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Research Article

Mycobacterium bovis in Swine: Spoligotyping of Isolates from Argentina

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A total of 143 *Mycobacterium bovis* isolates of pigs, from the most productive swine area in Argentina, were typed by spoligotyping. Twenty-two different spoligotypes were identified, and 133 (93%) isolates were grouped into 12 clusters. One of them, designed SB0140, was the most frequent because it held 83 (58%) isolates. This spoligotype also grouped 362 (43%) out of 841 isolates from previously typed cattle and, thus, constitutes the most frequent in our country. In addition, 135 (94%) isolates revealed spoligotypes identical to those of cattle, showing an epidemiological link. On the other hand, there were seven novel spoligotypes, six of which were also unique since they had only one isolate each. This study aimed to identify the spoligotypes of *M. bovis* isolated from pigs to contribute to a better understanding of the distribution of bovine tuberculosis in the main productive area of Argentina.

1. Introduction

Bovine tuberculosis (BTB) is a major livestock disease in Latin America. About 70% of the cattle bred are held in areas with high disease prevalence and nearly 17% in areas virtually free from tuberculosis [1].

In Argentina, between 1969 and 2004, an average of 10 million bovine carcasses was annually submitted to official veterinary inspection. During that period, the percentage of animals condemned for tuberculosis decreased from 6.7% to 1.2% [1]. Regarding human tuberculosis, the disease caused by *Mycobacterium bovis* and that caused by *M. tuberculosis* are clinically indistinguishable. A recent work has described an incidence ranging from 0.7% to 6.2% in a main milk region of Argentina where most patients were closely related to rural activities, and a much lower national prevalence [2]. Between 1980 and 2003, the incidence of tuberculosis in Santa Fe, a province with 21% of the national dairy herd and 34% of the nation's milk production, declined from 48.1 to 20.7 per 100,000 [1]. However, the number of cases due to *M. bovis* remained stable, thus suggesting the relatively constant risk of infection [1]. In the Muñiz Hospital of Buenos Aires city, the percentage of *M. bovis* cases not associated with HIV infection significantly decreased from 0.95% between 1981 and 1991 to 0.22% between 2000–2006, whereas in the HIV/AIDS group the decrease was less pronounced (0.83% to 0.58%) [3]. The first case of person-to-person transmission of *M. bovis* in Argentina has been recently described [4].

In the United States, BTB was the most prevalent infectious disease in bovines and pigs between the XVIII century and the beginning of the XIX century, and the production loss was higher than that caused by all other diseases grouped [5].

Pigs are susceptible to *M. tuberculosis*, *M. bovis*, and the *M. avium* complex. In Argentina, the information about incidence and prevalence of tuberculosis in pigs is scarce. However, *M. bovis* is considered the primary cause of their

infection. The prevalence of lesions found in slaughterhouses from the Pampa Húmeda region during three samplings performed in 1986, 1995, and 1997, were 6.4%, 1.2%, and 0.1%, respectively, and *M. bovis* was the bacterial agent most frequently isolated in the three cases [6]. In countries where BTB has not been eradicated, such as in Argentina, pigs often become infected from cattle by oral route through ingestion of milk or other dairy products [6, 7]. Therefore, in Argentina, swine tuberculosis underwent a decline parallel to that observed in local cattle [1, 6, 8] because the eradication of BTB from cattle is the main action to avoid the transmission to swine and other hosts. Thus, the percentage of pigs condemned for tuberculosis decreased from 8.4 to 0.7% between 1969 and 2005 [9]. This panorama of the disease is also attributed to the modernization and intensification of breeding systems, which has improved the swine health conditions [9, 10]. Furthermore, in February 2009, pigs were incorporated to the National Control and Eradication Program of Bovine Tuberculosis, implemented for bovines since 1999 [11, 12]. This new resolution considers that those herds with negative skin test results with PPD once a year and the absence of tuberculosis lesions during carcass inspections in slaughterhouses are free of infection [11]. Although the skin test with PPD has a limited usefulness for individual animal diagnosis, it is a good tool to detect infected herds [13].

Approximately 80% of the swine production in Argentina is concentrated in the central region (Buenos Aires, Santa Fe, and Córdoba provinces), and it is closely related to maize production, because pigs are fed mainly on corn (65%) and soy flour (25%).

Molecular epidemiology is a helpful tool that contributes to the understanding of the dynamics of the distribution and spreading of BTB between the different hosts. One of the techniques that has improved and simplified the typing of the *M. tuberculosis* complex is spoligotyping [14]. Spoligotyping is a PCR-based method complemented with reverse line blot hybridization, in which the polymorphism consequence of rearrangements of the direct repeat (DR) region, which is composed of perfect direct 36-bp repeats and variable spacers [14]. The combination of the presence and absence of each 43 representative spacer sequences, shown with spots, represents a spoligotype.

The spoligotyping database of the Biotechnology Institute (BI) of INTA, Argentina, contains the spoligotypes of 1188 *M. bovis* isolates from different hosts from Argentina typed since 1996 and constitutes a valuable source of molecular epidemiology information that may help to contribute to the eradication program of bovine tuberculosis.

The aim of this work was to determine the *M. bovis* spoligotypes circulating among pigs from the main porcine productive region of Argentina.

2. Materials and Methods

A total of 143 isolates of *M. bovis* were obtained from culture of lymph nodes and viscera samples with tuberculosis compatible lesions from pigs from Buenos Aires ($n = 66$), Córdoba ($n = 44$), Entre Ríos ($n = 6$), La Pampa ($n = 3$), Mendoza ($n = 1$), and Santa Fe ($n = 23$) provinces (Figure 1), following a convenient sampling. Samples were obtained from the veterinary inspection of 35,000 pigs, between July 2007 and November 2008. The slaughterhouses involved in this sampling were located in Merlo, Moreno, and Tandil cities from Buenos Aires province. The samples were decontaminated using the Petroff's method and cultured in Lowenstein-Jensen and Stonebrink media at 37°C for 60 days [15]. The bacteriological typing of the isolates was performed based on the culture media, incubation temperature, growing time, colony morphology, and Ziehl-Neelsen stain.

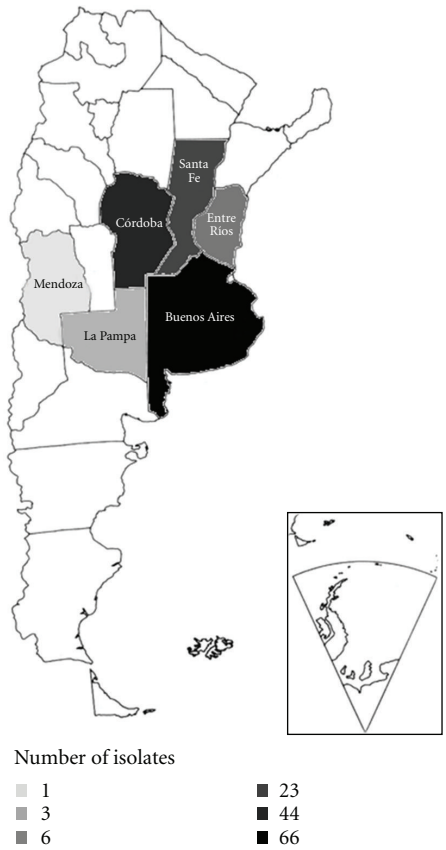

Figure 1: Map of Argentina. The provinces sampled are denoted in a gray scale according to the number of isolates.

Each *M. bovis* strain was isolated from independent animals without apparent epidemiological linkage. A loopful of colonies was transferred into a microcentrifuge tube containing 250 μL of distilled water and heated at 96°C for 45 min. Colonies were then centrifuged at 12,000 rpm for 10 min, and 5 μL of the supernatant was used for PCR to amplify the DR region. Spoligotyping [14] was carried out by using the spoligotyping kit (Isogen Biosolutions B.V., Ocimun Biosolutions Company, Ijsselstein, the Netherlands). *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* Bacillus Calmette-Guerin (BCG) (ATCC 27289) were included as reference strains in each spoligotyping

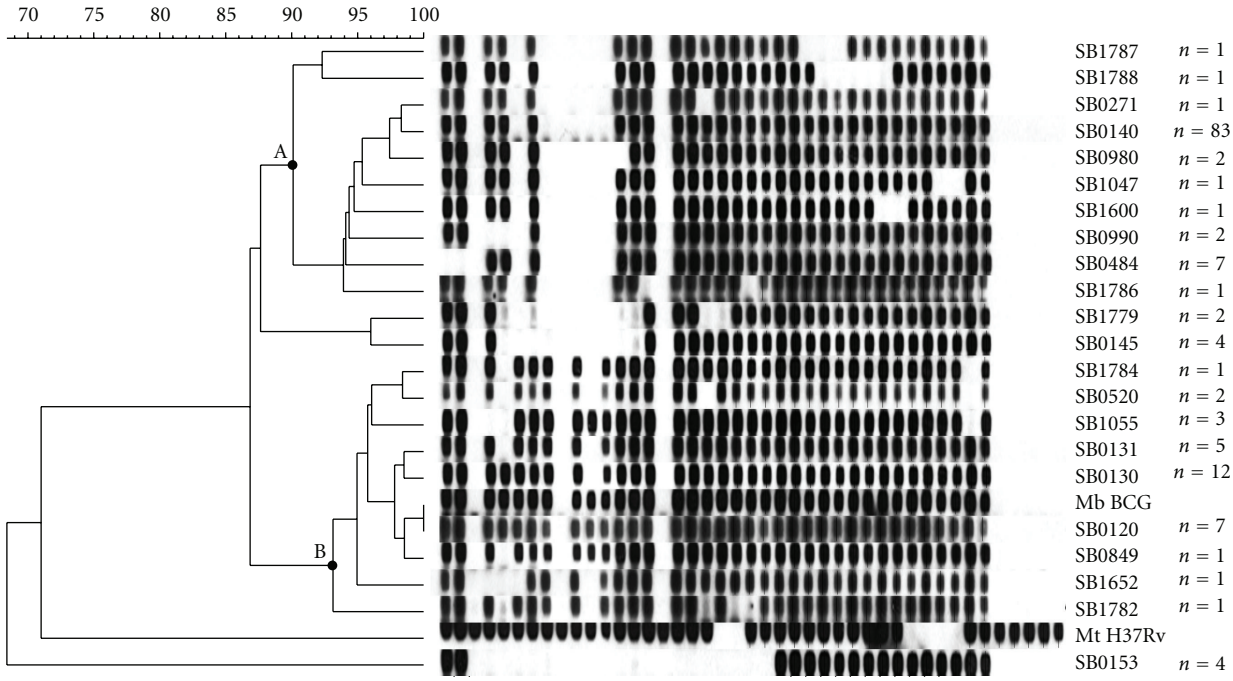

Figure 2: Dendrogram showing the relationship between 22 different spoligotypes identified among 143 *M. bovis* isolates of pigs from Argentina. The spoligotypes are detailed on the right of each pattern. The two families of spoligotypes were designated with letters A and B. *n*: number of isolates.

experiment. A cluster analysis of the spoligotype patterns was performed with the BioNumerics software (Windows NT, version 2.5; Applied Maths, Kortrijk, Belgium). The categorical coefficient was used to calculate the similarity of spoligotype patterns, and the UPGMA (unweighted pair-group method with arithmetic averages) method was applied to calculate a dendrogram. Clusters of isolates were defined as two or more *M. bovis* strains with identical spoligotypes. The different spoligotypes were compared against those included in the BI database, and each was assigned a number (SB code) according to the international database of the Veterinary Laboratories Agency (VLA), the United Kingdom (hosted on University of Sussex servers, http://www.mbovis.org/). When a spoligotype was not found in these databases, it was considered a novel spoligotype. Spoligotypes with only one isolate were regarded as unique spoligotypes. The discriminatory index (D) described by Hunter and Gaston and expressed by the formula of Simpson [16] was calculated to determine the discriminatory power of the spoligotyping in each province (http://insilico.ehu.es/) [17].

3. Results

All the isolates analyzed ($n = 143$) lacked spacers 3, 9, 16, and 39 to 43, characteristic of *M. bovis* strains. Twenty-two different spoligotypes were found among the 143 *M. bovis* isolates studied (Figure 2 and Table 1). One hundred thirty-three (93%) strains were grouped in 12 clusters; one of them (SB1779) was a novel spoligotype because it had not been previously reported among isolates of other hosts from Argentina or in the VLA database. The main cluster involved 83 (58%) isolates and showed spoligotype SB0140 followed by spoligotype SB0130 with 12 (8.4%) isolates. The remaining spoligotypes grouped seven or fewer isolates. Ten (7%) isolates were unique, and seven of them were also novel (SB0849, SB1600, SB1652, SB1784, SB1786, SB1782, and SB1779) (Figure 2 and Table 1). Additionally, the cluster with the novel spoligotype (SB1779) held two isolates from the cities of Río Cuarto and General Viamonte, both in Córdoba province, which are approximately 150 km from each other. The global discriminatory power of the spoligotyping in this study was 0.65. This index was also individually calculated for each province (Table 1), being 0.62, 0.80, 0.60, and 0.40, for Buenos Aires, Córdoba, Entre Ríos and Santa Fe, respectively. These provinces provided most isolates. These results are related to the size of the clusters and the number of spoligotypes.

The dendrogram grouped the spoligotypes into two main families designed A and B, related to a similarity higher than 90% and 93%, respectively (Figure 2). The inclusion of the two most frequent spoligotypes from Argentina (SB0140 and SB0130) in each family is the main feature of these families. Moreover, 70% and 23% of the isolates were grouped in families A and B, respectively.

4. Discussion

Five of the six provinces studied are located in the most productive swine area in Argentina. When we compared the spoligotypes in the BI database, we found that 135 (94%) isolates revealed spoligotypes identical to bovine isolates,

Table 1: Number of isolates of each spoligotype by origin. The novel spoligotypes are in italic. D: discriminatory index. S: number of spoligotypes.

Province number of isolates (%)	Number of isolates by spoligotype																						D	S
	0120	0130	0131	0140	0145	0153	0271	0484	0520	*0849*	0980	0990	1047	1055	*1600*	*1652*	*1779*	*1782*	*1784*	*1786*	1787	1788		
Buenos Aires 66 (46.2%)	5	7	1	40 (60.6%)	1	2		2	1			1	1	1	1	1		1		1			0.62	15
Córdoba 44 (30.7%)	1	5	3	19 (43.2%)	1	2	1	3			2			2			2		1		1	1	0.80	14
Entre Ríos 6 (4.2%)				4 (66.7%)				1	1														0.60	3
La Pampa 3 (2.1%)				2 (66.7%)				1															0.66	2
Mendoza 1 (0.7%)					1																		1	1
Santa Fe 23 (16.1%)	1		1	18 (78.3%)	1					1		1											0.40	6
Total 143	**7**	**12**	**5**	**83**	**4**	**4**	**1**	**7**	**2**	**1**	**2**	**2**	**1**	**3**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	0.65	22

showing an epidemiological link. Furthermore, seven of twenty two (32%) of the spoligotypes detected in pig isolates were detected in *M. bovis* human isolates. Conversely, seven (31.8%) spoligotypes had not been previously detected in cattle or other hosts from Argentina. Moreover, these spoligotypes were not reported in the VLA database. This finding could be due to the partial screening of the bovines with BTB in Argentina or to the existence of *M. bovis* clones circulating exclusively among pigs. Parra et al. [18] also described swine spoligotypes not detected previously in other hosts.

The main cluster detected (SB0140) grouped 43% of the 841 bovine *M. bovis* isolates from Argentina typed between 1996 and 2009 [19, 20]. This spoligotype is also frequent in Australia and New Zealand, which, like Argentina, introduced several cattle breeds in the 19th century from the United Kingdom, where this spoligotype is also prevalent [21]. Furthermore, spoligotype SB0140 was the most frequent in Buenos Aires (60.6%), Córdoba (43.2%), Entre Ríos (66.7%), and Santa Fe (78.3%) provinces, where most *M. bovis* isolates from pigs were obtained. Additionally, this spoligotype grouped 32.5, 38.9, 62.5, and 52.5% of bovine isolates of these provinces, respectively. These provinces concentrate most of the dairy farms from Argentina. Clustering of isolates has been described as an indication of active transmission of BTB [19]. Spoligotype SB0153, which was 68% related to the other types, was not integrated in families A and B and had been previously detected in only 2.7% of total *M. bovis* isolates from Argentina. Curiously, 34% of all the *M. bovis* isolates with spoligotype SB0153 belong to humans.

Taking into account the localization of most of the lesions along the digestive tract of sampled pigs (data not shown), we suggest that the infection via was the digestive route. This could be due to the fact that in Argentina pigs diets are usually supplemented with milk or other dairy derivates without thermal treatment.

Other authors have also found *M. bovis* spoligotypes of cattle in domestic pigs and other hosts (red deer and wild boar), which suggests transmission between species [18, 22, 23].

Spoligotyping is the best option for large-scale screening studies on the distribution of *M. tuberculosis* complex strains [14] and is used worldwide as the first-option typing method for *M. bovis*. Moreover, this technique is useful to identify new types from different host species [24, 25] and to detect preliminary transmission of TB between species. In order to trace transmission chains, it is necessary to perform complementary studies using more discriminatory typing methods such as the recently described VNTRs [26–29].

Future studies must be directed to evaluate the virulence and the fitness of these strains isolated from pigs, since transmission between different host species could be a selective force to increase the virulence of microorganisms. In a previous work carried out in a murine model of tuberculosis, we demonstrated that a particular *M. bovis* strain isolated from a wild boar was the most virulent compared to the *M. bovis* AN5 reference strain and other isolated from cattle and humans [30].

Considering the incidence of *M. bovis* in humans and the prevalence of the disease in cattle, especially in the provinces that hold most dairy herds, pigs could be an additional actor in the transmission chain of bovine tuberculosis to humans and cattle. This potential spillover could be controlled through the recent incorporation of swine to the National Control and Eradication Program of Bovine Tuberculosis of Argentina.

5. Conclusions

Most of the spoligotypes (68%) found in pigs had also been previously detected in cattle. There were seven novel spoligotype detected only in pigs. The most frequent spoligotype among *M. bovis* isolates studied from pigs (SB0140) was also the most prevalent in bovines from Argentina.

This work represents the first large-scale molecular typing study of *M. bovis* isolates from pigs carried out in Argentina and contributes to a better understanding of the features of tuberculosis in pigs in our country.

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