS*1245* positive). Of these remaining strains ($n = 36$), 30 (83·33%) were MAA (IS*901* positive), whereas six

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(16·66%) were MAH (IS*901* negative), following the selection criteria described by Eisenberg *et al.* [29].

Most ($n = 180$, 91·8%) of the isolates developed in ST, whereas the remaining 16 (8·2%) isolates developed in LJ. Furthermore, some ($n = 20$, 11·1%) of the isolates that developed in ST medium co-exist with two species (IS*6110* PCR- and IS*1245* PCR-positive). Conversely, all the isolates grown in LJ medium were IS*6110* PCR-negative. Thus, *M. bovis* was detected only in ST medium, whereas *M. avium* was found in LJ ($n = 16$) and ST ($n = 20$) media. Multiple ($n = 5$) spoligotypes were detected in the 20 isolates where co-existance of *M. bovis* and MAC was detected (Table 1). All the six MAH isolates grew in ST medium and in co-existence with *M. bovis*.

A MIRU-VNTR analysis revealed multiple patterns ($n = 8$) in 35 of the MAC-positive strains. Five different MIRU-VNTR patterns (referred to as INMVs) were observed (Table 1). The INMV1 and INMV2 patterns represented most isolates (48·5% and 28·5%, respectively), whereas INMV4, INMV3, and INMV5 grouped 14·2%, 5·7%, and 2·7% of the isolates, respectively. Most ($n = 34$, 97·14%) of the MAC isolates were grouped into four MIRU-VNTR clusters, whereas only one had a unique pattern (Table 1). Only one MAH strain did not belong to the INMV4 group. Furthermore, none of the patterns deduced for MAA matched those of MAH. In terms of the discriminatory power, locus X3 displayed the highest allelic diversity 0·63, followed by loci 25 and 32 with 0·29 for each (Table 2). Loci 292, 47, 7 and 10 lacked allelic diversity (Table 3).

We detected a significant ($P = 0·017$) spatial cluster of INMVs, which included four MAH-positive samples located in General Arenales (Fig. 2). Interestingly, under the null hypothesis of homogeneous distribution of INMVs, the expected number of MAH-positive samples in this region, was 0·57 (observed-to-expected ratio = 7).

DISCUSSION

In this study, we isolated strains of *M. avium* ($n = 36$) and *M. bovis* ($n = 180$) from granulomatous TB-like lesions collected from slaughtered domestic pigs. The predominant *Mycobacteria* in pigs depend on the epidemiological situation of TB in the country and the production characteristics of each region. An effective control of mycobacterial infections requires knowledge of the causative agent and its epidemiology as well as knowledge of interspecies transmission and biodiversity within the isolated strains [21].

Because bTB is endemic in Argentina, it was not surprising that *M. bovis* was the most frequently isolated bacterium in this study. However, a large proportion of MAC strains were also identified and the frequency of *M. avium* was larger (16·8%) than those reported in previous studies (6–7·5%) in Argentina [23, 24]. This finding may be explained, at least in part, by the decrease in the prevalence of bTB in cattle over the last 43 years, from 6·7% in 1969 to 0·3% in 2012 [30]. Such an epidemiological feature may have resulted in a relative increase in the proportion of MAC isolates from swine granulomatous lesions, as has been observed in countries in which bTB has been eradicated [1, 5]. Specifically in Argentina, TB prevalence in pigs decreased from 8·4% in 1969 to 0·3% in 2012 [30]. Therefore, we hypothesize that the reduction of competition between *M. bovis* and MAC probably allowed MAC to occupy more space in the chain of infection.

MAA and MAH are the two species of highest epidemiological importance in swine. MAA is the causative agent of avian TB and in turn birds, together with environments contaminated with infected bird faeces, are the main source of infection for pigs [5, 31]. On the other hand, MAH is the most prevalent opportunistic pathogen for pigs and humans, and has been isolated from many host species and environmental samples [1, 5, 17]. Furthermore, MAH has also acquired an increasing importance for public health in recent decades because of its ability to cause pulmonary disease, lymphadenitis in children and disseminated infections in immunocompromised patients [11]. In Argentina *M. avium* was reported as the major aetiological agent of clinically confirmed cases of non-tuberculous *Mycobacteria* in humans, but no differentiation of species and subspecies of MA has been assessed yet [32]. The use of molecular techniques had allowed the identification of a close genetic relationship between strains found in humans and swine, which suggest transmission between these two hosts or exposure to a common source of infection [17, 21, 22]. Thus, pigs could be considered as an additional source of transmission of *M. avium* to humans, especially to immunosuppressed populations [17, 21, 22]. MAH has previously been identified in two dogs (miniature Schnauzer) and one human in Argentina [33, 34].

As far as we know, this is the first report of MAH infection in pigs in Argentina. Most (66%) of the MAH isolates were clustered ($P < 0·01$) in one single

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Table 1. *MIRU-VNTR types detected in the 29* M. avium *subsp.* avium *and six* M. avium *subsp.* hominissuis *isolates. The co-existence of* M. avium *and* M. bovis *isolates is detailed. PCR and spoligotyping results are also shown*

	PCR				VNTR									Location	
Isolates	IS*1245*	IS*901*	IS*6110*	Spol.	292	X3	25	47	3	7	10	32	INMVR	Province	Department
M. avium subsp. *avium*	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Brandsen
	Pos	Pos	Neg	—	2	2	1	3	2	2	2	7	INMV3	Buenos Aires	Brandsen
	Pos	Pos	Neg	—	2	4	1	3	1	2	2	7	INMV2	Córdoba	Río Cuarto
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Córdoba	Gral. San Martín
	Pos	Pos	Neg	—	2	4	1	3	1	2	2	7	INMV2	Buenos Aires	Lobería
	Pos	Pos	Neg	—	2	4	1	3	1	2	2	7	INMV2	Córdoba	Marcos Juárez
	Pos	Pos	Neg	—	2	2	1	3	2	2	2	7	INMV3	Buenos Aires	San Andrés de Giles
	Pos	Pos	Neg	—	2	4	1	3	1	2	2	7	INMV2	Buenos Aires	Ayacucho
	Pos	Pos	Neg	—	2	4	1	3	1	2	2	7	INMV2	Buenos Aires	Rauch
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Córdoba	Gral San Martín
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Córdoba	Gral. San Martín
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Chivilcoy
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Gral. Pinto
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Córdoba	Marcos Juárez
	Pos	Pos	Neg	—	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Saladillo
M. avium subsp. *avium* and *M. bovis*	Pos	Pos	Pos	SB0130	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Bolívar
	Pos	Pos	Pos	SB0140	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Las Flores
	Pos	Pos	Pos	SB0140	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Tres Lomas
	Pos	Pos	Pos	SB0140	2	3	1	3	1	2	2	7	INMV1	Santa Fe	Gral. López
	Pos	Pos	Pos	SB0140	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	San Andrés de Giles
	Pos	Pos	Pos	SB0120	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Gral. Viamonte
	Pos	Pos	Pos	SB0140	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	San Andrés de Giles
	Pos	Pos	Pos	SB0140	2	4	1	3	1	2	2	7	INMV2	Buenos Aires	Lobería
	Pos	Pos	Pos	SB0140	2	4	1	3	1	2	2	7	INMV2	Buenos Aires	Ayacucho
	Pos	Pos	Pos	SB0859	2	3	1	3	1	2	2	7	INMV1	Córdoba	Rio IV
	Pos	Pos	Pos	SB0131	2	3	1	3	1	2	2	7	INMV1	Buenos Aires	Gral. Viamonte
	Pos	Pos	Pos	SB0140	2	4	1	3	1	2	2	7	INMV2	Santa Fe	Constitución
	Pos	Pos	Pos	SB0140	2	4	1	3	1	2	2	7	INMV2	Santa Fe	Constitución
	Pos	Pos	Pos	SB0140	2	4	1	3	1	2	2	7	INMV2	Buenos Aires	Rauch

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Table 1 (*cont.*)

	PCR				VNTR									Location	
Isolates	IS*1245*	IS*901*	IS*6110*	Spol.	292	X3	25	47	3	7	10	32	INMVR	Province	Department
M. avium subsp.	Pos	Neg	Pos	SB0140	2	3	4	3	1	2	2	9	INMV5	Buenos Aires	Gral. Rodríguez
hominissuis and	Pos	Neg	Pos	SB0140	2	2	4	3	1	2	2	9	INMV4	Buenos Aires	Arenales
M. bovis	Pos	Neg	Pos	SB0140	2	2	4	3	1	2	2	9	INMV4	Buenos Aires	Arenales
	Pos	Neg	Pos	SB0140	2	2	4	3	1	2	2	9	INMV4	Buenos Aires	Gral. Rodríguez
	Pos	Neg	Pos	SB0140	2	2	4	3	1	2	2	9	INMV4	Buenos Aires	Arenales
	Pos	Neg	Pos	SB0140	2	2	4	3	1	2	2	9	INMV4	Buenos Aires	Arenales

MIRU-VNTR, Mycobacterial interspersed repetitive unit, variable number of tandem repeats; PCR, polymerase chain reaction.

Table 2. *Allelic distribution of polymorphic MIRU-VNTR*

MIRU-VNTR	No. of alleles	More frequent alleles (no. of isolates)	Allelic diversity
X3	3	3 (18)	0·63
25	2	1 (29)	0·29
3	2	1 (33)	0·11
32	2	7 (29)	0·29

MIRU-VNTR, Mycobacterial interspersed repetitive unit, variable number of tandem repeats.
The discriminatory index of Hunter and Gaston was calculated using the formula of Simpson (http://insilico.ehu.es/mini_tools/discriminatory_power/).

Table 3. *MIRU-VNTR allelic distribution of* M. avium *subsp.* avium *and* M. avium *subsp.* hominissuis *isolates*

	No. of isolates with the specific MIRU allele						
Locus	1	2	3	4	7	9	Allelic diversity
M. avium subsp. *avium* ($n = 29$)							
32					29		0
292		29					0
7		29					0
10		29					0
25	29						0
47			29				0
X3		2	17	10			0·55
M. avium subsp. *hominissuis* ($n = 6$)							
32						6	0
292		6					0
7		6					0
10		6					0
25				6			0
47			6				0
X3		5	1				0·33

MIRU-VNTR, Mycobacterial interspersed repetitive unit, variable number of tandem repeats.

department from Buenos Aires province, General Arenales, which suggests a limited geographical distribution of MAH in the country. Other *M. avium* strains ($n = 30$) were classified as MAA (IS*901*) positive. Some characteristics of swine production in Argentina may favour the transmission of these two species of *Mycobacteria* in swine. Outdoor production systems are relatively common and, in farms with limited or absent biosecurity measures in place, pigs may have contact with wild birds and/or wild boars, which increases the opportunity for disease transmission.

For MLVA analysis we used the set of primers designed on the basis of the MAP genomic sequence [18] and detected multiple ($n = 5$) MLVA patterns. The most frequent pattern, referred to as INMV1, grouped 48·5% (17/35) of the isolates and included animals from the three sampled provinces (Buenos Aires, Córdoba, Santa Fe) (Table 1 and Fig. 2). The observed patterns were different compared to those found associated with poultry, cattle, and humans from Argentina [34]. However, the absence of such patterns may be due to the limited number of

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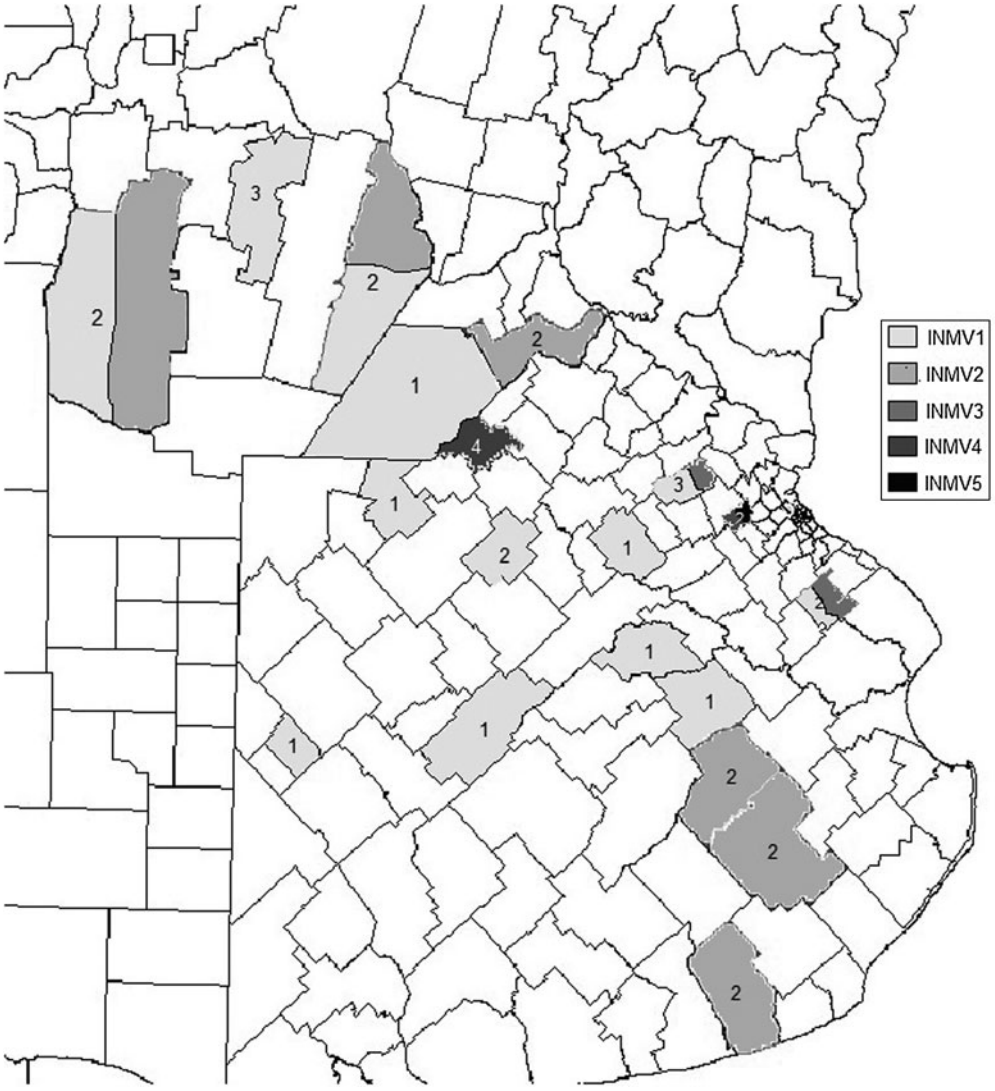

Fig. 2. Map of the central region of Argentina (Buenos Aires, Córdoba, Santa Fe, La Pampa, Entre Ríos provinces) showing the distribution of the isolates (ArcGIS, v. 10·0; ESRI, USA). Each department shows the number of isolates and the genotypes are denoted by greyscale. In General Arenales department, with four isolates, a significant spatial clustering of MAH was observed. INMV, INRA Nouzilly, MIRU-VNTR (numerical profile combinations).

isolates that are available and analysed using this technique [34].

The highest polymorphism was observed in MAA isolates compared to MAH strains, in contrast to that observed elsewhere [20, 22]. With the exception of one, all MAH isolates had the same INMV4 pattern. Loci 47, 292, 10, and 7 were monomorphic, whereas locus X3 was the most polymorphic, as previously suggested [20–22, 35]. The discriminatory power of the combined MIRU-VNTR was 0·68, lower than those obtained by other researchers 0·80 [22], 0·88 [20], 0·90 [35], and 0·92 [21]. This finding may be explained by the presence of clones in the concentrated production region of Argentina. Moreover, we identified allelic signatures associated with subspecies of MAC. For instance, MAA and MAS contained allele 1 of locus 25, whereas all MAH and MAP strains contained two or more repeats in this locus [20, 21, 35]. Allele 1 was present in all MAA samples, whereas, in MAH strains allele 4 was in locus 25.

As a result of this minimal overlap in the allelic distributions between the different subspecies, MIRU-VNTR-based genotype complexes perfectly correlated with the differentiation of MAH strains from MAA or MAS. Specifically, MAH strains are negative for IS*901*, whereas MAA or MAS are positive for this sequence. Locus 292 provides information to

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differentiate between MAP and MAA [18]. Thus, alleles 3 and 4 of locus 292 were exclusively restricted to, and shared by most of, MAP strains and were absent from the 35 isolates typed in the present study [18, 20]. This method is technically simple and thus the numerical genotype format may be a very useful tool for molecular characterization of isolates.

The results presented herein demonstrate that the differentiation of MAC strains using MIRU-VNTR is a convenient typing method capable of distinguishing the three main subspecies of the complex and providing new epidemiological knowledge on the strains [20].

The *M. bovis* strains identified in co-infection events were spoligotyped and multiple ($n = 5$) spoligotypes were observed. One of these spoligotypes (SB0140) grouped 80% (16/20) of the isolates and coincided with the most frequent spoligotype identified in cattle from Argentina [36], whereas the remaining spoligotypes ($n = 4$) only included one isolate each (Table 1).

Co-infection by two different *Mycobacteria* has been increasingly documented in human TB [37–39], and has incidentally been documented in swine [5, 39, 40]. However, the large proportion (10·2%) of co-infection events detected here suggests that this phenomenon should be further investigated.

This study contributes to the characterization of *M. avium* strains isolated from pigs in Argentina and, furthermore, is the first report on swine in the country. The relatively low genetic variation and limited spatial extension of MHA isolates suggest that infection is not extensive in the country. Additionally, we reported a proportion of *M. avium* and *M. bovis* co-infections larger than those previously reported. These results could contribute to the development of molecular epidemiological tools which may broaden the understanding of disease dynamics in swine. Furthermore, they may help to design and implement effective TB control programmes in Argentina.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Matlova L, *et al.*** Distribution of *Mycobacterium avium* complex isolates in tissue samples of pigs fed peat naturally contaminated with mycobacteria as a supplement. *Journal of Clinical Microbiology* 2005; **43**: 1261–1268.
2. **Naranjo *et al.*** Evidence of the role of European wild boar as a reservoir of *Mycobacterium tuberculosis* complex. *Veterinary Microbiology* 2008; **127**: 1–9.
3. **Arega SM, Conraths FJ, Ameni G.** Prevalence of tuberculosis in pigs slaughtered at two abattoirs in Ethiopia and molecular characterization of *Mycobacterium tuberculosis* isolated from tuberculous-like lesions in pigs. *BMC Veterinary Research* 2013; **6**: 9: 97.
4. **Mijs W, *et al.*** Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and M. *avium* subsp. *hominissuis* for the human/porcine type of M. *avium*. *International Journal of Systematic and Evolutionary Microbiology* 2002; **52**: 1505–1518.
5. **Agdestein A, *et al.*** A comparative study of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *hominissuis* in experimentally infected pigs. *BMC Veterinary Research* 2012; **8**: 11.
6. **Domingos M, Amado A, Botelho A.** IS*1245* RFLP analysis of strains of *Mycobacterium avium* subspecies *hominissuis* isolated from pigs with tuberculosis lymphadenitis in Portugal. *Veterinary Record* 2009; **164**: 116–120.
7. **Perez AM.** Swine tuberculosis. In: *Diseases of Swine*. School of Veterinary Science, UNR Rosario, 2005, pp. 192.
8. **Serraino A, *et al.*** Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques. *Journal of Clinical Microbiology* 1999; **37**: 2766–2771.
9. **Parra A, *et al.*** Epidemiology of *Mycobacterium bovis* infections of pigs and wild boars using a molecular approach. *Veterinary Microbiology* 2003; **97**: 123–133.
10. **Muwonge A, *et al.*** *Mycobacterium bovis* infections in slaughter pigs in Mubende district, Uganda: a public health concern. *BMC Veterinary Research* 2012; **8**: 168.
11. **Alvarez J, *et al.*** Epidemiological investigation of a *Mycobacterium avium* subsp. *hominissuis* outbreak in swine. *Epidemiology and Infection* 2010; **139**: 143–148.
12. **Lara GH, *et al.*** Occurrence of *Mycobacterium* spp. and other pathogens in lymph nodes of slaughtered swine and wild boars (*Sus scrofa*). *Research in Veterinary Science* 2011; **90**: 185–188.
13. **Hermans PW, *et al.*** Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *Journal of Clinical Microbiology* 1990; **28**: 2051–2058.

14. **Kamerbeek J, *et al.*** Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology* 1997; **35**: 907–914.
15. **Guerrero C, *et al.*** A novel insertion element from *Mycobacterium avium*, IS*1245*, is a specific target for analysis of strain relatedness. *Journal of Clinical Microbiology* 1995; **33**: 304–307.
16. **Kunze ZM, *et al.*** IS*901*, a new member of a widespread class of atypical insertion sequences, is associated with pathogenicity in *Mycobacterium avium*. *Molecular Microbiology* 1991; **5**: 2265–2272.
17. **Johansen TB, *et al.*** New probes used for IS*1245* and IS*1311* restriction fragment length polymorphism of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *hominissuis* isolates of human and animal origin in Norway. *BMC Microbiology* 2007; **7**: 14.
18. **Thibault VC, *et al.*** New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* strains: comparison with IS*900* and IS*1245* restriction fragment length polymorphism typing. *Journal of Clinical Microbiology* 2007; **45**: 2404–2410.
19. **Thibault VC, *et al.*** Combined multilocus short-sequence-repeat and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing of *Mycobacterium avium* subsp. *paratuberculosis* isolates. *Journal of Clinical Microbiology* 2008; **46**: 4091–4094.
20. **Radomski N, *et al.*** Determination of genotypic diversity of *Mycobacterium avium* subspecies from human and animal origins by mycobacterial interspersed repetitive-unit-variable-number tandem-repeat and IS*1311* restriction fragment length polymorphism typing methods. *Journal of Clinical Microbiology* 2010; **48**: 1026–1034.
21. **Tirkkonen T, *et al.*** Comparison of variable-number tandem-repeat markers typing and IS1245 restriction fragment length polymorphism fingerprinting of *Mycobacterium avium* subsp. *hominissuis* from human and porcine origins. Acta Veterinaria Scandinavica. Published online: 10 May 2010. doi:10.1186/1751-0147-52-21
22. **Pate M, *et al.*** MIRU-VNTR typing of *Mycobacterium avium* in animals and humans: heterogeneity of *Mycobacterium avium* subsp. *hominissuis versus* homogeneity of *Mycobacterium avium* subsp. *avium* strains. *Research in Veterinary Science* 2011; **91**: 376–381.
23. **de Kantor IN, Lesslie IW.** Isolation and classification of mycobacteria from lymph nodes of swine in Argentina. *Boletín de la Oficina Sanitaria Panamericana* 1974; **77**: 495–499.
24. **Perez A, *et al.*** Trend of swine tuberculosis and bromatological validity of inspection for detection in areas of intensive production from Argentina. *Revista Argentina de Medicina Veterinaria* 2002; **83**: 14–18.
25. **Barandiaran S, *et al.*** *Mycobacterium bovis* in swine: spoligotyping of isolates from Argentina. *Veterinary Medicine International*. Published online: 19 April 2011. doi:10.4061/2011/979647.
26. **Jorge M, *et al.*** *Diagnostic Manual of Mycobacteria of Veterinary Importance*, 1st edn. Buenos Aires: Asociación Argentina de Veterinarios de Laboratorios de Diagnóstico (AAVLD), 2005, pp. 20–46.
27. **Jung I, Kulldorff M, Richard OJ.** A spatial scan statistic for multinomial data. *Statistics in Medicine* 2010; **29**: 1910–1918.
28. **Kulldorff M.** SaTScan: software for the spatial and space-time scan statistics. 2009 (http://www.satscan.org/).
29. **Eisenberg T, *et al.*** Outbreak of reproductive disorders and mycobacteriosis in swine associated with a single strain of *Mycobacterium avium* subspecies *hominissuis*. *Veterinary Microbiology* 2012; **159**, 69–76.
30. **SENASA.** Status of bovine tuberculosis in Argentina. 2012 (http://www.senasa.gov.ar//Archivos/File/File6832-TubBRepAa.pdf).
31. **Moravkova M, *et al.*** Genetic IS*901* RFLP diversity among *Mycobacterium avium* subsp. *avium* isolates from four pheasant flocks. *Journal of Veterinary Science* 2013; **14**: 99–102.
32. **Imperiale B, *et al.*** Disease caused by non-tuberculous mycobacteria: diagnostic procedures and treatment evaluation in the North of Buenos Aires province. *Revista Argentina de Microbiología* 2012; **44**: 3–9.
33. **Martinez Vivot M, *et al.*** First isolation of *Mycobacterium avium* subspecies *hominissuis* in a miniature schnauzer in Argentine. XVIII Congress of Argentinian Veterinary Association of Laboratory Diagnostics (AAVLD), Mercedes, Corrientes, Argentina, 2010.
34. **Gioffré A, *et al.*** Exploiting multiple-locus variable number tandem repeat analysis to evaluate strain diversity of M. *avium* complex in Argentina. 11th International Colloquium on Paratuberculosis, Sydney, Australia, 2012.
35. **Inagaki T, *et al.*** Comparison of a variable-number tandem-repeat (VNTR) method for typing *Mycobacterium avium* with mycobacterial interspersed repetitive-unit-VNTR and IS*1245* restriction fragment length polymorphism typing. *Journal of Clinical Microbiology* 2009; **47**: 2156–2164.
36. **Zumárraga MJ, *et al.*** Understanding the relationship between Mycobacterium *bovis* spoligotypes from cattle in Latin American Countries. *Research in Veterinary Science* 2013; **94**: 9–21.
37. **Suffys P, *et al.*** Detection of mixed infections with *Mycobacterium lentiflavum* and *Mycobacterium avium* by molecular genotyping methods. *Journal of Medical Microbiology* 2006; **55**: 127–131.
38. **Huyen MN, *et al.*** Mixed tuberculosis infections in rural South Vietnam. *Journal of Clinical Microbiology* 2012; **50**: 1586–1592.
39. **de Kantor IN, *et al.*** Tuberculous infection in cattle not detected by slaughterhouse inspection. *Zentralblatt fur Veterinarmedizin. Reihe B. Journal of Veterinary Medicine* 1987; **34**: 202–205.
40. **Bartos M, *et al.*** Identification of members of *Mycobacterium avium* species by Accu-Probes, serotyping, and single IS*900*, IS*901*, IS*1245* and IS*901*-flanking region PCR with internal standards. *Journal of Microbiological Methods* 2006; **64**: 333–345.