Full Length Research Paper

Genetic diversity of *Mycobacterium bovis* in Jalisco, Mexico: Tracing back sources of infection

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Bovine tuberculosis (bTB) is a disease of cattle that presents risk to public health, causing severe economic losses to the livestock industry and difficulty in eradication because of its complex epidemiology. The aim of this study was to identify relationships between *Mycobacterium bovis* strains from cattle in the State of Jalisco, and those of other States of México. Molecular fingerprints of 337 *M. bovis* isolates from Jalisco, and 1152 from other States of México were included in the study. Isolates were obtained from tubercles between 1997 and 2015. Evolutionary relationship was determined throughout spoligoforest (www.emi.unsw.edu.au/spoltools/). From 337 isolates from Jalisco, 59 spoligotypes were obtained, ten of them included 48% of all isolates in the state. Five spoligotypes were common to beef and dairy cattle. The molecular analysis showed eight clusters in a philogenetic three: one with three subclusters of nine isolates each, all from dairy cattle; four with two isolates, including dairy and beef cattle. All spoligotypes from Jalisco have been reported in other states, four of the most frequent ones: SB0673, SB0971, SB0669 and SB0140, were the same as in other states. The most frequent spoligotypes of *M. bovis* found in Jalisco were also the most frequent ones in other parts of Mexico. However, there is no evidence to conclude that Jalisco is the source of infection to other states since no information on movement and destination of cattle could be documented.

Key words: Tuberculosis, *Mycobacterium bovis*, spoligotyping, cattle, Jalisco, molecular epidemiology.

INTRODUCTION

Bovine tuberculosis (bTB) is an infectious disease caused by *Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis* complex, which also includes *M. tuberculosis*, *M. cannettii*, *M. africanum*, *M. bovis*, *M. pinnipedii*, *M. caprae* and *M. microti.* Bacilli in this group are 99.9% genetically similar at the nucleotide level with identical 16S rRNA sequences (Boddinghaus et al., 1990; Sreevatsan et al., 1997) but with different host

preferences; *M. bovis* has the broadest host range, causing disease in a wide range of mammals, including humans (O'Reilly and Daborn, 1995; Blischak et al., 2015). The proportion of cases due to *M. bovis* in humans in the last two decades has been variable, ranging from 0.5 to 13% depending on the study population, and it is estimated that nearly 10 million people are affected by tuberculosis worldwide every year (Müller et al., 2013; Olea-Popelka et al., 2017; Perea-Razo et al., 2017). Transmission to humans occurs by inhalation of infectious droplets from infected cattle, and consumption of contaminated unpasteurized dairy products (de la Rua-Domenech et al., 2006).

bTB causes direct and indirect economic losses to the livestock industry, infected animals have poor production performance, die or are culled prematurely. Free trade of animals and animal products in affected areas is prohibited, especially for exportation (Bawinek and Taylor, 2014; El-Sayed et al., 2016).

Like many countries, Mexico has a national program for the control and eradication of tuberculosis (NOM-031-ZOO-1995). This program is based on tuberculin testing and culling of reactors; however, after about two decades, the success has been partial, prevalence in beef cattle has been reduced to low levels (<0.5%) in 85% of the national territory, but in dairy cattle prevalence remains in about 16% (Plan Estratégico de la Campaña Nacional de la Tuberculosis Bovina en México, 2008-2012; Milián-Suazo et al., 2016). Poor participation of dairy farmers in the program, who are not willing to eliminate reactors is one of the main reasons. Nevertheless, the elimination of bTB in Mexico is a high priority task.

Another reason that has hampered the complete success of the bTB program in Mexico, and in other developing countries is the lack of a good system to trace back sources of infection and the indiscriminate movement of animals. Fortunately, in the last ten years, the arrival of molecular techniques to genotype strains of *M. bovis* has enormously supported epidemiological studies focused on detecting areas of risk. Because of simplicity and the low levels of DNA required in the analysis, spoligotyping is one of the methods most frequently used for studying genetic relationship between strains, and the spatial and temporal distribution of *M. bovis* (Kamerbeek et al., 1997; Rodríguez-Campos et al., 2011).

Spoligotyping detects presence or absence of spacers of the Direct Repeat (DR) locus in the *M. bovis* genome (Supply et al., 2006). The DR region contains a large number of DR´s of 36 bp interspersed by spacers from 35 to 41 bp in length. These repeats are present in isolates of the *M. tuberculosis* complex only, and it has been shown that this region is variable (Kemmerbeek et al., 1997). Presence or absence of DR´s allows phylogenetic analysis to determine genetic relationship between individual or groups of strains (Acosta-Salinas et al., 2009;

Jagielski et al., 2014).

Molecular genotyping suggests that isolates with similar fingerprints are epidemiologically related and differ from those epidemiologically unrelated (Maslow and Mulligan, 1993); however, the desirable characteristic for typing is related to its stability within the strain and its diversity within the species (Kemerbeek et al., 1997; Zhou et al., 2011; Kim et al., 2017). Strains with the same spoligotype are assumed to be individuals recently derived by clonal replication from a single ancestral cell; therefore, epidemiological related strains should have higher genetic similarity than those no related (Rodríguez-Campos et al., 2011; Milián et al., 2016). Furthermore, spoligotyping has been used successfully in epidemiological studies in many countries (Gibson et al., 2004; Parra et al., 2005; Duarte et al., 2010; Rodríguez et al., 2010; Skuce et al., 2010; Ruettger et al., 2012; Mwakapuja et al., 2013).

Bacilli of the *M. tuberculosis* complex are clonal, exchange of DNA between individual does not exist. Therefore, it is widely accepted that spoligotypes provide enough information to estimate recent evolution events to perform phylogenetic analysis for epidemiological purposes (Supply et al., 2006), and together with MIRU-VNTR has been recognized as the new gold standard for molecular epidemiological investigations of TB (Jagielski et al., 2014). Currently, there are many reports about the spatial and temporal distribution of *M. bovis* in different geographic areas around the world; however, no information on the role of specific geographic areas in the dissemination of bTB is available.

Therefore, the objective of this study was to use spoligotyping patterns to better understand the population structure of *M. bovis* in cattle in Jalisco, and to evaluate the role of this state as a source on infection for other regions in Mexico.

MATERIALS AND METHODS

Isolates data

Data from a total of 337 *M. bovis* isolates from cattle in the State of Jalisco, and 1,152 from other states in Mexico between 1997 and 2015 were included in the study. Isolates were obtained directly from bTB suspicious tissue collected from carcasses in slaughterhouses, and cultured in Stonebrink and Lowenstein-Jensen with pyruvate. Briefly, tissue samples were first decontaminated with 1:1000 solution of sodium hypochlorite and then macerated and decontaminated with a 10% solution of hydrochloric acid. DNA for spoligotyping was obtained by the CTAB-chloroform method, according to de Almeida et al. (2013). Briefly, a total of 500 µL of suspended colonies in TE 1X buffer was transferred into lysozyme (10 mg/ml) and incubated at 37°C for 1 h. Then, proteinase K and sodium dodecyl sulfate 10% were added, and the suspension was incubated at 65°C for 30 min. Subsequently, a solution consisting of a mixture of NaCl and CTAB (NaCl 5 M and CTAB 10%) was added, and the suspension was incubated for 30 min at 65°C. DNA was then extracted with chloroform/isoamyl alcohol (24:1). The supernatant was transferred to a new tube and isopropanol was added. The suspension was

Table 1. The most frequent *M. bovis* spoligotypes in Jalisco and other States of Mexico, by breed.

State	Breed	Spoligotype (SB)										Other	Total
		0673	0971	0669	0140	0145	0121	0663	0269	1116	0119		
Jalisco	Dairy	35	14	30	15	18	10	10	8	7	9	163	319
	Beef	0	0	0	1	0	0	2	0	0	0	2	5
	Unknown	2	0	0	0	0	0	0	1	2	1	7	13
	Total	37	14	30	16	18	10	12	9	9	10	172	337
Other States	Dairy	148	60	67	75	66	70	27	21	13	11	284	842
	Beef	8	19	13	4	0	0	7	5	10	1	24	91
	Unknown	22	33	13	14	10	4	4	7	10	3	99	219
	Total	178	112	93	93	76	74	38	33	33	15	407	1152
Grand total		215	126	123	109	94	84	50	42	42	25	579	1489

kept at -20°C for 2 h and centrifuged for 15 min at 14,000 g. The pellet was washed with 500 µL of 70% ethanol and centrifuged for 5 min at 14,000 g, and 50 µL of TE buffer was added.

Spoligotyping

Spoligotyping was performed according to Kamerbeek et al. (1997). The DR region was amplified using the primers DRa (GGTTTTGGGTCTGACGAC, 5_ biotinylated) and DRb (CCGAGAGGGGACGGAAAC). The amplified product was hybridized to a nylon membrane to which 37 spacer sequences from *M. tuberculosis* H37Rv and 6 spacer sequences from *M. bovis* BCG were covalently bound (Isogen Bioscience BV, Maarssen, The Netherlands). For the detection of hybridizing DNA, chemiluminescent ECL detection system (Amersham Biosciences; Piscataway, NJ) was used, followed by exposure to X-ray film (Kodak) for 45 min. Spoligotypes were named according to the website *M. bovis* spoligotype database (www.mbovis.org).

Phylogenetic analysis

Spoligotyping data were converted to binary character data (absent=0 and present=1) for the 43 probe hybridization positions. Genetic relationship between spoligotypes was determined by using the algorithm MIRU-VNTRplus available in www.miru-vntrplus.org. Evolutionary relationship was determined throughout spoligoforest in the spolTools webpage (www.emi.unsw.edu.au/spoltools/) for all spoligotypes clustering at least three isolates. Spoligoforest provides an evolutionary genetic tree showing the most probable relationship of all the spolgotypes in the data set (Reyes et al., 2008). This algorithm uses a model that considers mutations by irreversible deletions of spacers and assigns probabilities to the lengths of these deletions. The number of isolates in the cluster determines the size of each node in the tree. Edges between nodes reflect evolutionary relationships between spoligotypes with arrowheads pointing to descendants. Spoligotypes from Jalisco were matched to spoligotypes from other States of Mexico to determine the level of dissemination of *M. bovis* in the country.

RESULTS

Out of 337 isolates from Jalisco, a total of 59

spoligotypes were obtained, ten: SB0673, SB0971, SB0669, SB0140, SB0145, SB0121, SB0663, SB0269, SB1116, and SB0119 included 48% of all isolates from Jalisco; grouping between nine and 37 isolates. Ten spoligotypes grouped between two and three isolates, and 39 were orphans. When comparing spoligotypes from Jalisco with those from other states, it was found that the most frequent spoligotypes in Jalisco were also the most frequent ones in other States of Mexico. Five spoligotypes from Jalisco were common to beef and dairy cattle, suggesting related strains between these two breeds (Table 1).

Out of the 1,152 isolates from states other than Jalisco, a total of 159 spoligotypes were obtained, which included 56% of all isolates in the data set, 98 were orphans. The ten most frequent spoligotypes in Jalisco were also the ten most frequent ones in other parts of Mexico; two hundred and sixty-six were not found in the www.mbovis.org data set. From all the isolates in the data set, 1,161 came from dairy, and 96 from beef cattle; the rest had not information for this variable (Table 1).

Figure 1, shows the phylogenetic tree of spoligotypes from Jalisco, containing groups of at least three isolates each. Eight clusters were formed in this tree: one with three subclusters with nine isolates each, all from dairy cattle. Four with two isolates, including dairy and beef cattle, and three with one subcluster; one including an isolate from beef cattle. Isolates from other states matching spoligotypes from Jalisco are described in Table 2. All spoligotypes from Jalisco have been reported in other States, four of the most frequent spoligotypes in Jalisco: SB0673, SB0971, SB0669 and SB0140, were also the most frequent ones in other states. Some spoligotypes are common to dairy and beef cattle, suggesting related strains of bTB between these two breeds.

Figure 2 shows the spoligoforest hierarchical layout of isolates from Jalisco, where the continuity of lines indicates the weight of the hypothetical evolutionary
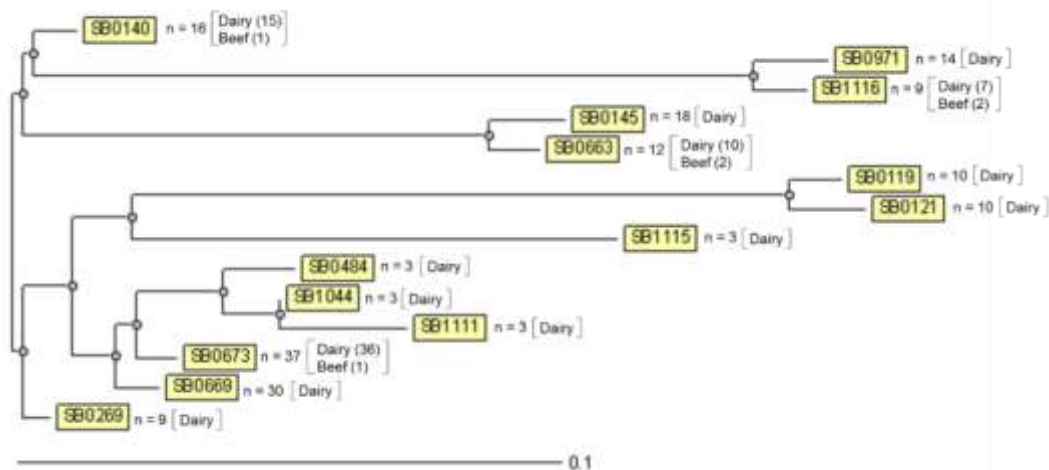
Figure 1. Phylogenetic tree of *M. bovis* isolates obtained from cattle in the State of Jalisco. Number in brackets indicates the number of isolates per breed.

relationship between spoligotypes; continuous line indicates stronger relationship. The spoligoforest shows two trees with connected components. The biggest tree, the one with the largest number of branches, is rooted by strain SB0140, suggesting this as the oldest strain in the tree.

A total of 141 isolates descended from SB0140, in four clearly defined clusters with 9 to 37 isolates each. The hypothetical evolutionary relationship between spoligotypes SB0140 and SB0673, the spoligotype with the largest cluster in the data set is strong, suggesting a small number of changes in the DR region sequence. Spoligotype SB0669, the second largest cluster, has a strong relationship with spoligotype SB0673 but is not directly connected to spoligotype SB0140, suggesting a new evolutionary route.

Spoligotypes SB0145, SB0269 and SB0971 all directly descended from SB0140. SB0145 and SB0971 have their own evolution route since other spoligotypes are derived from them (SB0663 and SB1116, respectively). From the second tree, rooted by spoligotype SB0121, only one lineage is formed, giving origin to strain SB0119.

Figure 3 shows the spoligoforest hierarchical layout of isolates from states apart from Jalisco in Mexico. Like the Jalisco´s spoligotypes, the other States spoligoforest shows two separated trees, originating also from SB0121 and SB0140. The only big difference between the two spoligoforests is the presence of spoligotype SB0971 with a large number of isolates in a separated branch, suggesting a new genetic line. As before, spoligotype SB0140 seems to be the older spoligotype in the country.

DISCUSSION

Spoligotypes SB0673 and SB0669 were the most frequent spoligotypes in Jalisco. These spoligotypes SB0971 and SB0669 were also the most frequent ones in the rest of the country; however, this fact does not necessarily mean that Jalisco is the source of infection since no epidemiological evidence connecting strains from different sources in Mexico could be obtained. It is known that Jalisco is an important source of dairy replacements to other regions in Mexico under especial circumstances; however, this could not be confirmed due to the lack of information on movement of cattle. It is known from personal communication that Jalisco acted as a source of dairy heifers for other regions in the years 2003 to 2006, when Mexico closed the border to the importation of cattle from the United States (US) and Canada because of the bovine spongiform disease outbreak; the US and Canada are the main sources of replacements for dairy in Mexico. It was not known, however, what the distribution of *M. bovis* strains in the country was before that event, for comparison.

Beef and dairy cattle are maintained under different conditions in Mexico. Dairy cattle are raised in close intensive settings with a large number of cattle per square meter; in some regions it is possible to observe 10,000 cattle in a single unit operation. On the contrary, beef cattle are raised in open extensive areas with a low number of cattle per hectare. Because of this, the prevalence of bTB in dairy cattle is higher ≈16% (range 0 to 40%) than in beef cattle ≈0.5% (range 0 to 1%) (Plan Estratégico de la Campaña Nacional de la Tuberculosis Bovina en México, 2008-2012). Therefore, infected dairy populations are a risk to bTB-free or bTB-low prevalence areas of beef cattle. Fourteen of the isolates from beef cattle had spoligotypes SB0673 or SB0669, two of the most frequent spoligotypes in dairy cattle, suggesting transmission between breeds. From the epidemiological point of view, this is relevant because transmission from

Table 2. Frequency and relationship of *M. bovis* isolates from Jalisco and other States of Mexico by breed.

| Spoligotypes from Jalisco | Number of isolates | State (number of isolates by State for States other than Jalisco) | | Unknown |
		Dairy	Beef	
SB0673	182	Ags (9), BC (3), Chih (6), Coah (16), EdoMex (17), Gto (9), Hgo (17), Qro (66), Sin (2), SLP (1), Ver (1), Zac (1).	Chih (1), Dgo (1), Gto (2,), Zac (4).	Ags (7), Coah (2), Col (1), Gro (1), Mich (1), Mor (7), Qro (7).
SB0971	109	Ags (6), BC (1), Chis (1), EdoMex (10), Gto (8), Hgo (8), Mich (1), Qro (22), SLP (1), Ver (1).	Gto (1), Mich (1), Qro (15), Son (1), Ver (1).	Ags (8), EdoMex (7), Gto (3), Gro (1), Mor (1), Nay (2), Qro (7), SLP (2),
SB0669	173	Ags (11), BC (1), Coah (10), Col (1), Dgo (3), EdoMex (18), Gro (2), Gto (5), Hgo (9), Qro (4), SLP (2), Tlax (1).	Gto (2), Mich (1), Nay (8) y Zac (2).	Gro (2), BL (1), Pueb (4), SLP (3), Son (1), Zac (1).
SB0140	90	Ags (10), Chih (8), Coah (2), Edo Mex (5), Gto (3), Hgo (9), Mich (3), Qro (33), Zac (2).	Gto (1), Mich (1), Zac (2).	Ags (6), EdoMex (2), Gto (2), Mor (1).
SB0145	74	Ags (4), BC (14), EdoMex (16), Gto (4), Hgo (5), Qro (20), Ver (2), Zac (1).	--	Ags (2), Nay (1), Qro (2), Sin (2), Ver (1),
SB0121	74	Ags (1), BC (1), Chih (29), Coah (2), EdoMex (3), Hgo (20), Qro (14).	--	Mich (3), Nay (1).
SB0663	37	Ags (5), BC (10), Chih (1), Dgo (1), Edo Mex (3), Gto (2), Qro (4), Zac (1).	Mor (1), Nay (4), Son (1) y Ver (1).	Sin (1), Son (1), Ver (1).
SB1116	33	Ags (5), Col (1), Dgo (1), Edo Mex (2), Mich (1), Qro (2), Zac (1).	Gro (1), Nay (1), Ver (8).	Pue (2), SLP (3), Sin (1), Tamps (2),Ver (2).
SB0269	33	Ags (5), Coah (1), Dgo (1), Edo Mex (2), Gro (1), Hgo (3), Qro (2), Ver (6).	Camp (3), Gto (1), Sin (1).	Col (1), Nay (1), Pue (2), SLP (3).
SB0119	14	Chih (4), Coah (2), Dgo (1), Hgo (3),y NL (1).	Gto (1).	Col (1), Sin (1).
SB0484	10	Edo Mex (3), Gto (1), Hgo (3) Qro (1), SLP (2).	--	--
SB1044	9	Edo Mex (3), Hgo (2), Qro (2).	--	Qro (1), Zac (1).
SB1115	1	Ags (1)	--	--
SB1111	1	Edo Mex (1)	--	--

dairy to beef cattle might jeopardize the goals of the national program to eradicate bTB, and the exportation of calves, an important source of currency for Mexican farmers. In this study, it is not known however if beef animals found infected are from cattle for beef operations or from dairy operations, where sometimes, beef bulls are used for breeding cows with reproductive problems, and beef calves are kept for fattening in the same farm.

Even though clustering of isolates occurred, the diversity of spoligotypes is wide. This agrees with previous reports (Cobos-Marín et al., 2005; Santillán-flores et al., 2006; Reyes et al., 2008; Pérez-Guerrero et al., 2008; Bobadilla-del Valle et al., 2015; Sandoval-Azuara et al., 2017), where in spite of studying samples from different and specific regions of Mexico, the diversity of strains has been evident, suggesting an intense and continuous exchange of animals, and new genetic

lines emerging as a consequence of the high prevalence of bTB in dairy cattle.

The spoligoforest demonstrates all possible relations of spoligotypes under the assumption of spoligotype mutation, with genetic instability ranging from 10 to 20 years (Brosch et al., 2002; Gutiérrez et al., 2005; Smith et al., 2006). In the data set, the largest root of the tree was spoligotype SB0140. Spoligotype SB0140 has infected cattle, deer, badgers and people in
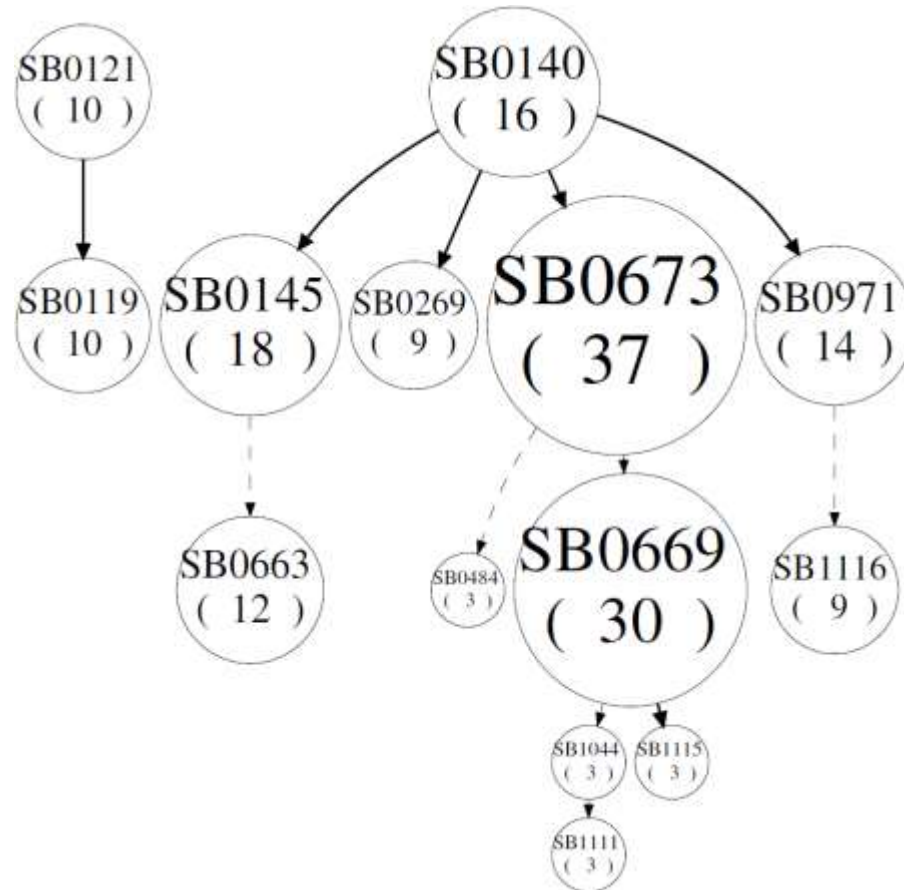
Figure 2. Spoligoforest of *M. bovis* spoligotypes obtained from cattle in Jalisco, Mexico. Nodes are labeled with the SB identifier as indicated in *mbovis.org*; numbers in parenthesis indicate cluster size. Lines between nodes reflect hypothetical evolutionary relationships among spoligotypes with arrows denoting descendence. Continuous lines indicate stronger relationship.
Source: http://spoltools.emi.unsw.edu.au/

Ireland (de la Rua-Domenech et al., 2006; McLernon et al., 2010), and cattle in the United Kingdom (de la Rua-Domenech et al., 2006; McLernon et al., 2010). It has also been reported as the most frequent spoligotype in pigs (Barandiaran et al., 2011), cattle and cats in Argentina (Zumárraga et al., 2009), and humans in the United States (Rodwell et al., 2008) and Mexico (Bobadilla-del Valle et al., 2015).

Spoligotype SB0140 has been studied profoundly in the United Kingdom (Smith et al., 2003). It was concluded that the frequency of strains with SB0140 in that country cannot be explained by random drift without selection. It has been concluded that some genotypes increase in number in a specific region in a "clonal expansion" by selection of favorable mutations when some cells find new host species or new geographical regions. In Mexico, it is believed that both situations are possible at least for the most frequent spoligotypes: selection of favorable mutations due to the high prevalence of *M. bovis* in the population and ecological opportunity by the indiscriminate movement of animals between regions.

Clusters with similar or highly similar *M. bovis* spoligotypes are considered the result or recent transmission, and that the orphans arise from migration or reactivation of acquired infections (Luciani et al., 2008). However, other factors may be involved in that clustering, that is, sampling and the mutation rate of the molecular marker used in fingerprinting (Tanaka and Francis, 2005). In the current study, both clustering and a high frequency of orphan spoligotypes occur. Clustering might well be a consequence of the conditions in which dairy cattle are maintained, in high density populations and orphans, the result of the indiscriminate movement of animals between regions or the high prevalence of the disease, which gives rise to new genetic lineages.

Something that is clear from the current study is that molecular information itself is not enough to explain the epidemiology of a disease. In the present study, no data on movement of animals from Jalisco could be obtained in spite of intensive search of data, and this is clearly a
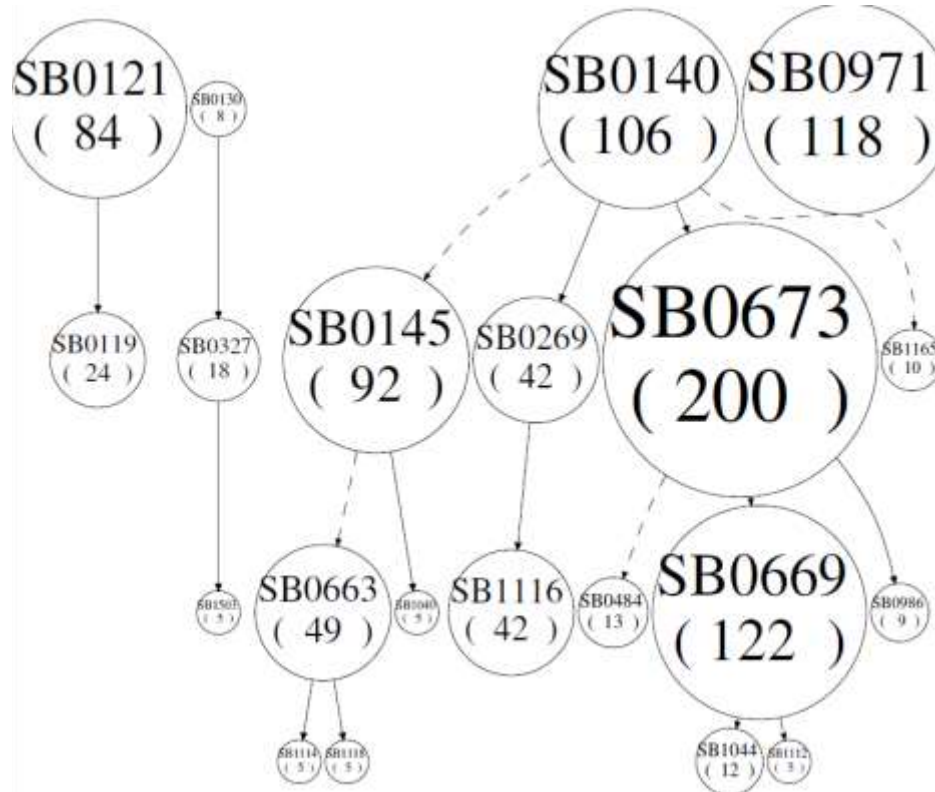
Figure 3. Spoligoforest of *M. bovis* spoligotypes obtained from States other than Jalisco in Mexico. Nodes are labeled with the SB identifier as indicated in *mbovis.org*; numbers in parenthesis indicate cluster size. Lines between nodes reflect hypothetical evolutionary relationships among spoligotypes with arrows denoting descendence. Continuous lines indicate stronger relationship.
Source: http://spoltools.emi.unsw.edu.au/

weakness of this study. This suggests that more knowledge on epidemiological methodologies, especially databases maintenance by bodies responsible for animal health care in Mexico, is required.

Conclusions

The most frequent spoligotypes of *M. bovis* found in Jalisco are also the most frequent ones in other parts of Mexico. However, there is no evidence to conclude that Jalisco is the source of infection since no information on movement and destination of animals could be documented. It is believed that similarity of spoligotypes around the country is in fact due to the indiscriminate movement of animals. The long history of bTB in Mexican herds, which favors the increase and dissemination of new and existing *M. bovis* strains in the population could be another reason.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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