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 (IS6110) (n = 160) or M. avium (IS1245) (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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Molecular epidemiology of TB in swine

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 $-20\,^{\circ}\mathrm{C}$ 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 $37\,^{\circ}\mathrm{C}$ 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].
Molecular typing

The AFB-positive isolates were tested by PCR for species-specific identification of Mycobacteria. The PCR-targeted sequences were IS6110 for M. tuberculosis complex (CMTB) [13]; IS1245 for M. avium [15], and IS901 for MAA and MAS, specifically [16]. The IS6110-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 IS6110 and IS1245 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 (IS6110), M. avium (IS1245), 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 (IS6110) positive with typical spoligotypes, including 143 isolates described elsewhere [28]; and 36 (16.66%) remaining strains were MAC (IS1245 positive). Of these remaining strains (n = 36), 30 (83.33%) were MAA (IS901 positive), whereas six
(16.66%) were MAH (IS901 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 (IS6110 PCR- and IS1245 PCR-positive). Conversely, all the isolates grown in LJ medium were IS6110 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-existence 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 MAH 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 birds 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
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.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>PCR</th>
<th>VNTR</th>
<th>Location</th>
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<tbody>
<tr>
<td></td>
<td>IS1245</td>
<td>IS901</td>
<td>IS6110</td>
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<td><em>M. avium</em> subsp. <em>avium</em></td>
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* M. avium subsp. *avium* and *M. bovis* | Pos | Pos | Pos | SB0130 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | Bolivar |
|          | 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 | Buenos Aires | Santa Fe |
|          | Pos | Pos | Pos | SB0140 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | Gral. López |
|          | Pos | Pos | Pos | SB0140 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | San Andrés |
|          | Pos | Pos | Pos | SB0120 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | Constitución |
|          | Pos | Pos | Pos | SB0140 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | Constitución |
|          | Pos | Pos | Pos | SB0140 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV2 | Buenos Aires | Constitución |
|          | Pos | Pos | Pos | SB0140 | 2 | 4 | 1 | 3 | 1 | 2 | 2 | 7 | INMV2 | Buenos Aires | Constitución |
|          | Pos | Pos | Pos | SB0140 | 2 | 4 | 1 | 3 | 1 | 2 | 2 | 7 | INMV2 | Buenos Aires | Constitución |
|          | Pos | Pos | Pos | SB0859 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | Ciudadela |
|          | Pos | Pos | Pos | SB0131 | 2 | 3 | 1 | 3 | 1 | 2 | 2 | 7 | INMV1 | Buenos Aires | Ciudadela |
|          | Pos | Pos | Pos | SB0140 | 2 | 4 | 1 | 3 | 1 | 2 | 2 | 7 | INMV2 | Buenos Aires | Ciudadela |
|          | Pos | Pos | Pos | SB0140 | 2 | 4 | 1 | 3 | 1 | 2 | 2 | 7 | INMV2 | Buenos Aires | Ciudadela |
|          | Pos | Pos | Pos | SB0140 | 2 | 4 | 1 | 3 | 1 | 2 | 2 | 7 | INMV2 | Buenos Aires | Ciudadela |

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department from Buenos Aires province, General Arenales, which suggests a limited geographical distribution of MAH in the country. Other \textit{M. avium} strains (\(n = 30\)) were classified as MAA (IS901) positive. Some characteristics of swine production in Argentina may favour the transmission of these two species of \textit{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

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|l|l|l|l|l|l|l|l|l|}
\hline
Isolates & PCR & & & & & & & & & & & \\
\hline
& IS1245 & IS901 & IS6110 & Spol. & & & & & & & & \\
\hline
\textit{M. avium} subsp. \textit{avium} & Pos & Neg & Pos & SB0140 & 2 & 3 & 4 & 3 & 1 & 2 & 2 & 9 & INMV5 \\
\textit{M. avium} subsp. \textit{hominissuis} and \textit{M. bovis} & Pos & Neg & Pos & SB0140 & 2 & 2 & 4 & 3 & 1 & 2 & 2 & 9 & INMV4 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|l|l|l|l|l|}
\hline
\textit{M. avium} subsp. \textit{avium} (\(n = 29\)) & & & & & & & & \\
\hline
Locus & 1 & 2 & 3 & 4 & 7 & 9 & Allelic diversity & \\
\hline
X3 & 2 & 17 & 10 & 0.55 & \\
25 & 29 & 0 & \\
3 & 1 (33) & 0.11 & \\
32 & 7 (29) & 0.29 & \\
\hline
\end{tabular}
\end{table}

\begin{table}
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\begin{tabular}{|l|l|l|l|l|l|l|l|l|}
\hline
\textit{M. avium} subsp. \textit{hominissuis} (\(n = 6\)) & & & & & & & & \\
\hline
Locus & 1 & 2 & 3 & 4 & 7 & 9 & Allelic diversity & \\
\hline
X3 & 5 & 1 & 0.33 & \\
25 & 6 & 0 & \\
47 & 6 & 0 & \\
\hline
\end{tabular}
\end{table}

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

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|l|l|l|l|l|}
\hline
\textit{M. avium} subsp. \textit{avium} and \textit{M. avium} subsp. \textit{hominissuis} isolates & & & & & & & & \\
\hline
Locus & 1 & 2 & 3 & 4 & 7 & 9 & Allelic diversity & \\
\hline
X3 & 2 & 17 & 10 & 0.55 & \\
25 & 29 & 0 & \\
3 & 1 (33) & 0.11 & \\
32 & 7 (29) & 0.29 & \\
\hline
\end{tabular}
\end{table}
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 IS901, whereas MAA or MAS are positive for this sequence. Locus 292 provides information to

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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).
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 \textit{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 \textit{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 \textit{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 \textit{M. avium} and \textit{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.

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