Analysis of bovine tuberculosis transmission in Jalisco, Mexico through whole-genome sequencing

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Abstract

Introduction: Bovine tuberculosis, caused by *M. bovis*, is endemic in Mexico and has had a big impact on public health. Jalisco is considered to be an important dairy region in the country, accounting for approximately 19% of the total milk production. Within Jalisco, the region of Altos Sur holds the largest proportion of the cattle inventory of the state. **Material and Methods:** To determine the frequency of bovine tuberculosis in Altos Sur, Jalisco, as well as *M. bovis* genetic diversity, sampling of tissue (lymph nodes, lungs, and liver) from Holstein cattle was performed in four abattoirs belonging to three municipalities of this region (Tepatitlán de Morelos, San Miguel el Alto, and Arandas). Spoligotyping and whole-genome sequencing were carried out to assess the genetic relationships of *M. bovis* strains circulating in this area, as well as a comparison to isolates from other places in Mexico. **Results:** Prevalence was 15.06%, and distribution similar among the three municipalities. The most frequent spoligotypes were SB0673, SB121, and SB0145. Whole-genome sequencing revealed three main clades (I, II, III), but isolates did not show clustering by region. **Conclusion:** Phylogenetic analysis suggested ongoing transmission between herds of the different regions, and no unique source of infection was determined. This hinders efforts under the national program for the control and eradication of the disease, so serious attention must be paid to rural regions such as Altos Sur in order to improve its success.

Keywords: *M. bovis*, bovine tuberculosis, spoligotyping, whole-genome sequencing, Mexico.

Introduction

Tuberculosis (Tb) remains a major cause of death worldwide, with approximately two billion people infected with the tubercle bacilli (30). *Mycobacterium bovis* (*M. bovis*), a member of the *M. tuberculosis* complex (MTBC), is the pathogen responsible for causing the bovine tuberculosis (BTb) disease in cattle, though it can infect a very wide variety of mammals, including man (9). In North America, BTb has been nearly eradicated in Canada, such that over the past decade there have only been sporadic localised outbreaks (22); the US has very low prevalence (0.001%), except for the white-tailed deer population, which represents a problematic reservoir (10). In Mexico, however, BTb prevalence has been reported at 16% in dairy regions (15). Tuberculosis caused by *M. bovis* in humans in Canada and the US has been noted at less than 1.4%, but in Mexico, it has been recorded at as high as 26.2% by a retrospective study performed in the third level health centre which analysed cases of Tb in humans during the years 2000–2015 (27). In Mexico, infection with *M. bovis* represents an important risk to public health when fresh milk from infected animals is consumed by people, but pasteurisation can significantly contribute to solving this problem (6).

The milk traditionally consumed in Mexico is fresh (unpasteurised), and such is also used for the production of artisan cheeses. The region of Altos de Jalisco, which comprises Altos Sur and Altos Norte, plays an important role in this industry, as it is

responsible for as much as 50% to 80% of total direct sales. It is among the four most important milk production regions in Mexico, contributing 18.3% of the national milk production, even though individual animal productivity is low, ranging from 6.5 to 7.5 litres/cow/day (1). Production units are relatively small and diverse and consist mainly of family-run operations. Also, this region holds 13.4% of the national inventory of cattle, and alone accounts for 47% of the cattle inventory for Jalisco. This makes Altos de Jalisco the densest dairy region (cows/km^2) in Mexico, leading Central Jalisco, which is the second most important dairy region in Mexico, holding 2.4 times fewer animals per km^2. Furthermore, Altos de Jalisco has the highest number of registered dairy organisations. By the end of the 1990s, their number stood at 330. Additionally, a large number of world-class agro-industrial companies, such as Nestle, Parmalat, and Yoplait, are established here, as well as nationally important ones like La Concordia, Lácteos Deshidratados de México, Lechera Guadalajara, and Alprodel. This has had a major influence on the establishment of quality standards for the industry (1).

Given the lack of research, particularly in this region, that can produce situational information on BTb, and given the importance of this region as a milk producer for Mexico, there is a strong need for new tools that can be used in the control of this disease. Epidemiological investigations are of great value in these circumstances, as authorities rely on a deep understanding of disease dynamics and pathogen diversity in order to develop adequate strategies for the control and eradication of the disease. Seeking to contribute, the present project aimed to determine the frequency of BTb in Altos Sur, as well as *M. bovis* genetic diversity through spoligotyping and whole-genome sequencing (WGS).

Material and Methods

Study area. For this project, four abattoirs in the region of Altos Sur (Fig. 1), in the state of Jalisco, Mexico, were included for sampling. These were within three main municipalities: Tepatitlán de Morelos, San Miguel el Alto, and Arandas. These abattoirs were chosen because most of the cattle from the region are taken here for slaughter, so they are representative of the region.

Sample collection. Convenience sampling was performed for the collection of tissue samples with or without typical BTb lesions from Holstein cattle. Various samples from each animal were obtained at post-mortem inspection of the carcass from the following tissues: lungs, liver, and lymph nodes of the upper respiratory tract, such as maxillary, retropharyngeal, and mediastinal. Post-mortem inspection was carried out according to the guidelines of the Manual for the Sanitary Inspection of Cattle Suspected of Suffering From Bovine Tuberculosis, and under the supervision of an official licensed veterinarian.

The tissue samples were kept in air-tight plastic bags, which were labelled with the animal's ID, age, gender, and place of origin, and with the date of sample collection. In order to properly conserve the samples, they were stored at −20°C until further analysis.
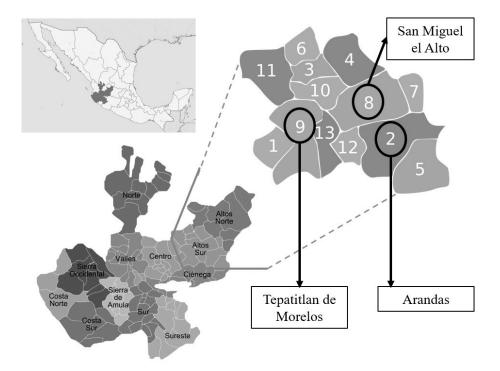

Fig. 1. Map of the state of Jalisco, Mexico, indicating the three municipalities (Arandas, San Miguel el Alto, and Tepatitlán de Morelos) included in this study

Table 1. Contribution of the total number of samples by municipality

Municipality	Number of samples	Percentage of the total sample size
Tepatitlán de Morelos	230	69
Arandas	55	17
San Miguel el Alto	47	14
TOTAL	332	100

Table 2. Type of tissue sampled which showed typical BTb lesions

Type of tissue	Number of samples with lesions	%
Lymph nodes		
Maxillary	2	2.9
Retropharyngeal	18	26.4
Mediastinal	22	32.4
Lung	3	4.4
Liver	1	1.5
Multiple tissue lesions		
retropharyngeal and maxillary	1	1.5
retropharyngeal and mediastinal	9	13.2
retropharyngeal and lung	3	4.4
retropharyngeal, mediastinal and lung	4	5.9
maxillary and mediastinal	1	1.5
mediastinal and lung	4	5.9
TOTAL	68	100

A total of 332 bovine tissue samples were obtained from three municipalities in the Altos Sur region of the state of Jalisco, in Mexico. As shown in Table 1, Tepatitlán de Morelos (TM) contributed the most samples (230), followed by Arandas (A) (55), and San Miguel el Alto (SMA) (47).

From the total number of tissues sampled, only 68 (20.5%) showed lesions typical of BTb. The distribution of the lesions by type of tissue is shown in Table 2. As seen in the table, mediastinal and retropharyngeal lymph nodes were the most-affected tissues, representing 32.4% and 26.4%, respectively, of the total tissues with lesions typical of BTb. Lung tissue also represented a fair amount of the total tissues with lesions, either as the single tissue affected (4.4%), or in combination with other types (4.4%, 5.9%, and 5.9%). Liver tissue represented the least amount of tissue affected, with only a 1.5% share of total tissue with lesions. Also, lesions were present either in a single type of tissue, or in several, such as lung and a combination of the different lymph nodes.

Mycobacterial isolation. Analysis was performed at the Laboratory of Animal Health and Environmental Microbiology of the Natural Sciences Department in the Autonomous University of Queretaro. The different tissues were pooled in order to have one sample per animal, *i.e.* lung and lymph node tissues were mixed together to make one sample for that specific animal. Samples were decontaminated using the modified Petroff´s method (29), and culture was executed in Stonebrink solid medium for the isolation of *Mycobacterium bovis*. Cultures were incubated at 37°C for 8 to 12 weeks, and weekly checks were performed to verify the progress of cultures.

DNA extraction. Once growth of culture was confirmed, cells were harvested using a sterile inoculating loop and deposited in a 1.5 mL Eppendorf tube with 100 µL of 1X TE buffer (100 mM of tris-HCl pH 8 and 10 mM of EDTA) for posterior DNA extraction. DNA extraction was performed by the phenol-chloroform method (CTAB method) described previously (3). DNA was resuspended in nuclease-free water to evaluate concentration and purity with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA integrity was evaluated through electrophoresis in an agarose gel (3%) for 120 min at 45 V. For storage, DNA was kept at −20°C until further use.

PCR. An initial PCR reaction was performed in a C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycle parameters: an initial denaturing cycle for 15 min at 96°C, then 33 cycles of 1 min each at 96°C, followed by an annealing cycle for 1 min at 55°C, an extension cycle for 30 s at 72°C, and a final extension cycle for 10 min at 72°C. PCR products were stored at 4°C until further use. The primers used, which are complementary to the DVR regions, were as follows: forward (DRa) 5´-GGTTTTGGGTCTGACGAC-3´ and reverse (DRb) 5´-CCGAGAGGGGACGGAAAC-3´ (Mapmygenome, Hyderabad, India). Two DNA isolates, one from strain AN5 and the other from H37Rv were used as positive controls. DNA integrity and PCR products were analysed through gel electrophoresis in agarose gel (3%) submerged in TAE 1X buffer (120 min, 45 V) including loading buffer (Gel Red 1:100) and bromophenol blue. Gels were posteriorly visualised with a Gel Doc XR+ UV digital

photodocumentation system (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories).

Spoligotyping. In order to prepare the hybridisation membrane (Mapmygenome), it was washed twice with distilled water for 15 min, and then washed in 125 mL of 0.1% SSPE 2X/SDS at 60°C, prior to placing it in a MiniBlotter (Immunetics, Boston, MA, USA). The PCR products (20 μL) were diluted in 150 μL of 0.1% SSPE 2X/SDS, brought to boiling point during 10 min, and then set on ice. Each PCR product was micropipetted into the MiniBlotter, carefully avoiding the formation of bubbles inside the lanes. Once all the samples were loaded, the MiniBlotter was wrapped in aluminium foil and heat-treated in a hybridisation oven at 60°C for 45 min. After this, the membrane was washed using 150 μL of 0.5% SSPE 2X/SDS on the rocker plate of the hybridisation oven for 15 min at 42°C. Posteriorly, a solution of streptavidin/peroxidase (1:2,000) was poured onto the membrane, immersing it completely, and it was incubated at 42°C for 1 h. After the incubation, the membrane was washed in 125 μL of 0.5% SSPE 2X/SDS at 42°C for 10 min and washed twice at room temperature for 5 min. The membrane was then placed on an acetate sheet, luminol was applied onto the membrane, and then it was covered with another acetate sheet. This was then incubated at 37°C for 1 min. Still within the acetate sheets, the membrane was placed on an x-ray film (Kodak, Rochester, NY, USA) inside an x-ray cassette, this being done inside a dark room to avoid harming the x-ray film. The x-ray film was developed according to the manufacturer's specification. Finally, to prepare the membrane for storage, it was washed twice in 10% SDS at 80°C for 30 min, followed by two more washes in EDTA, pH 8.0, at room temperature for 15 min. The membrane was stored in 10 mL of EDTA, pH 8.0, at 4°C until further use.

For the determination of evolutionary relationships based on spoligotypes, spoligoforests were built using SpolTools (http://spoltools.emi.unsw.edu.au/). These provide a visualisation of the most probable genetic relationships among the spoligotypes in a given sample. The method makes use of a model that considers deletions of spacers an irreversible event and assigns probabilities to the lengths of these deletions (total number of spacers deleted), so that one spoligotype is inferred to arise from only one specific parent spoligotype.

Whole-genome sequencing and variant calling. Sequencing was performed at the USDA National Veterinary Services Laboratory. To obtain the whole genome sequences of the 34 *M. bovis* isolates, 20 ng of total DNA was used to perform sequencing on a MiSeq instrument (Illumina, San Diego, CA, USA) using 2 × 250 paired-end chemistry and the Nextera XT library preparation kit (Illumina), according to the manufacturer's instructions. The bioinformatics pipeline vSNP used for this study was created by the National Veterinary Services Laboratories belonging to the United States Department of Agriculture (https://github.com/USDA-VS; Iowa, USA) and has been used in numerous studies. Quality of reads was analysed by the FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were aligned to the reference genome AF2122/97, NCBI accession number NC_002945.4, using BWA (http://bio-bwa.sourceforge.net/) and Samtools (http://samtools.sourceforge.net/). A depth of coverage of 100X was targeted. Binary sequence alignment map (BAM) files were processed based on the Genome Analysis Toolkit (https://software.broadinstitute.org/gatk/) best practices workflow. Single nucleotide polymorphisms (SNPs) were called using GATK's HaplotypeCaller Software (https://software.broadinstitute.org/gatk/documentation/tooldocs/4.0.8.0/org_broadinstitute_hellbender_tools_walkers_haplotypecaller_HaplotypeCaller.php) outputting them to variant call files (VCF). Results were filtered using a minimum QUAL score of 150 and AC = 2. VCF files of closely related samples were grouped, and SNPs were output to two formats: an aligned fasta file (a text-based format for representing the nucleotide sequences using single-letter codes); and a formatted Excel worksheet. SNPs were also visually validated using IGV (https://software.broadinstitute.org/software/igv/), by checking each SNP's location and coverage aligned to the reference genome.

Phylogenetic reconstruction. A total of 196 *M. bovis* whole-genome SNP sequences were used for analysis, 34 obtained from this study and 162 previously reported (19). A maximum likelihood phylogenetic tree was plotted with RAxML (https://cme.h-its.org/exelixis/web/software/raxml/) software using a GTR-CAT model, optimised for visualisation using FigTree v1.4.3 (https://github.com/rambaut/figtree/releases/tag/v1.4.4).

Results

From the total animals sampled, 50 were positive for *M. bovis*, confirmed by culture, which represents a total prevalence of 15.06%. Table 3 shows the positive and negative cases by municipality. Tepatitlán de Morelos presented the highest prevalence (9.9%), and San Miguel el Alto and Arandas showed similar prevalence, with 2.7% and 2.4%, respectively.

Spoligotyping. From the positive cases, 10 spoligotype patterns were determined (Table 4). All patterns showed the absence of spacers 3, 9, 16, and 39–43. The most frequent spoligotype was SB0673 (32%), followed by SB0121 (26%), and SB0145 (14%). There were three orphan (singleton) spoligotypes: SB1177, SB1023, and SB0872.

According to the spoligoforest, the spoligotypes are split into five different clusters (Fig. 2). SB0121 seems to be the precursor of SB1177; SB0130 appears

as the most prolific, giving rise to SB1758 and SB0145, the latter of which also gave rise to SB0872. SB0971 is directly related to SB1023. However, SB0673 and SB1216 show no genetic relationship to any other spoligotype. From 50 *M. bovis* isolates, 10 different genotypes were obtained. This shows the high genetic diversity of *M. bovis* within the Holstein bovine population in the region.

Tepatitlán de Morelos had the most spoligotype diversity, accounting for 8 out of the total 10. San Miguel el Alto followed with 6 spoligotypes (Fig. 3).

The least genetic diversity was seen in Arandas, with only 4 spoligotypes. Between municipalities, at least 3 spoligotypes were shared: TM and SMA shared SB0673, SB0121, SB0145, and SB0971; SMA and A shared SB0673, SB0121, SB0145, and SB1758; and A and TM shared SB0673, SB0121, and SB0145. Three spoligotypes were shared between all three municipalities: SB0673, SB0121, and SB0145. Exclusive spoligotypes to each municipality were SB0130, SB1216, SB1177, and SB1023 for TM and SB0872 for SMA; there were none for A.

Table 3. Prevalence of BTb by municipality as confirmed by positive isolation of *M. bovis* in culture

Municipality	Positive cases	Negative cases	Total animals sampled	Regional prevalence	Total prevalence
Tepatitlán de Morelos	33	197	230	14.3	9.9
San Miguel el Alto	9	38	47	19.1	2.7
Arandas	8	47	55	14.5	2.4
TOTAL	50	282	332		

Table 4. Spoligotype patterns obtained for isolates of *M. bovis* from the region of Altos Sur in Jalisco, Mexico. The total number of isolates per spoligotype is in parentheses

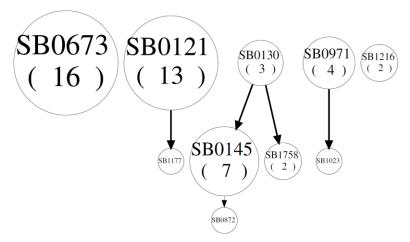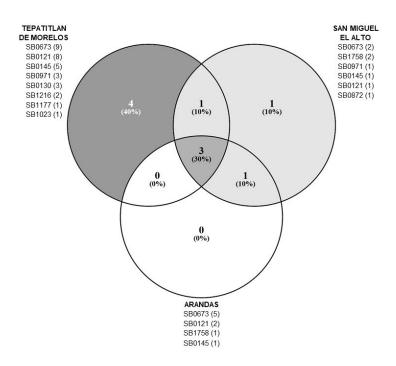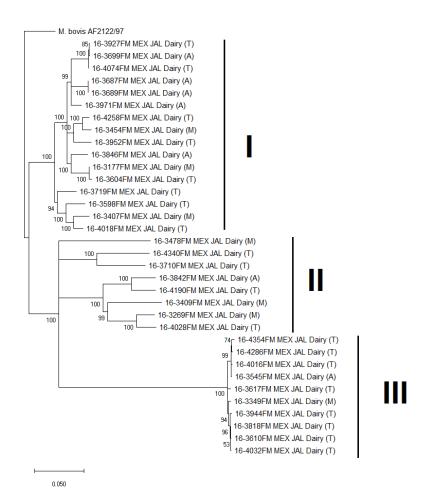| Spoligotype	Pattern
SB0673 (16)	□■□■■□■□□□□■■■□■■■■■■■■■■■■■■■■■■■□□□□□
SB0121 (13)	■■□■■■■■□■■■■■□■■■□■■■■■■■■■■■■■■□□□□□
SB0145 (7)	■■□■□□□□□□□□■□■■■■■■■■■■■■■■■■■□□□□□
SB0971 (4)	■■□■□■□□□□■□■■■■■■■■■■□□□□□□■■□□□□□
SB0130 (3)	■■□■□■■□■□■■■■■■■■■■■■■■■■■■■■■□□□□□
SB1758 (2)	■■□■■■■■□■□■■■■■■■■■■■■■■■■■■■□■■□□□□□
SB1216 (2)	□□□■■■■■□■□■■■■■■■■□□□■■■■■■■□□□□□
SB1177 (1)	■■□■□■■□□■□■■■□■■■□□□■■■■■■■■□□□□□
SB0872 (1)	■■□□□□□□□□□□□□□■■■■■■■■■■■■■■■■■□□□□□
SB1023 (1)	■■□■■□■□□□□■■■□□□□□□□□□□□□□□□□□□□□□□□□

Fig. 2. Spoligoforest including all the *M. bovis* spoligotypes obtained from Holstein cattle in the region of Altos Sur of Jalisco, Mexico. The size of each node is an increasing function of the number of isolates (*i.e.*, the cluster size); edges between nodes reflect evolutionary relationships between spoligotypes with arrowheads pointing to descendants

Fig. 3. Distribution of *M. bovis* spoligotypes by municipality in the region of Altos Sur of Jalisco. The total number of isolates per spoligotype is in parentheses

Fig. 4. Evolutionary analysis by maximum likelihood using as input the concatenated SNP sequences from WGS of 34 *M. bovis* isolates from three municipalities of the region Altos Sur of Jalisco, Mexico: (T) Tepatitlán de Morelos, (A) Arandas, and (M) San Miguel el Alto. The tree is rooted to the reference *M. bovis* AF2122/97 (accession number NC_002945.4)

Whole-genome sequencing. Whole genome sequences for 34 *M. bovis* isolates for the region of Altos Sur of Jalisco were obtained. All the metadata concerning these sequences can be found in Supplementary Table 1. A maximum-likelihood phylogenetic tree was built to obtain the genetic relationships between the isolates (Fig. 4).

There are three major clades (I, II, and III) into which the isolates can group together. Only one clade

(III) was composed primarily of isolates from Tepatitlán de Morelos, only two isolates in the clade originating outside the region, one from Arandas and one from San Miguel el Alto. The other two major clades include isolates from all three municipalities. The group containing the most isolates was Clade I (14), followed by Clade III (9) and Clade II (7). Table 5 shows the genetic diversity within each clade, with reference to the spoligotypes obtained.

Table 5. Genetic diversity within the three major clades obtained by WGS of *M. bovis* isolates from the region of Altos Sur in Jalisco, Mexico

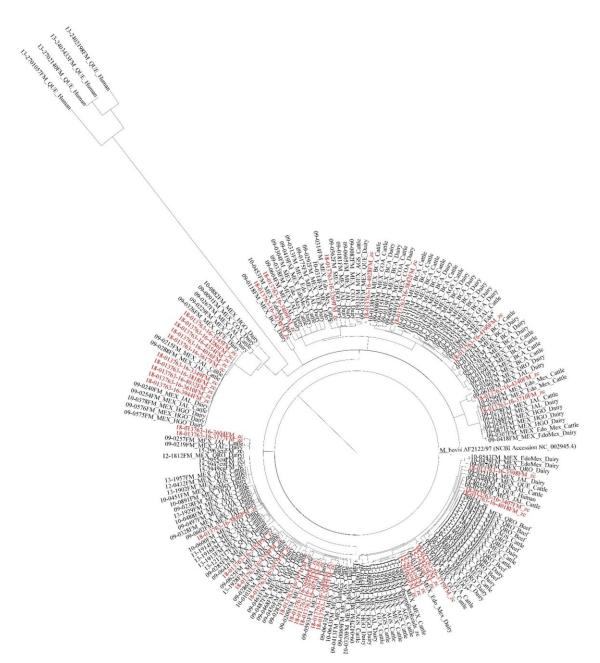| Clade	Isolates	Spoligotypes	Municipalities
I	16	SB0673, SB0971, NF	Tepatitlán de Morelos, Arandas, San Miguel el Alto
II	8	SB0130, SB0145, SB1758, SB1216, SB0872	Tepatitlán de Morelos, Arandas, San Miguel el Alto
III	10	SB0121, SB1177	Tepatitlán de Morelos, Arandas, San Miguel el Alto

Fig. 5. Maximum likelihood phylogenetic reconstruction of the whole-genome SNP sequences of 196 *M. bovis* isolates from Mexico (Altos Sur isolates are in red)

Clade II showed the most diversity, even though it included the fewest isolates. Clade III was the least diverse, with only two spoligotypes within it. Furthermore, isolates within Clade III were the most closely related, differing, on average, by only 1–3 SNPs. On the other hand, isolates from Clade II show the greatest genetic distance between them, with an average difference of 6–13 SNPs. Supplementary Table 2 shows the SNPs associated with each of the isolates included in the study. The total number of validated SNP loci was 2,511. The length of the SNP sequences for each isolate ranged from 205 to 547, and Clade I isolates had the smallest SNP sequences, ranging from 205 to 368. Sequences of Clades II and III were above 477 SNPs in length.

We performed an analysis including the 34 *M. bovis* genomes obtained in this study, plus 162 previously reported sequences (under NCBI BioProject PRJNA449507). Supplementary Table 3 provides a list of the accessions for each genome included, which also appear as a maximum likelihood phylogenetic reconstruction in Fig. 5. The three main clades can be seen, and once again Clade I is the largest group, followed by Clade II and Clade III. Most of the isolates (27/34) are directly related to previously reported Jalisco isolates; however, the remaining ones show close genetic relationships to isolates from other places, mainly Baja California, Coahuila, and Estado de Mexico. The most diversity can be seen in Clade I; it includes isolates from many states (Jalisco, Queretaro, Aguascalientes, Estado de Mexico, Guanajuato, etc.). Clade II is mostly composed of isolates from Baja California and Jalisco, and Clade III are mostly from Jalisco.

Discussion

Spoligotyping has been used widely in Mexico and other countries as a means to obtain *M. bovis* fingerprints to analyse the genetic diversity of strains, and as an important tool in veterinary epidemiology for tracing sources of infection (7, 12, 13, 21, 26). However, though it can be a very practical technique because of its adequate discriminatory power and cost-effectiveness, new methodologies for the study of BTb are being put in place, such as whole-genome sequencing (16, 20, 24). Similarly, the present project aimed to determine the frequency of BTb in Altos de Jalisco, as well as *M. bovis* genetic diversity through spoligotyping and whole-genome sequencing (WGS), as this is an important dairy area in Mexico.

According to the National Programme for the control of Bovine Tuberculosis (NPBTb), in the state of Jalisco, BTb divides the terrain into two areas by the status of the disease: a) control (average prevalence 1.98%) and b) eradication (average prevalence 0.06%). The region of Altos Sur falls within the Control Area, and in this study, we obtained a higher prevalence (15%) than the one reported by the NPBTb. Furthermore, this region falls within the 33% of the national territory classified as Not Accredited by the USDA under the United States–Mexico Joint Strategic Plan for Collaboration on Bovine Tuberculosis. According to this, Jalisco is divided into five regions (A1, A2, A4, B1, and B), with a reported prevalence ranging from 0.07 to 0.26, which also differs from the prevalence obtained in this study. It is possible that infected herds are not reported to the authorities, and thus disease prevalence could be underestimated. Smaller, family-run operations, which often involve less than 100 animals, avoid reporting the disease because they fear having to cull their herd, and it often happens that this represents their only means of living.

Official BTb (NPBTb) surveillance by federal authorities consists mainly of two strategies: 1) tuberculin skin testing and 2) slaughter-house surveillance. The tuberculin test consists of injecting the animal with a bovine purified protein derivative (PPD bovis) intradermally and measuring the reaction (inflammation) at the injection site. As it is a specific delayed-type hypersensitivity response, animals having been exposed to or infected with *M. bovis* tend to have a positive response. Any swelling, sensitivity, or increase in thickness of the skin is considered to be a positive response. However, exposure to environmental mycobacteria may cause false-positive responses. Through slaughter-house surveillance, however, a sample of tissue (generally if visible lesions – granulomas – are present) is sent to a reference laboratory for culture and histopathology. Even though the latter may be a more passive strategy, it gives a more convincing perspective on the sanitary status of the animals in a particular region. Once the pathogen is isolated, molecular methods may be used to evaluate genetic diversity, and consequently, trace infection. Spoligotyping and whole-genome sequencing provide molecular fingerprints for *M. bovis*, so when cattle from different farms show exact or similar fingerprints, it can serve as an indication that they have a common source of infection (13, 20, 24, 28).

The higher amount of samples obtained from Tepatitlán de Morelos is accounted for by the fact that more animals are transported here for slaughter, due to the owners' preferences. In fact, of the four abattoirs included in the study, two of them are considered to be within the municipality of Tepatitlán de Morelos. Therefore, Tepatitlán accounted for 69% of the samples obtained.

Regarding the proportion of positive cases per municipality, all three were similar: Tepatitlán de Morelos had 14%, San Miguel el Alto 19%, and Arandas 14%. However, Tepatitlán contributed the most to the overall prevalence of the Altos Sur region, with 9.9%. San Miguel el Alto and Arandas contributed almost equally with 2.7% and 2.4%, respectively. In general, the prevalence for the region of Altos Sur was 15.06%, which is higher than the one reported by

official surveillance (1.98%) but similar to that reported previously (15) for high milk production regions in Mexico (16%). There is a lack of studies for this region in particular, but BTb prevalence for Jalisco has been reported at 0.07%, 0.09%, 0.22%, 0.26%, and 0.35% (25).

It is also important to mention that the total amount of positive cases reported in this study may be under-represented. False negative results may be possible due to the stage of infection in the animal. For example, initial infection may show no lesions, and the burden of mycobacteria may not be sufficient to obtain positive culture. Furthermore, even though extreme care was taken to perform culture and PCR, there may exist certain substances that can inhibit bacterial growth and PCR amplification.

Concerning lesion distribution, lymphatic node tissue was the most affected (45/68; 66.1%), as seen in Table 2. Previous studies in Mexico had similar findings (4, 15). This is clearly associated with how the infection enters and spreads throughout the bovine organism. The main routes of infection are nasally *via* aerosols and orally by ingestion of contaminated material, such as feed and water (17).

We found 10 different spoligotype patterns in this study out of a total of 50 *M. bovis* isolates. This represents the high genetic diversity of the pathogen in the region of Altos Sur, in Jalisco, Mexico. Other studies have also found high genetic diversity on the national (6, 8, 12) and local scales (24). The most frequent spoligotypes were SB0673 (32%) and SB0121 (26%), which coincides with reports made by Gutierrez-Reyes *et al*. (7) and Milian-Suazo *et al*. (13). On the other hand, contrary to what some studies reported, which was that SB0140 was the most frequent (5, 21), we did not find this spoligotype in the region of Altos Sur. In one study that also analysed *M. bovis* isolates from Jalisco (5), out of 14 spoligotypes found only four coincided with those found in our research (SB0673, SB0121, SB0145, SB0971); these are the most common and frequently reported for Mexico (13). Five spoligotypes found in this study (SB1758, SB1216, SB1177, SB0872, and SB1023) have not been previously reported in the literature. The appearance of these "new" strains may be a consequence of the accumulation of non-lethal mutations caused by the natural evolution of the microorganism (23). It has been described before that the occurrence of singletons may be a consequence of the high prevalence of the disease in the population (7), and the role of time, BTb being a chronic disease (12). Both contribute to increasing the chances of an *M. bovis* strain evolving into new genetic lineages.

It is important to mention that in this study only Holstein cattle were sampled, and because this breed is mainly used for milk production in Mexico, it is necessary to explore the risk posed to public health by this zoonosis. The most frequent spoligotypes found in the cattle in this study have also been recovered from

human cases of TB in Mexico (24, 18) and other countries (21). Even though the risk of transmission of *M. bovis* to humans can be reduced through pasteurisation of milk, a revered feature of Mexican culinary culture is the production of dairy products, mainly cheeses, with fresh (unpasteurised) milk. One study was able to recover *M. bovis* from a group of soft fresh cheese samples from Mexico (8). Also, there exist certain risk factors which can make an individual more susceptible to becoming ill from infection with *M. bovis*, which are mainly factors causing immunodeficiency, such as HIV infection or diabetes, glucocorticoid use, or smoker status. Age can also play an important role, and though it might seem logical to think that children and elderly people are at risk, studies have shown that the predominant age range of victims of the disease is from 30 to 65 (27).

At least three spoligotypes were shared between the municipalities (SB0673, SB0121, and SB0145). The region of Altos Sur is a small area within the state of Jalisco, so it is reasonable to observe these results. Arandas and San Miguel el Alto are adjacent to each other, which would suggest that these municipalities may share genotypes, and they did in SB0673, SB0121, SB0145, and SB1758. The latter is exclusively shared between these two. Tepatitlán de Morelos, being the largest municipality, had the most animals sampled, and thus showed the most genetic diversity, holding 8/10 (80%) of the genotypes found in this study. In all, these results suggest movement of infected animals between farms; in this region, it is common for large farms to sell animals that have declining production to smaller farms, which opens the door for the exchange of infected animals.

With regards to genetic relationships between strains, spoligotyping was not useful for making clear connections among all the genotypes found (Fig. 2). This is where whole-genome sequencing demonstrates higher resolution power, as shown by other authors (11, 23). From Fig. 4, we can clearly see that there are three main groups of *M. bovis* circulating in the region of Altos Sur, and the small SNP differences between isolates of the same clade suggest transmission between animals (11, 23). This cannot be determined by spoligotyping alone due to the possibility of homoplasy (2). Unfortunately, these results do not confirm a unique source of infection for this region. Furthermore, when compared to isolates from other parts of the country (Fig. 5), many isolates from Jalisco show close genetic relationships to isolates from Baja California, Coahuila, Guanajuato, Estado de Mexico, and Aguascalientes. Compliance with the Mexican Norm NOM-031-ZOO-1995 (NPBTb) demands that animal movements are restricted, so infected animals may not be transported to low- or zero-prevalence areas. Of the states mentioned above, only Coahuila remains in a low-prevalence area, unlike Jalisco and the other states, which are designated as Control Areas. Mexico has imported cattle from South America and

Europe in the past, and previous studies (14) have found that spoligotypes are shared between these regions, suggesting introduction of infection. Unfortunately, *M. bovis* infection in Mexican cattle is long-standing, and aggravated by the problem that small farms pose regarding the lack of reporting of infections. However, one study showed significant genetic differentiation between isolates from the central region of Mexico (Queretaro, Guanajuato, Aguascalientes, Estado de Mexico, Hidalgo, and Jalisco) and isolates in the northwest (Baja California), which suggests that the NPBTb for the control of bovine tuberculosis is successful (19).

In this regard, it is critical to implement strategies that improve the success of the NPBTb on the local scale. The region of Altos Sur is an example of the other rural areas in the country that need urgent surveillance. Additionally, it is important that farmers be educated on the jeopardy to people's health and farmers' livelihoods of this common zoonotic disease so that they can assess the risks of introducing unknown cattle into their herds, and most importantly, so that milk from known infected animals is strictly pasteurised, and not used for the production of any type of dairy product, thus eliminating the risk of contagion to humans.

BTb in the region of Altos Sur, in Jalisco, has a higher prevalence (15.06%) than that reported for the whole of Jalisco by official authorities. The use of raw milk from infected animals in this region for dairy products such as fresh milk cheese poses a great risk to public health, nationally and internationally. Spoligotyping and WGS confirmed transmission among herds from different municipalities of the region, hindering efforts by the NPBTb. Urgent attention must be dedicated to rural regions such as Altos Sur in order to make control and eradication efforts more efficient.

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