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Bovine tuberculosis in domestic pigs: Genotyping and distribution of isolates in Argentina

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ABSTRACT

Bovine tuberculosis is caused by *Mycobacterium bovis* and affects primarily cattle, among many other mammal species. In this study, 250 isolates of *M. bovis* collected from pigs slaughtered in Argentina were typed by spoligotyping. Over half of the isolates (66%) grouped into two spoligotypes.
Moreover, SB0140 was the most frequent spoligotype detected in the three performed samplings. In addition, 195 isolates were typed through variable number of tandem repeats (VNTR) by selecting 7 loci (MIRU 16–26–31 and ETR A–B–C–D). The relationship among the patterns was performed using a goeBURST algorithm and the main clonal complexes grouped 110 isolates (56%). Although pigs shared genotypes with cattle (n = 21), some patterns were detected only in pigs (n = 14). These findings suggest the pig as a source of *M. bovis* infection to cattle.

1. Introduction

Bovine tuberculosis (BTB) is caused by *Mycobacterium bovis* (*M. bovis*), a member of *Mycobacterium tuberculosis* complex (MTBC). *M. bovis* infects a wide range of wild and domestic mammals, which play different epidemiological roles in the transmission and persistence of the disease; its epidemiology depends on several factors such as genetics, population density, disease prevalence and ethological characteristics (Nugent et al., 2011; Pesciaroli et al., 2014). Pigs are susceptible to different mycobacteria, including *Mycobacterium avium*, which is the main species identified in countries where BTB is very low or is eradicated (Agdestein et al., 2012; Eisenberg et al., 2012). By contrast, in developing countries, where the disease is endemic in cattle, *M. bovis* is the most frequently isolated species in pigs (O'Reilly and Daborn, 1995; Parra et al., 2003; Muwonge et al., 2012).

The prevalence of BTB is estimated by detection of macroscopic tuberculosis lesions during the slaughterhouse inspection and, in Argentina, is 0.3% (SENASA, 2014). Usually, pigs are considered a final host or spillover in the transmission of the BTB; however, no molecular study has described the function and distribution of the disease in this species (Nugent et al., 2011). In Argentina, we have studied the diversity of spoligotypes of *M. bovis* from pigs (Barandiaran et al., 2011). In other work we have assessed tuberculosis produced by *M. avium* complex and also by *M. bovis*. We have was detected a significant number of animals coinfected with these two species (Barandiaran et al., 2015).

The genotyping of *M. bovis* in different hosts has contributed to a better understanding of the transmission of BTB and to the identification of wild reservoirs contributing with BTB control (de Lisle et al., 2001).

The molecular typing methods most commonly used for the members of the MTBC are the spoligotyping and the VNTR (McLernon et al., 2010). Spoligotyping has demonstrated to be a rapid and profitable cost-effective first-line typing method of *M. bovis* (Zumárraga et al., 2013). Furthermore, typing by VNTR is feasible and has a good power of discrimination for the strains (Roring et al., 2004; Allix et al., 2006; McLernon et al., 2010).

Because the discriminatory power of spoligotyping is moderate, a group of strains with the same type are not necessary identical. In this context, the aim of this study was to perform the molecular typing using spoligotyping and VNTR (MIRU–ETR) typing to assess the genetic diversity and geographical distribution of *M. bovis* isolates from pigs in Argentina. This study will contribute with the knowledge of the epidemiology of the BTB in pigs from our country, where these data are still limited.

2. Materials and methods

2.1. Samples

The *post-mortem* inspection of pig carcasses was performed in three slaughterhouses located at the Buenos Aires province; two samplings

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were made: 2007–2009 and 2011–2013. Thirty eight isolates obtained between 1994 and 1996 were also studied.

The pigs came from Argentinian provinces: La Pampa, Santa Fe, Córdoba, Entre Ríos, Buenos Aires and Mendoza. These areas centralized most part (80%) of livestock production in the country. The presence of macroscopic granulomatous lesions was considered as a criterion for selection of samples. The head and mesenteric lymph nodes as well as the liver and the lung were routinely examined; gross lesions in other locations were also recorded. Approximately 10 g of the lymph nodes with gross pathological lesions was ground in a sterile bag and transported to the laboratory in insulated boxes with ice packs at 4 °C.

2.2. Bacterial cultures

The tissue sample was ground with sterile sand and water in a mortar, subsequently decontaminated according to Petroff's method using 4% NaOH, and cultured in Stonebrink media at 37 °C for 60 days (Jorge et al., 2005). The developed colonies were subjected to Ziehl–Neelsen stain for detection of acid-fast bacilli (AFB).

2.3. Molecular identification of M. tuberculosis complex

The DNA was obtained from colonies by suspending them in 250 μL of distilled water and then heated at 96 °C for 45 min. After centrifugation at 12,000 *g* for 5 min, 10 μL of the supernatant was used as template for PCR. The positive Ziehl–Neelsen micobacteria were analyzed by IS*6110*-PCR to identify the MTBC as described by Hermans et al. (1990).

2.4. Molecular typing

2.4.1. Spoligotyping

To detect and type *M. bovis* isolates spoligotyping (Kamerbeek et al., 1997) was carried out by using the spoligotyping kit (Isogen Biosolutions B.V., Ocimum Biosolutions Company, Hyderabad, India) to identify *M. bovis* and to differentiate intraspecies. The scanned images of the films were analyzed using BioNumerics (Version 3.5, Applied Maths, Sint-Martens-Latem, Belgium). The patterns were compared with the *M. bovis* spoligotypes stored at the database of the Institute of Biotechnology, INTA, Argentina, and at the www.mbovis.org database from the Animal and Plant Health Agency (APHA), United Kingdom. The spoligotypes were named according to the APHA code (SB).

2.4.2. VNTR typing

VNTR typing was performed to increase the degree of differentiation of the isolates. We selected MIRU 16, 26 and 31 (Supply et al., 2000) because these MIRUs have shown the highest discrimination power among the *M. bovis* isolates from Argentinian cattle, in accordance with other publications (Roring et al., 2004; Boniotti et al., 2009; McLernon et al., 2010). The MIRUs were amplified using the primers MIRU 16 (F TCGGTGATCGGGTCCAGTCCAAGTA and R CCCGTCGTGCAG CCCTGGTAC), MIRU 26 (F TAGGTCTACCGTCGAAATCTGTGAC and R CATAGGCGACCAGGCGAATAG) and MIRU 31 (F ACTGATTGGCTTCATA CGGCTTTA and R GTGCCGACGTGGTCTTGAT). The PCR thermal profile consisted of an initial denaturation at 96 °C for 3 min and 35 cycles of denaturation, annealing and extension of 96 °C for 1 min, 55 °C for 1 min and 72 °C for 45 s, respectively. Six microliters of the PCR product was subjected to horizontal electrophoresis in a 3% agarose gel (Ultra Pure Agarose 1000, Invitrogen, USA) with ethidium bromide (0.5 μg/mL) in 1× TBE buffer for 4 h at 95 V. A 100 bp DNA Ladder (Promega Corp., USA) was used as a molecular marker. The size of the amplicons was estimated by comparing with the molecular weight marker and by using the image analysis program BioNumerics (Applied Maths, Belgium). We limited the analysis to the exact tandem repeat ETR-A to D (Frothingham and Meeker-O'Connell, 1998). Multiplex PCRs were used combining primer pairs: ETR-A/B and ETR-C/D. The PCR mix was prepared in 96-well plates with the Hot Start Mastermix kit (Qiagen, Germany). For each multiplex mixture, only one primer of each pair was tagged with a different fluorescent dye ETR-A (F; AAAT CGGTCCCATCACCTTCTTA-FAM and R; CGAAGCCTGGGGTGCCCGCGAT TT), ETR-B (F; GCGAACACCAGGACAGCATCAT-JOE and R; GGCATGCC GGTGATCGAGTGG), ETR-C (F; GTGAGTCGCTGCAGAACCTGCAG-(HEX) and R; GGCGTCTTGACCTCCACGAGTG) and ETR-D (F; CAGGTCACAACG AGAGGAAGAGC-FAM and R; GCGGATCGGCCAGCGACTCCTC). The PCR thermal profile for the two multiplex reactions consisted of an initial denaturation at 95 °C for 12 min; 35 cycles of denaturation, annealing and extension of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min, respectively, and a final extension of 72 °C for 10 min.

Polymerase chain reaction amplifications were subjected to capillary electrophoresis analysis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The molecular marker used was GS500-250 (ROX). The allele was established using the GenMapper software version 3.7 (Applied Biosystems Foster City, CA, USA).

The VNTR genotype of a strain, which represents the number of repeat elements at each locus, is presented as a series of four integers between 1 and 12 separated by hyphens. The variants of an integer were marked by an asterisk (*).

2.5. Discriminatory power

The discriminatory index (D) described by Hunter and Gaston and expressed by the formula of Simpson was calculated to determine the discriminatory power of spoligotyping and VNTR (MIRU and ETR) techniques (http://insilico.ehu.es) (Hunter and Gaston, 1988).

2.6. Genetic relationship of the strains

To analyze and visualize the hypothetical relationship of genetic patterns of the strains, we have applied a goeBURST algorithm using the PhyloViz free software (Francisco et al., 2012). The combination of each spoligotype with the VNTR (MIRU and ETR) was denoted with a type number (ST). For the spoligotyping, each spacer was considered as a character.

3. Results

Three hundred and ten samples with lesions compatible with tuberculosis (LCTB) were collected. The samples were cultured and development of colonies was observed in two hundred fifty samples.

All the isolates were positive for the Ziehl–Neelsen staining and IS*6110*-PCR.

3.1. Spoligotyping

The isolates were obtained from six different provinces, Buenos Aires (n = 132), Córdoba (n = 60), Santa Fe (n = 45), Entre Ríos (n = 8), La Pampa (n = 4) and Mendoza (n = 1) (Fig. 1). All isolates were positive for IS*6110*-PCR and showed the characteristic pattern of the *M. bovis* strains by spoligotyping. The isolates were distributed into 35 spoligotypes. Most (n = 143) of the 250 isolates of *M. bovis* have been previously described by Barandiaran et al. (2011) and were included in this study to complete the typing with VNTR and with a comparison with patterns of cattle and humans.

The novel spoligotypes (SB2192, SB2189 and SB2350) were incorporated into the spoligotype database of the APHA (http://www.mbovis.org).

Most of the isolates (93.2%: 233/250) grouped in 18 clusters, with at least two isolates, whereas a small minority (6.8%: 17/250) were unique (Table 1 and Fig. 1). The main spoligotype was SB0140 and grouped 56.8% (142/250) of the isolates. Seventeen patterns were unique, of which five were described previously in cattle from Argentina and 12 were reported for the first time in the country (SB1788, SB1784, SB1786, SB1600, SB0849, SB0859, SB1247, SB2192 SB2189, SB0121,

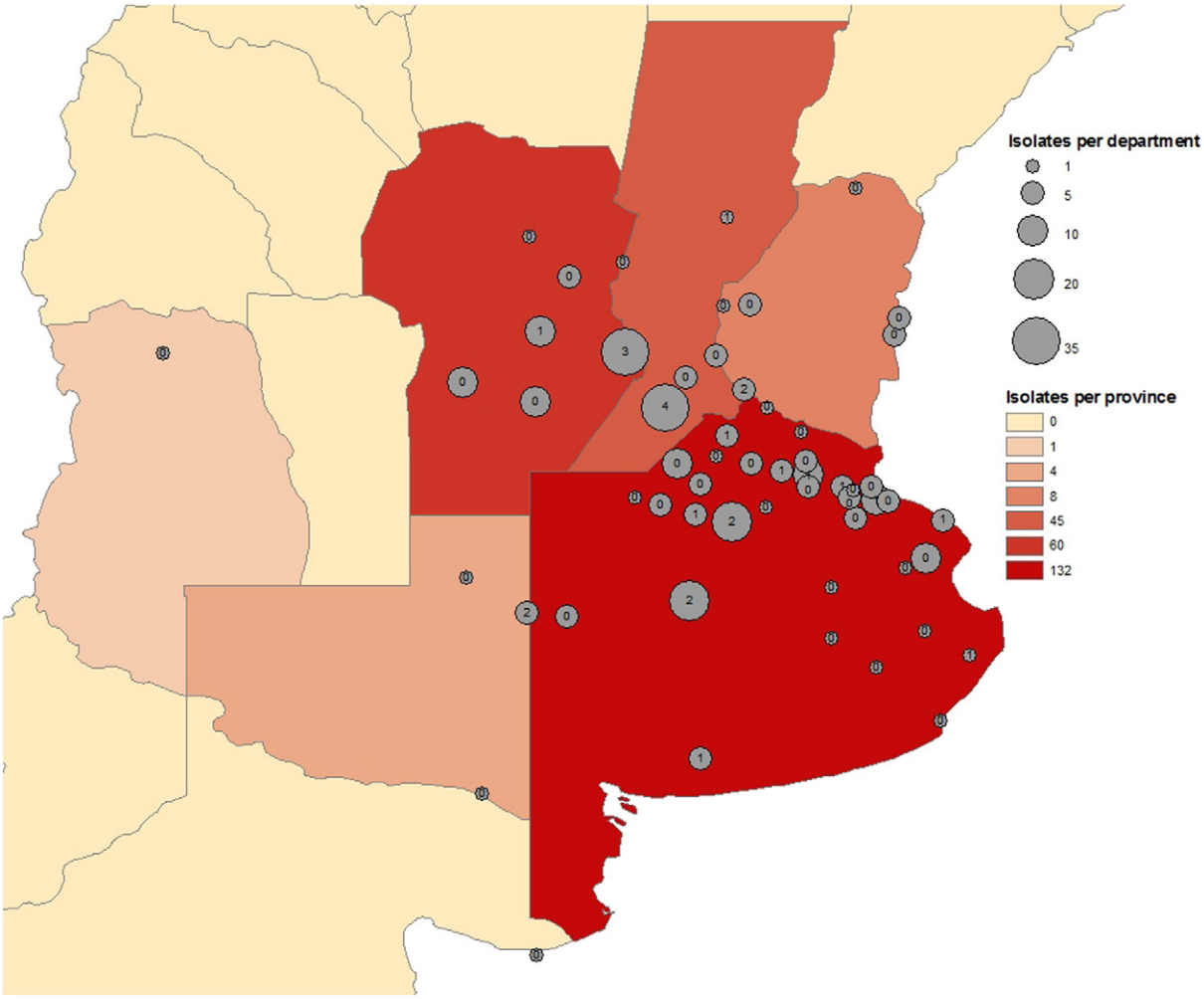

Fig. 1. Geographical origins of the 250 *M. bovis* isolates typed by spoligotyping. The number of isolates from each province was denoted in color. The size of the gray circle is related with the number of isolates by department. The number of isolates clustered with the most frequent pattern (ST41) is detailed inside of the gray circle (ArcGIS, v. 10·0; ESRI, USA).

SB0141 and SB2350). Additionally, four clusters, one with three isolates and the others with two isolates, were present in pigs but not in cattle (SB1779, SB1652, SB0290 and SB0486). All animal clustering were from relatively proximate farms. Comparing the patterns of the three temporal samplings, we detected 5 spoligotypes present in the three periods (Fig. 1). The most frequent spoligotype in cattle from Argentina (SB0140) was prevalent in the three temporal samplings. Table 1 shows the spoligotypes detected in each sampling.

Table 1
Spoligotypes detected among the 250 *M. bovis* isolates.
The novel spoligotypes are in bold, whereas the isolates not previously observed in cattle or/and not previously detected in Argentina are in gray.

Spoligotype	Period			Spoligotype	Period			Spoligotype	Period		
	1994–1996	2007–2009	2011–2013		1994–1996	2007–2009	2011–2013		1994–1996	2007–2009	2011–2013
SB0140	20	106	16	SB1652		2		SB0859		1	
SB0130	6	17	0	SB1787		2	1	SB2189		1	
SB0484	2	9	1	SB0486		2		SB1066	1		
SB0120	1	8	1	SB1779		2	1	SB1600		1	
SB0145	2	3	1	SB0520		2		SB0849		1	
SB0990		5		SB0980		2		SB1784		1	
SB0131		5		SB1048	1			SB1786		1	
SB0153	1	3	1	SB0121	1			SB0271		1	
SB1055		3		SB2192		1		SB1047		1	
SB1049	3			SB1247		1		SB1788		1	
SB0856			1	SB0269			3	SB0141			1
SB0290			2	SB2350			1				

3.2. Spoligotyping and VNTR (MIRU and ETR) combined typing

The combination of the three methods yielded 89 different patterns (single types, ST) among the 195 *M. bovis* typed isolates (Table 2).

The main spoligotype SB0140 was divided in 34 different types of VNTR. The most common cluster was ST41 (SB0140, MIRU 2–5–3, ETR 6–5–5–4*) with 26 (13.3%) isolates, followed by ST47 (SB0140, MIRU 2–5–3, 7–5–5–4*) with 25 (12.8%) isolates (Table 2 and Fig. 1). The difference among these two clusters is due to ETR A. Finally, mixed isolates were observed in one animal from General López, province of Santa Fe, with two different patterns on loci MIRU 16–26–31 and ETR A.

3.3. Discriminatory power of the techniques and allelic diversity at each locus

The global discriminatory index (D) obtained with the spoligotyping and the seven VNTRs combined was 0.96 (Table 3). In addition, the most polymorphic loci were ETR A (D = 0.68), MIRU 26 (D = 0.41) and ETR B (D = 0.31). The lower diversity indexes were obtained with ETR C (0.10) and D (0.07).

3.4. Genetic relationship of the strains

To show the hypothetical relationship between the *M. bovis* isolates, we built a clonal complex (CC) analysis using goeBURST algorithm taking into account the 43 character types of the spoligotypes and the 7 VNTR (MIRU–ETR) loci. The CC was performed setting the maximum difference allowed within a group as single locus variant (SLV). A total of 8 CC and 23 singletons were detected among the 89 ST. The main CC involved 32 ST and grouped 110 isolates (56%). In this group, the putative founder genotype (PFG) was ST41 (Fig. 2).

Table 2
Clustered ST detected among the 195 *M. bovis* isolates typed by spoligotyping and VNTR (MIRU and ETR).

ST	Spoligotypes	MIRU 16	MIRU 26	MIR U31	ETR A	ETR B	ETR C	ETR D	No. isolates
5	SB1055	3	5	3	3	3	5	4*	3
8	SB0120	3	4	3	6	5	5	4*	4
10	SB0131	2	6	3	7	5	5	4*	2
14	SB0130	2	3	3	7	5	5	4*	3
16	SB0130	2	5	3	7	5	5	4*	2
19	SB0130	2	6	3	6	5	5	4*	2
20	SB0130	2	6	3	5	5	5	4*	2
22	SB0130	2	6	4	6	5	5	4*	2
23	SB0484	2	5	3	7	4	5	4*	2
24	SB0484	2	5	3	7	5	5	4*	4
25	SB0484	2	5	3	5	5	5	4*	2
26	SB0484	2	5	3	6	5	5	4*	3
31	SB0140	2	4	3	7	5	5	4*	2
33	SB0140	2	5	3	4	5	5	4*	2
35	SB0140	2	5	3	4	7	5	4*	2
37	SB0140	2	5	3	5	5	5	4*	16
40	SB0140	2	5	3	6	4	5	4*	2
41	SB0140	2	5	3	6	5	5	4*	26
44	SB0140	2	5	3	6	9	5	4*	3
47	SB0140	2	5	3	7	5	5	4*	25
51	SB0140	2	6	3	6	5	5	4*	4
52	SB0140	2	6	3	7	5	5	4*	2
56	SB0140	3	5	3	6	5	5	4*	3
61	SB0520	2	6	3	7	5	5	4*	2
62	SB0980	2	5	3	7	5	4	4*	2
63	SB0990	2	5	3	4	5	5	4*	4
68	SB1779	2	5	3	6	5	4	4*	2
81	SB1049	2	5	3	7	5	5	4*	3
82	SB0130	2	5	3	6	5	5	4*	2
88	SB0153	2	5	3	7	5	5	4*	2
89	SB0145	2	5	3	6	5	5	4*	2

4. Discussion

The endemic presence of BTB in cattle and pigs in Argentina limits the economic potential of the productive sectors as well as the international trade. These limitations affect negatively the profitability of farms and the quality of animal products and sub products. In this study we aimed to gain some insights into the current situation of *M. bovis* infection in pigs from the main productive area from Argentina. For this purpose, we obtained and typed 250 isolates, from samples with LCTB from pigs inspected in different slaughterhouses from Argentina. Most of the isolates (93.2%) were clustered by spoligotyping (Table 1). The VNTR (MIRU–ETR) analysis demonstrated the existence of a large genomic diversity within pig population, where 70.25% (137/195) were kept in groups (Table 2).

The combination of all the molecular markers yielded a discriminatory index of 0.96. However, when only the most polymorphic loci (MIRU 26, ETR A and ETR B) were selected combined to spoligotyping, the value was almost invariable (0.95) at the complete scheme of analysis (Table 3). This behavior is similar to that observed by other authors who recommend a reduced panel of VNTRs to type *M. bovis* (Roring et al., 2004; Allix et al., 2006; Boniotti et al., 2009; McLernon et al., 2010).

Seventy eight percent of the clustered spoligotypes of pigs were detected in cattle, which suggests the active transmission of BTB between the two species as described by Pesciaroli et al. (2014). Furthermore, the first (SB0140) and second (SB0130) most common spoligotypes detected in cattle (Zumárraga et al., 2013) showed the same frequency in pigs.

Only the patterns of VNTR-ETR found in cattle could be compared because of the scarce analysis reports with VNTR-MIRU available in the country. From this analysis, the most frequent ETR pattern (7–5–5–4*) followed by 6–5–5–4* had been previously identified in cattle (Shimizu et al., 2014). In the isolates from cattle of Argentina, all the analyzed strains hold the allelic variant 4* in ETR D (Shimizu et al., 2014), as we have observed in pigs in this study.

Secondly, the presence of circulating clones is evident, since strains from the same region maintain the same genotype. Regarding goeBURST analysis, the main clonal complex grouped 56% of the isolates and involved at 32 ST. The putative founder of the CC was ST41. This ST was related with 12 ST and also was the most frequent (n = 26) among the 195 studied isolates, although other authors have described that the PFG was not the most frequent genotype (Rodriguez-Campos et al., 2013).

ST5 grouped 3 animals that came from the same department, whereas ST61 and ST62, with 2 isolates each, came from pigs from the same province. In addition, 80% (4/5) of the isolates grouped in ST63 belonged to the bordering provinces. Moreover, ST81, a very rare ST in cattle from Argentina (Zumárraga et al., 2013), came from pigs from the same region. These genetic and spatial clusters would indicate the presence of clones within these geographic areas.

The comparison of the genotypes of pigs and bovines from Argentina suggests that bovines are the main source of *M. bovis* infection for pigs. Further, most of the observed lesions were in the digestive tract due to feeding with contaminated dairy products (O'Reilly and Daborn, 1995; Nugent et al., 2011). Also, in other countries, the prevalence of BTB in pigs is associated with the prevalence in cattle (Corner, 2006; Nugent et al., 2011). However, we cannot discard the possible role of the pig in maintaining the disease among the cattle. Moreover, we have detected genotypes particular of pigs in the country, twelve with a single isolate each and four clustered. One of them, the spoligotype SB0486, was detected in the APHA database as corresponding to a sample taken in 1995 from an Argentinian pig coming from the same slaughterhouse. This spoligotype has not been detected in Argentinian cattle in the last 19 years. The presence of new *M. bovis* genetic profiles among pigs suggests that the BTB is self-maintained in this population (Pesciaroli et al., 2014; Di Marco et al., 2012).

Table 3
Discriminatory power of spoligotyping and VNTR (MIRU and ETR).

	Spoligotyping	VNTR (MIRU 16, 26, 31 and ETR A, B, C, D)	Spoligotyping and VNTR (MIRU 16, 26, 31 and ETR A, B, C, D)	Spoligotyping and VNTR (MIRU 26 and ETR A, B)
Patterns	30	58	89	79
Unique types	17	34	58	46
Clusters	13	24	31	33
Main cluster	110	41	26	28
Clustered isolates	179 (91.32%)	162 (82.65%)	138 (70.40%)	150 (76.53%)
Discriminatory index	0.67	0.91	0.96	0.95

Furthermore, we have also observed disseminated lesions. This characteristic would favor the elimination of the bacillus increasing the likelihood of transmission between pigs of the same species, between pigs and cattle, and as environmental pollution (Di Marco et al., 2012).

Molecular epidemiological studies in Mediterranean Spain suggest that crossinfection occurs between wild boar and domestic pigs in the absence of cattle (Parra et al., 2003; Naranjo et al., 2008). Therefore, in Spain wild boar are considered important maintenance host and a significant source of *M. bovis* infection in domestic cattle (Parra et al., 2003; Martín-Hernando et al., 2007; Naranjo et al., 2008; Santos et al., 2009). Moreover, in the last years, a *M. bovis* strain (SB0140) isolated from a wild boar from Argentina has been described as very virulent in the mouse model as well as in guinea pig and cattle (Aguilar León et al., 2009; Meikle et al., 2011). In certain areas of Portugal and Italy, the domestic pig is considered a possible reservoir of BTB as well (Boadella et al., 2012; Di Marco et al., 2012). On the other hand, in New Zealand, Australia and Great Britain, pigs are considered a terminal host (Corner, 2006, Nugent et al., 2011) and could have a sentinel role to monitor the presence of *M. bovis* in other species (Nugent et al., 2011). This eco epidemiologic scenario has not been studied in Argentina yet.

In the production systems of Argentina, pigs spend most of their productive life in the field, in most cases in contact with cattle. This behavior is more similar to that of the wild pigs seen in the Iberian Peninsula, contrary to what happens in intensive production conditions in developed countries, where they never have contact with cattle (Bailey et al., 2013).

In Argentina, 80% of the production is run by small families and swine activity is complementary to a primary dairy activity or meat-cattle production; facilities, furthermore, do not prevent fluid contact between these species and often share common spaces in the same establishment, thus increasing the rate of transmission (Perez et al., 2004; de Kantor and Ritacco, 2006). Moreover, it is necessary to stand out the importance of this zoonotic disease in its transmission by food, especially when cooking insufficiently contaminated meat, or when elaborating sausages with raw material from infected animals (Pate et al., 2008). Certainly, in most documented cases of *M. bovis* in humans in Argentina, the direct contact with animals or associated work activities was involved with the infection (Etchechoury et al., 2009).

In this study, the finding of a significant number of lesions detected in slaughterhouses with bacteriological and molecular *M. bovis* confirmation shows a potential risk of contracting the disease for human population, especially pig handlers. Furthermore, the presence of *M. bovis* is not always correlated with the presence of macroscopic lesions in the pig and its presence therefore is underestimated in many cases (di

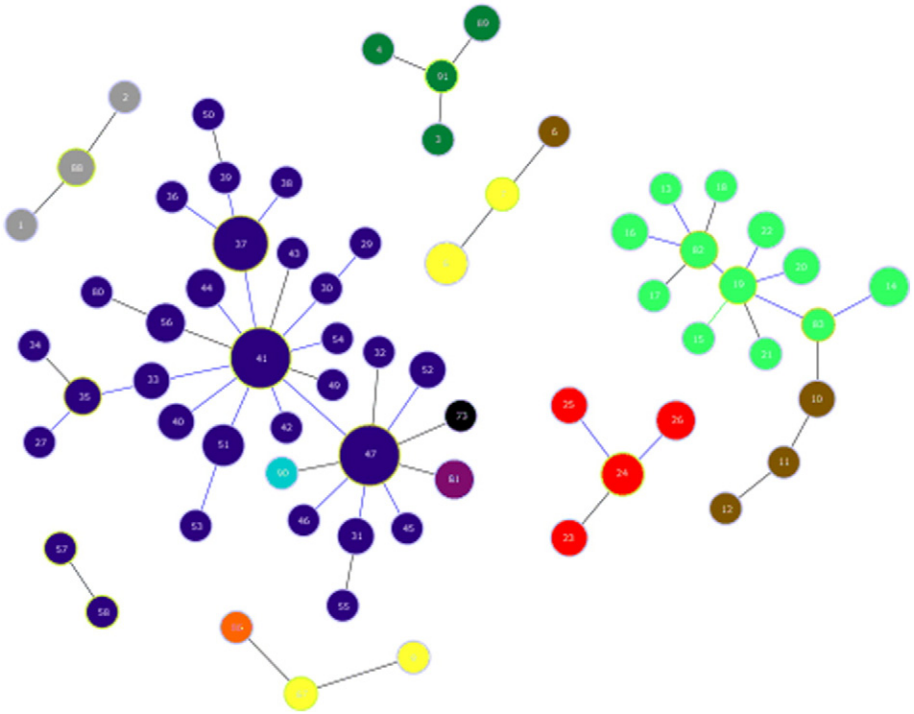

Fig. 2. *M. bovis* ST relationship among the 195 *M. bovis* isolates typed by spoligotyping and VNTR (MIRU–ETR) using goeBURST. Clonal complexes (CCs) were built based on ST linkage by SLV criteria. Singletons were excluded. Spoligotype distribution among CCs: Blue: SB0140; light green: SB0130; red: SB0484; yellow: SB0120; brown: SB0131; dark green: SB0145; purple: SB1049; gray: SB0153; light blue SB1066; orange: SB0121; and black: SB0271.

Marco et al., 2012). Pesciaroli et al. (2012) detected 4 animals with no visible lesions using IFN-γ and only in one case could isolate *M. bovis*. Moreover these researchers believe that bacterial culture of clinical samples cannot be considered the gold standard to diagnose *M. bovis* infection due to an intrinsic lack of sensitivity (Pesciaroli et al., 2012). We have detected nine spoligotypes from pigs shared with humans in Argentina: SB0140, SB0856, SB0269, SB0153, SB0145, SB0131, SB0130, SB1047 and SB0520. The two most common patterns in pigs are those observed in higher proportion in human samples.

The information from this study may contribute to a better understanding of the epidemiological role of pigs in the transmission of tuberculosis in Argentina. In addition, because pigs are susceptible to tuberculosis and free zones of the disease are delimited this species might act as sentinel species to monitor the presence of *M. bovis* in cattle.

This study is pioneer in the study of bovine tuberculosis in pigs in Argentina because it has provided enough molecular epidemiology information to contribute to the National Program of Control and Eradication of bovine tuberculosis. Future investigations will be necessary to assess the real epidemiological role the pigs play in our country.

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