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Understanding the relationship between *Mycobacterium bovis* spoligotypes from cattle in Latin American Countries

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ABSTRACT

Spoligotyping is the most frequently used method for genotyping isolates of *Mycobacterium bovis* worldwide. In the current work, we compared spoligotypes from 1684 *M. bovis* isolates from Argentina (816), Brazil (412), Chile (66), Mexico (274) and Venezuela (116), obtained from cattle, humans, pigs, wild boars, farmed deer, goats, buffaloes, cats, and wild animals. A total of 269 different spoligotypes were found: 142 (8.4%) isolates presented orphan spoligotypes, whereas 1542 (91.6%) formed 113 different clusters. In cattle, SB0140 was the most representative spoligotype with 355 (24.6%) isolates, followed by SB0121 with 149 (10.3%) isolates. Clustering of spoligotypes ranged from 95.2% in Argentina to 85.3% in Mexico. Orphan spoligotypes were also variable, ranging from 23.7% in Mexico to 4.1% in Brazil. A large proportion of spoligotypes were common to the neighboring countries Argentina, Brazil and Chile. In conclusion, despite the diversity of spoligotypes found in the five countries studied, there are major patterns that predominate in these neighboring countries. These clusters may reflect a long-lasting active transmission of bovine tuberculosis or common historical origins of infection.

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1. Introduction

In Latin America, especially in South America, meat and milk are essential products for internal consumption and exportation. Therefore, eliminating diseases that limit the trade of animals and their products is a priority in this region. The cattle population in Latin America has been estimated in 450 million heads, 51 of which are in Argentina, 189 in Brazil, 4.1 in Chile, 13.5 in Venezuela and 30 in Mexico (de Kantor and Ritacco, 2006). About

262 million cattle live in countries with more than 1% prevalence of tuberculosis (TB) (de Kantor and Ritacco, 2006).

Bovine tuberculosis (BTB) is an infectious disease mainly caused by *Mycobacterium bovis*, which affects a wide range of mammals, including humans (Amanfu, 2006). *M. bovis* is a member of the *M. tuberculosis* complex (MTBC), which also includes *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. microti*, *M. caprae* and *M. pinnipedii*.

Tuberculosis in cattle is endemic in Latin America; therefore, almost every country has a tuberculosis control and eradication program (some quite successful) dealing with this disease. In Argentina, for example, the proportion of carcasses condemned because of TB decreased from 6.7% in 1969 to 1.1% in 2005 (de Kantor and Ritacco, 2006; Torres, 2009). The prevalence of the disease is not uniform in this region: Chile is divided into two zones: the control zone in the north with 13.7% prevalence and the eradication

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zone in the south with 0.34% prevalence (SAG and Censo Agropecuario, 1997). A countrywide survey in Venezuela showed an average incidence of 0.09% of tuberculin reactors (WAHID, <http://www.oie.int/wahis/public.php?page=home>). In Mexico, the prevalence of TB in eradication areas has been reduced to 0.01%, whereas in central and south areas, the prevalence is about 2%; nevertheless, the prevalence in dairy areas is much higher (<http://www.senasica.gob.mx>). In Brazil, the prevalence of tuberculin reactors to the caudal fold tuberculin tests in 2004 ranged from 0.4% in the South-eastern region to 3.6% in the Northern region (Roxo, 2005; de Kantor and Ritacco, 2006). Under the assumptions of the programs, lesions identified at slaughter are then sent to the laboratory for microbiological analysis and, in some cases, for spoligotyping of the *M. tuberculosis* complex for epidemiological purposes.

In Argentina, the control strategy is based on the Caudal Fold Skin Test (CFT), mainly in dairy herds, and slaughter surveillance. In Mexico, the prevalence of TB is established by the CFT, and, in some cases, a double comparative tuberculin test is also carried out to avoid false positives. In Venezuela, a national bovine TB control Program is currently in place. Annually, about 300,000–500,000 cattle are tested with the CFT, and reactors (less than 0.04%) are sacrificed. *M. bovis* has been isolated from tuberculin-positive bovines after post-mortem inspection of reactors in the slaughterhouse. In Chile, BTB surveillance is applied at the slaughterhouse and during certification of BTB-free status of dairy herds. The test used to certify BTB-free herds is the CFT, performed by accredited veterinarians. Cattle that are classified as positive to the CFT test are re-tested using the comparative cervical tuberculin (CCT) test. Organ and/or tissue samples isolated during surveillance at the slaughterhouse are sent to the laboratory for confirmation. The official laboratory tests established by the Chilean Livestock and Agricultural Service (SAG) are culture and/or Real Time TaqMan PCR specific for *M. bovis* (Roche-Chile). Brazilian policies regarding the control and eradication of BTB include the National Plan for the Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT), written in 2001 and reviewed in 2004, which is based on slaughter of all animals reactive to the tuberculin tests.

Currently, methods for genotyping *M. bovis* and other *M. tuberculosis* complex species include RFLP (Otal et al., 1991; Collins et al., 1993) and VNTR (Haddad et al., 2001); however, up to now, spoligotyping is the best option for large-scale screening studies on the distribution of *M. bovis* strains (Kamerbeek et al., 1997). Theories of molecular fingerprinting establish that epidemiologically related isolates have similar fingerprints that differ from those epidemiologically unrelated (Maslow et al., 1993). A desirable characteristic for typing is related to its stability within a strain and its diversity within a species. Although Spoligotyping may fulfill these characteristics in some cases, other typing methods such as VNTR have to be performed to discriminate isolates, especially in settings where one spoligotype is largely predominant.

Previous studies have shown a wide diversity of *M. bovis* spoligotypes in cattle populations in Latin American countries. For example, different spoligotypes of *M. bovis* have been found in different regions of Argentina, Brazil, Uruguay and Mexico (Zumárraga et al., 1999; Milián-Suazo et al., 2002; Rodríguez et al., 2004). In some cases, the indiscriminate movement of animals between regions has been indicated as the most probable source of this diversity.

Therefore, the objective of this study was to perform an initial assessment of the regional distribution and population structure of *M. bovis* in some Latin American countries. The *M. bovis* spoligotypes obtained from isolates from cattle and other species from Argentina, Brazil, Chile, Mexico and Venezuela were included in the study.

2. Materials and methods

2.1. Isolates

A convenience sampling approach was undertaken. A total of 1684 *M. bovis* isolates were spoligotyped. Most of the spoligotypes included in the study have not been previously reported. Isolates were from Argentina ($n = 816$), Brazil ($n = 412$), Chile ($n = 66$), Mexico ($n = 274$), and Venezuela ($n = 116$).

About 202 out of the 816 spoligotypes of Argentina have been previously reported (Zumárraga et al., 1999; Zumárraga et al., 2009; Barandiaran et al., 2011). Of the Brazilian isolates, 113 out of the 412 have been already described with their spoligotypes (Rodríguez et al., 2004) whereas 65 of these 412 isolates had been described in another paper with no spoligotypes reported (Parreiras et al., 2004). Fifty-seven out of the 274 of the spoligotyped isolates from Mexico have been previously reported (Milián-Suazo et al., 2002; Cobos-Marín et al., 2005).

Spoligotypes in this study came from cattle (1445), humans (109), pigs (77), wild boars (3), farmed deer (6), goats (7), buffaloes (4), cats (24), and wild animals (9). All cattle and pig isolates came from slaughterhouses. Human isolates came from hospital labs that tested samples for TB. Goats, buffaloes and farmed deer isolates came from animals necropsied at the farms. Isolates from wild animals (armadillos, coatis, foxes, monkeys, opossums, pumas, rats and wild boars) came from animals necropsied in the field. Isolates from cats came from animals examined or necropsied at veterinary service units or at veterinary units at universities. The isolate from the puma came from an animal necropsied at a zoo.

A complete list of isolates and the epidemiological information collected with data about the spoligopattern, the spoligotype internal number, the original sample number, the country, state/department/province, location of slaughterhouse or isolation, host, microbiologist in charge of the isolation, and year of isolation is shown in [Supplementary Table S1](#).

The tissue samples were decontaminated and cultured in Stonebrink culture media and incubated at 37 °C. The DNA was prepared from colonies by suspending them in 200 ml of distilled water and boiling at 100 °C for 10 min.

2.2. Spoligotyping assays and analysis

A total of 1684 isolates were spoligotyped by all participating laboratories, following Kamerbeek et al. (1997). In the case of Argentina, Chile and Venezuela, data were provided by a single laboratory. In the case of Brazil and Mexico, data came from two different laboratories. Spoligotypes were performed either on Isogen (Temse, Belgium) membranes until 2005 or Ocimum (Hyderabad, India) membranes, after 2005. Spoligotypes were collected in a binary format in an excel database, which was then introduced into Bionumerics® (Version 3.5, Applied Maths, Sint-Martens-Latem, Belgium) to perform a cluster analysis by pairwise similarities and to obtain a similarity matrix with the Dice coefficient. A dendrogram was constructed with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The cophenetic coefficient was estimated with the same program to measure how faithfully a dendrogram preserves the pairwise distances between the original unmodeled data points.

Mycobacterium bovis DNA was prepared from colonies by suspending them in 200 µl of distilled water and boiling at 100 °C for 8 min.

Spoligotypes were compared with the *M. bovis* spoligotypes contained in the www.mbovis.org database from the University of Sussex, UK. For the purpose of this study, a unique spoligotype was defined as that observed only once in a country, an orphan spoligotype was that observed only once in the study, and an exclusive spoligotype was that observed in only one country.

Statistical analysis: The discriminatory index (D) described by Hunter and Gaston (1988) and expressed by the formula of Simpson was calculated to determine the discriminatory power of spoligotyping in each country (<http://insilico.ehu.es>) (Rodríguez et al., 2010). This index is given by the equation:

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

where N is the total number of strains in the sample population, s is the total number of types described, and x_j is the number of strains belonging to the j th type (Hunter and Gaston, 1988). This index is based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups (Hunter and Gaston, 1988).

The results were statistically treated by using contingency tables and chi-square test. The analysis was performed by GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

A total of 269 spoligotypes were obtained from the 1684 isolates studied, 142 (8.4%) of which were orphan, and 1542 (91.6%) formed 102 clusters of at least two isolates (Table 1). A total of 217 spoligotypes with a maximum of 8 isolates (0.5%), grouping a total of 72 (6.3%) isolates, including the orphan spoligotypes ($n = 142$), were found (Fig. 1). The higher diversity was observed in Venezuela.

As a next step, separated analysis per species or group of species was performed.

3.1. Cattle isolates

When compared to spoligotypes in the www.mbovis.org database, the largest cluster ($n = 355$, 24.6% of isolates) was identified as spoligotype SB0140 (Table 2), one of the most frequent spoligotype reported worldwide, especially in Europe, and a predominant spoligotype in the British Islands and countries where British breeds have been introduced (Smith et al., 2006, 2011). The second largest cluster ($n = 149$, 10.31%) was spoligotype SB0121, frequently reported in France (Haddad et al., 2001), Spain (Rodríguez et al., 2010) and Portugal (Duarte et al., 2008). The distribution of isolates by country and province is shown in Fig. 2. In every country, isolates were collected in at least five districts and in different locations within the district. Concerning the representation of regions, we assessed this point by comparing the distribution of cattle in the country, as reported by local authorities or by FAO, with sampling locations in every country (Supplementary Fig. S1). In Argentina, Brazil and Chile, sampling correlated with cattle density. In Venezuela, the sampling was centered only in a state with high cattle farming activities. In Mexico, sampling occurred in many but not all states. However, a representative sampling was not assured in any of the countries studied.

The presence of isolates in clusters within the different countries was overall high, ranging from 96.9% in Argentina to 95.1% in Brazil, 95.2% in Chile, 81.7% in Mexico and 89.3% in Venezuela (Table 2).

The frequency of isolates with orphan spoligotypes found in cattle was 4.1% ($n = 26$) for Argentina, 4.1% ($n = 17$) for Brazil, 4.7% ($n = 3$) for Chile, 18.8% ($n = 45$) for Mexico and 10.7% ($n = 11$) for Venezuela (Table 2). Mexico and Venezuela have a high proportion of orphan spoligotypes.

The proportion of spoligotypes clustered exclusive for the country and for cattle was 16.9% ($n = 10$) for spoligotypes of Argentina, 43.0% ($n = 22$) for those of Brazil, 37.5% ($n = 3$) for those of Chile,

30.9% ($n = 26$) for those of Mexico and 30.0% ($n = 6$) for those of Venezuela.

The percentage of isolates included in exclusive spoligotypes was highly variable, ranging from 71.8% in Venezuela to 9.23% in Argentina ($P < 0.05$) (Table 2). The most frequent exclusive spoligotype (SB1862) was observed in Venezuela, representing 26.2% of the isolates.

The analysis of temporal clustering is only valid in Argentina and Mexico. In the other countries, a significant sampling was performed in few years. At least 20 samples per year were taken in Argentina and Mexico between 1994 and 2009 and between 1996 and 2006, respectively. In Argentina, SB0140 was the most prevalent spoligotype every year, except in 1995, 1997, 2003 and 2007. In Mexico, the situation was much more fluctuating. SB1112 predominated in the samples of the first three years and SB0673 predominated in the ones of the last two years. In spite of the overall predominance of SB0121, it was the most identified type only in 2004 (data not shown).

3.2. Other farmed animals (buffaloes, goats, deer, pigs)

Isolates from 94 animals from species other than cattle, mostly from Argentina, were also typed. SB0140 grouped 55% of these isolates. Clustering in this species group was lower than in bovines. Interestingly, SB0295, predominant in cattle from Brazil, was present only in buffaloes from Argentina (Supplementary Table S1). Seven spoligotypes exclusive of pigs, most of which were orphans, were found (Table 2).

3.3. *Mycobacterium bovis* isolates from humans

The percentages of human isolates found were 7.6% ($n = 62$) in Argentina, 13.6% ($n = 35$) in Mexico and 10.3% ($n = 12$) in Venezuela (Table 2). From these, 57 (92%), 12 (32%), and 12 (17%) had spoligotypes found in cattle, suggesting zoonotic transmission. In Mexico and Venezuela the proportion of exclusive for the country and for human was much higher than in Argentina. Interestingly, 31 spoligotypes were exclusive of humans (SB1042, SB1413, SB1060, SB1043 and SB1046 in Argentina; SB1852, SB1851, SB1850, SB1849, SB1846, SB1841, SB1840, SB1839, SB1838, SB1837, SB1836, SB1834, SB1833, SB1831, SB1830, SB1829, SB1828, SB1827, SB1826, SB1825 and SB1824 in Mexico; and SB1866, SB1857, SB1856, SB1855 and SB1854 in Venezuela). In Mexico, a low level of clustering was observed and is particularly interesting that many orphan and exclusive spoligotypes from humans were found in the state of Querétaro (SB1824, SB1825, SB1826, SB1827, SB1828, SB1829, SB1830, SB1831, SB1834, SB1833, SB1836, SB1837, SB1838, SB1839, SB1840, SB1841, SB1849, SB1850, SB1851 and SB1852) and in Delta Amacuro in Venezuela (SB1854, SB1856 and SB1857). Moreover, in Mexico, SB0121, which accounts for 10.6% of cattle isolates, was not found in the 35 isolates included in this study.

3.4. Cat isolates

These isolates were collected only in Argentina. Eleven out of 24 isolates from urban cats presented spoligotype SB0140 (Table 2). The spoligotype SB1165 found in one cat in Argentina matched the spoligotype of seven isolates from cattle in Mexico. Two spoligotypes were orphan and exclusive of cats in Argentina (Table 2).

3.5. Wild animals (rat monkeys, opossums, pumas, coatis, wild boars, armadillos)

Nine isolates from these wild animals, most of which were from Argentina, were genotyped. Only two of these spoligotypes

Table 1
Distribution of spoligotypes in each country.

| | Total of isolates | Total of spoligotypes | Total of exclusive spoligotypes (%) <N° isolates and %> | Unique spoligotypes | | Clustered spoligotypes | | Total of clustered isolates (%) | Major spoligotype (N° of isolates and %) |
|-----------|-------------------|-----------------------|---|---------------------|-----------------------------|---------------------------|-----------------------------|---------------------------------|--|
| | | | | Orphans | Shared with other countries | Exclusive for the country | Shared with other countries | | |
| Argentina | 816 | 79 | 61 (77.2%) <152; 18.6%> | 42 | 2 | 19 | 16 | 780 (94.6) | SB0140 (367; 45%) |
| Brazil | 412 | 51 | 39 (76.5%) <116; 28%> | 17 | 3 | 22 | 9 | 392 (95.1) | SB0121 (120; 29.1%) |
| Chile | 66 | 8 | 6 (75%) <19; 28.8%> | 3 | 0 | 3 | 2 | 63 (95.5) | SB0140 (45; 68.2%) |
| Mexico | 274 | 105 | 95 (90.5%) <189; 68.9%> | 65 | 2 | 24 | 8 | 207 (75.5) | SB0121 (29; 10.6%) |
| Venezuela | 116 | 26 | 23 (88.5%) <107; 92.2%> | 15 | 1 | 8 | 2 | 100 (86.2) | SB1862 (27; 23.3%) |
| Total | 1684 | 269 | | 142 | 8 | 76 | 37 | 1542 (91.6) | |

Definition of terms: Exclusive: those isolates clustered or not, detected in only one country. Unique: those isolates detected once in only one country. Clustered: two or more isolates with the same spoligotype. Orphan: those isolates detected once in this study.

matched SB0140, the spoligotype most frequently found in cattle (Table 2).

3.6. Regional distribution of *M. bovis* across species and countries

A total of 1101 (65.3%) isolates had spoligotypes shared by at least two countries (Table 3). Twenty-six percent of the isolates ($n = 447$ with 17 spoligotypes) were observed in two countries. One spoligotype, SB0121 ($n = 150$), was observed in three countries: Brazil (29.1%), Mexico (10.6%) and only once in Venezuela. Two spoligotypes (SB0140 and SB0120) were shared by four countries, grouping 29.8% of the isolates ($n = 504$). SB0140 was found in all countries except Venezuela, whereas SB0120 (the BCG spoligotype) was found in all countries except Chile, although a limited number of isolates was studied in Chile.

SB0140 was the genotype most frequently observed in Argentina (45%) and Chile (68.2%), but it was considerably less frequent in Brazil (5.6%) and Mexico (4.7%). No spoligotype was observed in all five countries (Table 3).

Regarding spoligotypes shared by neighboring countries, i.e., Argentina, Brazil and Chile, only one (SB0140) was common to the three countries (Table 3). Argentina and Brazil shared ten spoligotypes (SB0120, SB0131, SB0140, SB0267, SB0274, SB0288, SB0295, SB0484, SB1033 and SB1055), Argentina and Chile shared two spoligotypes (SB0140 and SB0990), and Brazil and Venezuela shared two spoligotypes (SB0121 and SB0120). Comparing spoligotypes from non-neighboring countries, there were common spoligotypes between Chile and Brazil ($n = 1$), Brazil and Mexico

($n = 4$), Mexico and Chile ($n = 1$) and Mexico and Venezuela ($n = 2$) (Table 3).

It is interesting that 31%, 24% and 22% of all exclusive spoligotypes from Argentina, Mexico and Venezuela were found only in hosts other than cattle, suggesting either clonal circulation among non-cattle species or an incomplete coverage of relevant clones in cattle.

3.7. Discriminatory index

The discriminatory index of spoligotyping estimated in this study was 0.76 for Argentina, 0.85 for Brazil, 0.51 for Chile, 0.97 for Mexico, and 0.86 for Venezuela. These results are related to the size of the clusters found in each country; however, it seems that in Chile the usefulness of the genotyping method is limited.

3.8. Relationship between spoligotypes

To estimate the relationship between spoligotypes, a dendrogram (Fig. 3) was built using Bionumerics®. Three main families of spoligotypes, designated A, B and C, were found.

Spoligotypes of family A ($n = 103$) had a similarity of more than 83% (72% of cophenetic coefficient) and grouped 849 (50.4%) isolates (Argentina 63%, Brazil 12%, Chile 94%, Mexico 61% and Venezuela 46%). The most prevalent spoligotype (SB0140) and the spoligotypes with prevalences between 0.6% and 2.5% (SB0145, SB0484, SB1862, SB1861, SB0673, SB0269, SB1033, SB0663, SB1112, SB1186, SB0971 and SB1186) belonged to this family (Fig. 3). Interestingly,

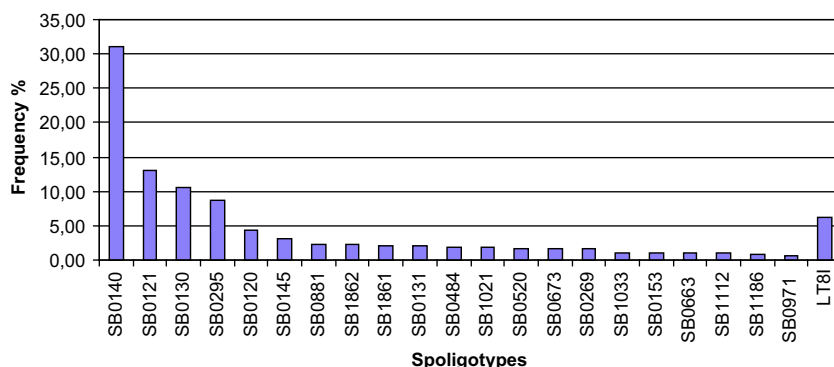


Fig. 1. Frequency of each spoligotype. LT81: Spoligotypes with a frequency lower than 0.5% (≤ 8 isolates). Spoligotypes are denoted by the spoligotype code under bars.

Table 2
Distribution of spoligotypes isolates in each country per species or group of species and clustering parameters: Unique and clustered and frequency of the main spoligotype.

| Species | Total of isolates | Total of spoligotypes | Total of exclusive spoligotypes of each species (%) <N° isolates and %> | Unique spoligotypes | | Clustered spoligotypes | | Shared with other countries | Total of clustered isolates (%) | Major spoligotype (N° of isolates and %) |
|-----------|-------------------|-----------------------|--|---------------------|--|---------------------------|--------------------------------------|-----------------------------|---------------------------------|--|
| | | | | Orphans | Exclusive for the country Found in other species | Exclusive for the country | Exclusive for the country and specie | | | |
| Argentina | 628 | 59 | 36 (61%) <58; 9.23% > | 26 | 1 | 16 | 10 | 15 | 609 (96.9) | SB0140 (280; 44.6%) |
| Brazil | 412 | 51 | 39 (76.5%) <116; 28% > | 17 | 0 | 22 | 22 | 9 | 392 (95.1) | SB0121 (120; 29.1%) |
| Chile | 63 | 8 | 6 (75%) <19; 30% > | 3 | 0 | 3 | 3 | 2 | 60 (95.2) | SB0140 (42; 66.6%) |
| Mexico | 239 | 84 | 71 (84.5%) <130; 54.4% > | 45 | 1 | 29 | 26 | 8 | 194 (81.7) | SB0121 (29; 12.1%) |
| Venezuela | 103 | 20 | 18 (90%) <74; 71.8% > | 11 | 0 | 7 | 6 | 2 | 92 (89.3) | SB1862 (27; 26.2%) |
| Total | 1445 | 222 | 170 | 102 | 2 | 77 | 67 | 36 | 1347 (93.2) | |
| Argentina | 90 | 23 | 8 (34.8%) <9; 10% > | 7 | 2 | 3 | 1 | 7 | 77 (85.5) | SB0140 (50; 55%) |
| Chile | 3 | 1 | 0 (0%) <0; 0% > | 0 | 0 | 0 | 0 | 1 | 3 (100.0) | SB0140 (3; 100.0%) |
| Venezuela | 1 | 1 | 0 (0%) <0; 0% > | 0 | 1 | 0 | 0 | 0 | 0 (0) | SB1021 (1; 100%) |
| Total | 94 | 25 | 8 | 7 | 3 | 3 | 1 | 8 | 80 (85.1) | SB0140 (23; 37%) |
| Argentina | 62 | 15 | 5 (33.3%) <5; 8.1% > | 5 | 3 | 1 | 0 | 3 | 51 (82.2) | SB0140 (3; 8.5%) |
| Mexico | 35 | 28 | 21 (75%) <23; 65.7% > | 20 | 2 | 3 | 1 | 2 | 12 (34.0) | SB1846 (3; 8.5%) |
| Venezuela | 12 | 7 | 5 (71.4%) <9; 75% > | 3 | 0 | 2 | 2 | 1 | 8 (66.6) | SB1958 (4; 33.3%) |
| Total | 109 | 50 | 31 | 28 | 5 | 6 | 3 | 6 | 71 (65.1) | |
| Argentina | 24 | 10 | 3 (30%) <3; 12.5% > | 3 | 2 | 1 | 0 | 2 | 17 (70.8) | SB0140 (11; 45.8%) |
| Argentina | 12 | 7 | 2 (28.6%) <3; 25% > | 1 | 1 | 1 | 1 | 2 | 7 (58.3) | SB0120 (3; 25%) |

Definition of terms: Exclusive: Those isolates clustered or not, detected in only one country. Unique: Those isolates detected once in only one country. Clustered: Two or more isolates with the same spoligotype. Orphan: Those isolates detected once in this study.

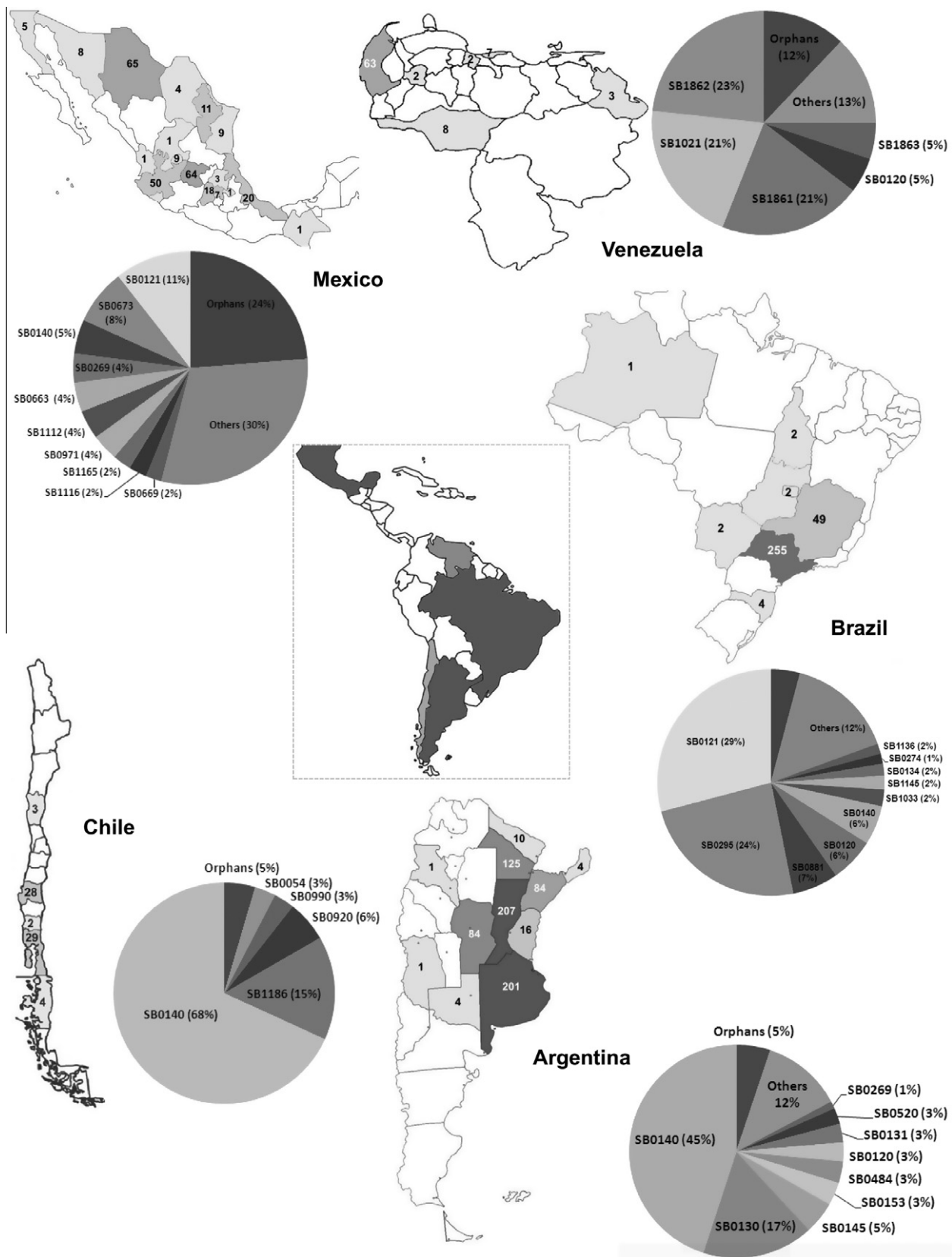


Fig. 2. Map of Argentina, Brazil, Chile, Mexico and Venezuela. Latin America is shown in the center with the five countries studied here in gray. The number in the shaded provinces represents the number of isolates obtained. Pie charts show the distribution of major spoligotypes in each country.

SB1186, which is highly related to SB0140, appeared only in Chile. Within this group of spoligotypes, we identified a subfamily (A1) of 14 spoligotypes involving more than 86% similarity (70% of

cophenetic coefficient) with 17 isolates from Mexico. All the spoligotypes of subfamily A1 were characterized by the lack of spacers 2, 10 and 15.

Table 3

Shared spoligotypes between the countries. N = total number of strains analyzed per country. Indicated for the countries is number of isolates with this spoligotype and the prevalence of this particular spoligotype in the country in%.

| SB N° | Argentina N = 816 | Brazil N = 412 | Chile N = 66 | Mexico N = 274 | Venezuela N = 116 | Total N = 1684 |
|--------|----------------------|-------------------|-----------------|-------------------|----------------------|-------------------|
| SB0807 | 0 | 1 (0.2%) | 0 | 2 (0.7%) | 0 | 3 (0.2%) |
| SB0288 | 2 (0.2%) | 2 (0.5%) | 0 | 0 | 0 | 4 (0.2%) |
| SB0990 | 2 (0.2%) | 0 | 2 (3%) | 0 | 0 | 4 (0.2%) |
| SB0274 | 1 (0.1%) | 6 (1.5%) | 0 | 0 | 0 | 7 (0.4%) |
| SB0267 | 7 (0.8%) | 1 (0.2%) | 0 | 0 | 0 | 8 (0.5%) |
| SB1031 | 6 (0.73%) | 0 | 0 | 0 | 2 (1.7%) | 8 (0.5%) |
| SB1044 | 3 (0.4%) | 0 | 0 | 5 (1.8%) | 0 | 8 (0.5%) |
| SB1165 | 1 (0.1%) | 0 | 0 | 7 (2.5%) | 0 | 8 (0.5%) |
| SB1055 | 7 (0.9%) | 3 (0.7%) | 0 | 0 | 0 | 10 (0.6%) |
| SB0663 | 2 (0.2%) | 0 | 0 | 11 (4%) | 0 | 13 (0.8%) |
| SB1033 | 3 (0.4%) | 10 (2.4%) | 0 | 0 | 0 | 13 (0.8%) |
| SB0269 | 9 (1.1%) | 0 | 0 | 11 (4%) | 0 | 20 (1.2%) |
| SB0131 | 23 (2.8%) | 4 (1%) | 0 | 0 | 0 | 27 (1.6%) |
| SB0484 | 27 (3.3%) | 1 (0.2%) | 0 | 0 | 0 | 28 (1.7%) |
| SB0145 | 40 (4.9%) | 0 | 0 | 5 (1.8%) | 0 | 45 (2.7%) |
| SB0120 | 23 (2.8%) | 26 (6.3%) | 0 | 1 (0.4%) | 6 (5.2%) | 56 (3.3%) |
| SB0295 | 3 (0.4%) | 99 (24%) | 0 | 0 | 0 | 102 (6%) |
| SB0130 | 138 (16.9%) | 0 | 0 | 1 | 0 | 139 (8.2%) |
| SB0121 | 0 | 120 (29.1%) | 0 | 29 (10.6%) | 1 (0.9%) | 150 (8.9%) |
| SB0140 | 367 (45%) | 23 (5.6%) | 45 (68.2%) | 13 (4.7%) | 0 | 448 (26.6%) |
| Total | 664 | 296 | 47 | 85 | 9 | 1101 |

Family B had 105 spoligotypes, similar in more than 84% (74% of cophenetic coefficient) and held 748 (44.4%) isolates (Argentina 33%, Brazil 88%, Chile 6%, Mexico 21% and Venezuela 45% of the total isolates of each country). Spoligotypes SB0120, SB0121, SB0130, SB0131, SB0295, SB1021, SB0520 and SB1055 belonged to this family, with frequencies between 0.6% and 8.9% (Figs. 1 and 3). Some of these strains showed a strong local distribution: SB0520 was found in the province of Córdoba in Argentina, SB0130 in the north of Argentina, and, 99 out of 102 isolates with SB0295 were detected in Brazil. Subfamily (B1), which consisted of 51 isolates from Brazil, classified into nine spoligotypes, grouped together with more than 86% of similarity (80% of cophenetic coefficient). Only two of these spoligotypes were orphans. The largest cluster of this family was SB0881, grouping 27 isolates from Brazil (Figs. 1 and 3). Most of the spoligotypes of this family were characterized by the lack of spacers 21 and 26–30.

Family C had 31 isolates grouped in 10 spoligotypes with more than 88% of similarity (cophenetic coefficient 92%). All isolates of this family were from Mexico and represented 11% of the isolates from this country; five of them were orphan spoligotypes. The largest cluster of this family (SB0971) held 10 isolates (Figs. 1 and 3). Most spoligotypes of this family were characterized by the lack of spacers 8–12 and 31–36.

4. Discussion

To our knowledge, this is the first report analyzing the spoligotype distribution of *M. bovis* isolates from several countries in Latin America. In spite of the large diversity of spoligotypes, a high degree of clustering was observed. The number of isolates in clusters (91.6%) and the size of the largest clusters suggest active transmission of *M. bovis*, although at different levels among countries. Association between clustering and transmission is frequently assumed for *M. tuberculosis* (Fok et al., 2008; Metcalfe et al., 2010; Jagielski et al., 2010) and *M. bovis* (Parra et al., 2005). In a similar study carried out in Spain, less percentage of clustering was observed (Rodríguez et al., 2010).

Spoligotype SB0140, frequent in Argentina and Chile but much less frequent in Brazil and Mexico and absent in Venezuela, called

our attention. Isolates with this spoligotype have also been frequently observed in the United Kingdom, New Zealand, Australia and Ireland (www.mbovis.org). It is known that Argentina has a historical tradition of importing British breeds of cattle, more intensively at the beginning of the past century. These data suggest the presence of BTB in these imported animals (Cataldi et al., 2002).

Spoligotype SB0121 was most prevalent in Brazil and Mexico but was not detected in Argentina, a country neighboring Brazil. This spoligotype was also prevalent in previous studies carried out in Brazil (Zanini et al., 2005; Rodríguez et al., 2004). The link between spoligotypes could be associated with the fact that, before 1946, some breeds of cattle (Zebú) were transferred from Brazil to Mexico. Thus, the finding of this spoligotype in Mexico could be due to an older, rather than recent, movement of infected cattle from Brazil to Mexico (Cobos-Marín et al., 2005). Argentina and Brazil shared the highest number of spoligotypes (10), in contrast to that observed for Argentina and Chile (2). This may be because Argentina and Chile are separated by the Andes, a topographical characteristic that makes exchange of animals extremely difficult, as opposed to the border between Argentina and Brazil which consists of rivers with lots of bridges and areas of land.

Because spoligotyping is less discriminative than VNTR in genotyping *M. bovis*, it is not possible to conclude that spoligotypes represent clones and that isolates are epidemiologically related. However, spoligotyping has shown to be a good tool to identify genetically distant relationships between strains of *M. bovis* (Zumárraga et al., 1999). We understand that deletions of spacers in the Direct Repeats region might be a pathway in the process of evolution of the species, and that this might originate new genetic lines, rising spoligotypes similar to those observed in other regions with no epidemiological relationship (homoplasy). This might be the case for the few spoligotypes shared by countries with no recent history of cattle exchange, as it is the case of Mexico, Brazil and Venezuela. Therefore, it is clear that if no appropriate epidemiological information is available to support the molecular findings, the interpretation of the results could be misleading.

Considering the distribution of predominant spoligotypes in Argentina, Brazil and Mexico, it is possible to speculate that after

the introduction of European breeds in these countries of Latin America, the clonal structure of *M. bovis* reflected that of Europe at the moment of cattle introduction. This is suggested by the fact that the most predominant spoligotypes in the countries studied

here reflect the predominance of *M. bovis* spoligotypes in European countries where cattle originated. For instance, SB0140 the most predominant spoligotype in Argentina (45%) reflects the predominance of *M. bovis* spoligotypes in the UK (36%) and Ireland (51%)

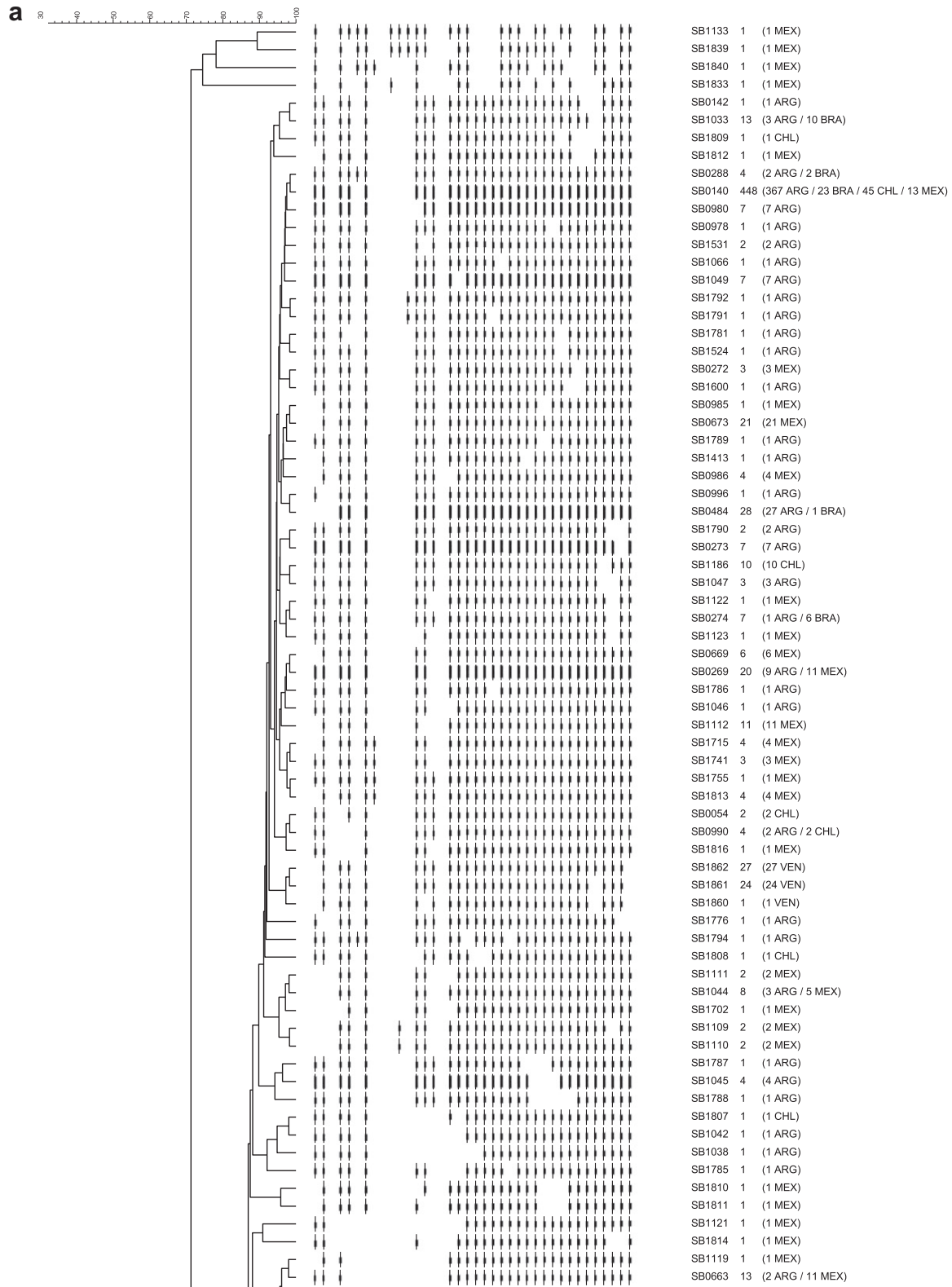


Fig. 3. Dendrogram showing the relationship between the 269 spoligotypes detected among the 1684 *M. bovis* isolates typed. Families of spoligotypes were built selecting the branches with spoligotype similarities higher than 83%.

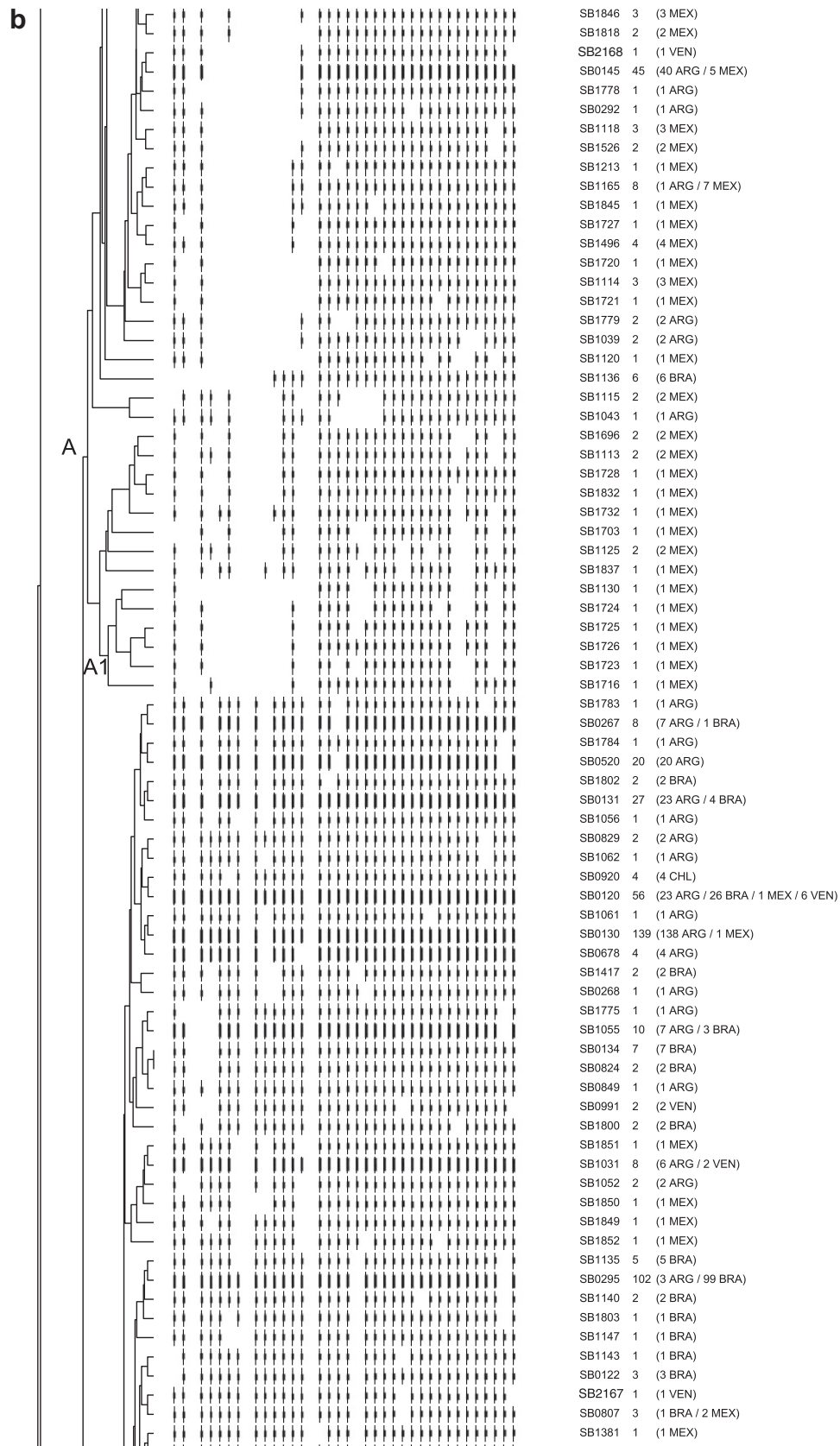


Fig. 3 (continued)

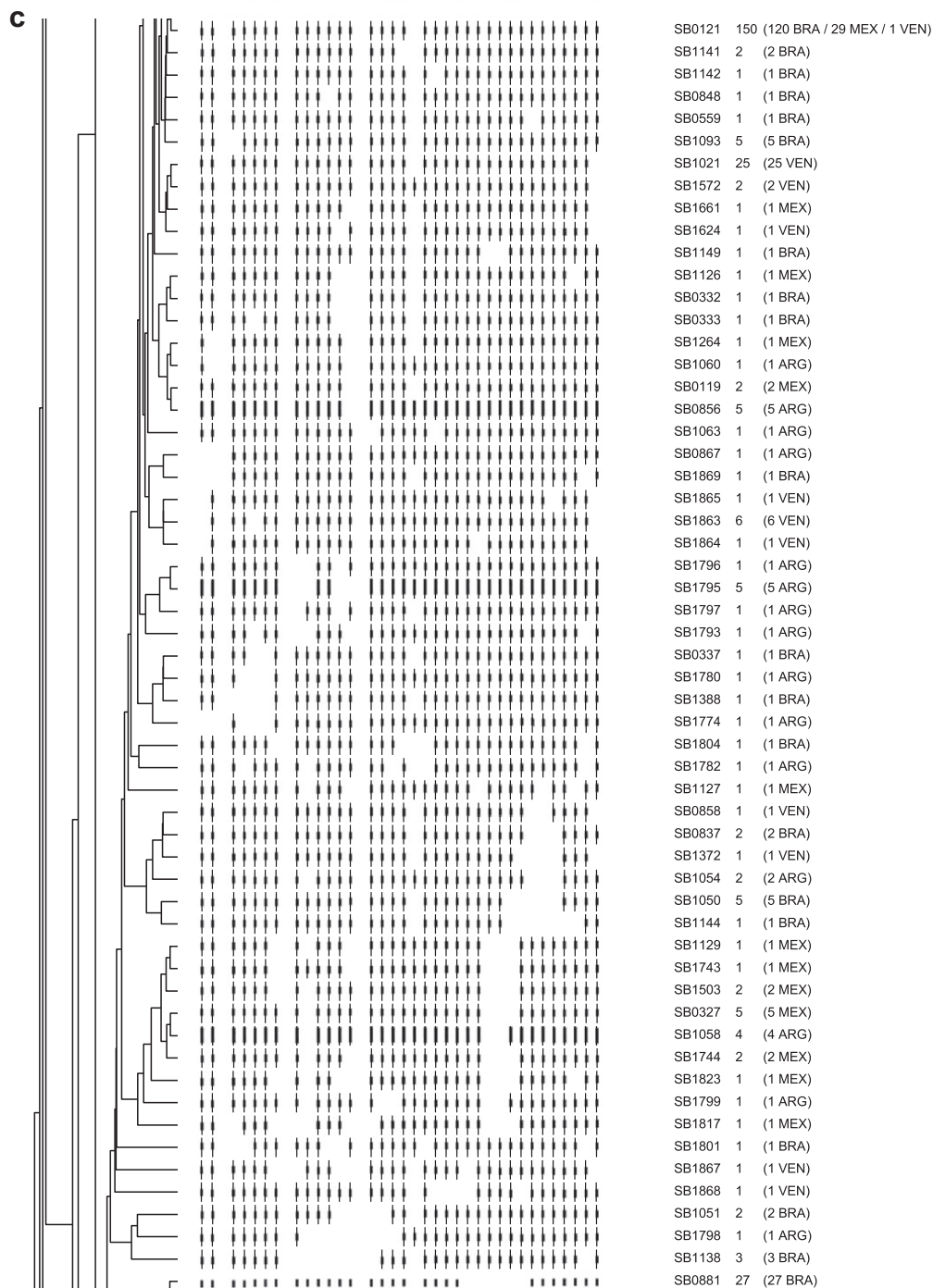


Fig. 3 (continued)

(Smith et al., 2011), whereas SB0121 the most predominant spoligotype in Brazil (29.1%) and Mexico (11%) reflects the predominance of *M. bovis* spoligotypes in Spain (29.6%) (Rodríguez et al., 2010) and Portugal (26.3%) (Duarte et al., 2008). However, after these highly predominant spoligotypes, the second and third most representative spoligotypes in Brazil and Argentina ranked differently compared to the spoligotypes of their countries of origin. In Argentina, SB130 is the second most predominant spoligotype, with a prevalence of 16.9%. This spoligotype has only a frequency of 1.1% in the UK, ranking 11th in that country. The second most predominant spoligotype in the UK (SB0263) does not even appear

in Argentina. Whereas in the UK, SB0274 (13%), SB0129 (6%), SB0673 (6%), SB0275 (4%), and SB0272 (3%), which are third to seventh in the ranking, with a frequency going from 13% to 3%, in Latin America, those spoligotypes are much less represented. The second (SB0295, 24%) and third (SB0881, 6.6%) most predominant spoligotypes in Brazil differ in prevalence in Spain and Portugal. Whereas SB0295 has a prevalence of around 5%, SB0881 has not been reported (Noel Smith, personal communication). We can observe that the predominant spoligotypes in every country studied here are related to those European countries having a preferential economic relationship in the second half of the 19th century, i.e.

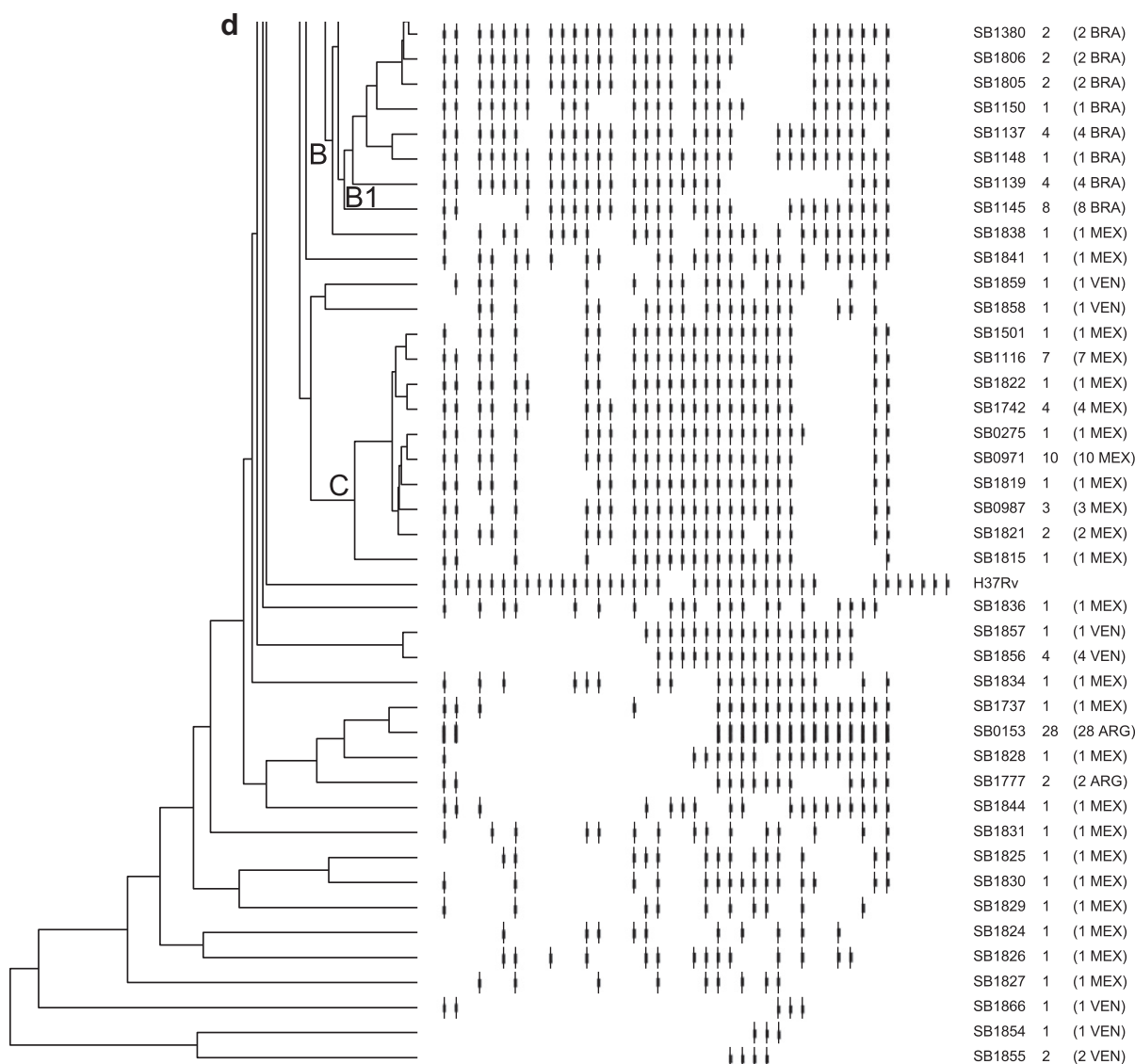


Fig. 3 (continued)

Argentina and Chile with the UK and Venezuela, Mexico with Spain, and Brazil with Portugal. With the present knowledge, it is not possible to establish if the proportion of strains introduced in Latin America should have been the same as that of the original country. These results suggest that there was a change in the population structure, either in Europe or in Latin America, some years after the initial introduction of elite cattle breeds from Europe.

As already mentioned, although *M. bovis* is the main cause of TB in cattle, it is frequently associated with TB in other mammalian hosts, including humans. In our study, 14% ($n = 239$) of the isolates came from non-cattle hosts, 109 had human source and showed spoligotypes identical to those found in isolates from cattle, suggesting transmission between species. There is international agreement that the proportion of human cases of TB owing to *M. bovis* is underestimated since many laboratories make no effort to cultivate *M. bovis*; *M. bovis* requires pyruvate as a source of carbon in the culture medium, which is not used in culturing *M. tuberculosis* (Thoen et al., 2006). In some countries, the risk of transmission of TB from cattle to humans is especially high; for example, in Mexico, 30–40% of the amount of milk produced is sold as raw milk (Pérez-Guerrero et al., 2008). The first case of *M. bovis* multidrug-resistance

person-to-person transmission among HIV-negative people was reported in Argentina (Etchechoury et al., 2010). This finding shows the importance of the application of molecular strategies to identify patients infected with *M. bovis*, especially in regions with high prevalence of bovine tuberculosis. The fact that isolates were obtained between 1997 and 2009 brings about the concern of temporality. However, the average genetic stability of the direct repeat markers has been estimated as 10–20 years. During this period, the strains are expected to keep their DR region unchanged (Clifton-Hadley et al., 1998; Duanmu and Douglas, 1999; Niemann et al., 1999; Soini et al., 2000). Therefore, the temporal difference in the sampling does not modify our hypothesis about one species being the possible source of infection to the other in the same geographic area.

Most studies that address the molecular epidemiology of *M. bovis* include the analysis of those isolates available at the time, which are not a representative sample of isolates in the country but rather a convenience sample. It is possible that in a convenience sample, the low frequency or absence of some spoligotypes for a given geographic location is the consequence of the low number of isolates obtained rather than of their real absence in the population. However, it is understood that a random sampling of

spoligotypes in any geographic location is not feasible since the distribution of such population is unknown. Therefore, we can be sure of the presence and frequency of any spoligotype, but not of its absence.

We recognize that the weakness of our study is a lack of more complete epidemiological information to draw better conclusions of the results. In the countries studied in the present work, it is common that even when the information is available, this information is not provided to the scientist.

The spoligotype most frequently found in cattle in Argentina (SB0140) was also the most frequently found in human isolates (37%). This situation was different in Mexico and Venezuela, where the most frequent spoligotypes in cattle (SB0121 and SB1862, respectively) were not those found in human isolates. The low number of spoligotypes common to humans and cattle could be due to the low number of cattle isolates genotyped in Mexico and Venezuela. Human *M. bovis* isolates from Brazil and Chile were not available. In the last 10 years, no human cases of *M. bovis* have been detected by the National Institute of Health of Santiago (de Kantor et al., 2008).

In conclusion, despite the high diversity of spoligotypes in the five countries, there are major patterns predominating in Argentina, Brazil and Chile. These clusters may reflect a long-lasting active transmission of BTB in the region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.rvsc.2012.07.012>.

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