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Whole Genome Sequencing for Detection of Zoonotic Tuberculosis in Queretaro, Mexico

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Abstract

A total of 2,736 samples, sputum, urine, and other fluids, collected from 1,154 tuberculosis suspicious patients in Queretaro, Mexico were included in the study. Acid-fast staining and culture in selective mediums, Stonebrink and Lowenstein-Jensen, were performed in all samples. Genotyping of isolates was performed by spoligotyping and single nucleotide polimorfism (SNP) whole genome sequencing. *Mycobacterium bovis* spoligotypes and SNP-types obtained were compared to those of cattle found in a database. Twenty-one (1.8%) isolates of *Mycobacterium* were obtained by culture, all from sputum; two (13%) were identified as *M. bovis* by spoligotyping, SB0673 and SB0971, which are frequently found in cattle in Mexico. From the isolates´ total, 15 were whole genome sequenced, confirming two as *M. bovis*. The SNP patterns of the two *M. bovis* isolates from human were similar to those found in cattle in different parts of Mexico.

Keywords: *M. bovis*; Tuberculosis; Spoligotyping; Molecular epidemiology, Queretaro

Introduction

Tuberculosis (TB) is a disease that affects both humans and animals. Humans are predominantly infected by *Mycobacterium tuberculosis* whereas animals by *M. bovis*; however, both are susceptible to both species, with no significant clinical, radiological or pathological differences [1]. *Mycobacterium tuberculosis* and *M. bovis* are 99.9% similar at the nucleotide and 16S rRNA sequences, virtually identical [2-4]. *M. bovis* has usually been neglected as an important pathogen in human tuberculosis; nevertheless, different studies in different populations have shown that *M. bovis* has an important role in human tuberculosis [5-9]. In past decades, retrospective studies on stored samples reported that TB due to *M. bovis* is between 8 and 10% [5-7]. Recent studies in Mexico, in a dairy zone with high prevalence of the disease in cattle (17%), a prevalence of 7% was found in samples from symptomatic patients [8]. More recently, in San Diego, *M. bovis* was found in 45% of tuberculosis cases in children, 6% in adults, most in patients with Hispanic background [9]. From 1995 to 2009, at the Occidental Medical Center of Guadalajara, Jalisco, Mexico, from 124 cases, 35 (28%) were due to *M. bovis*; 26 extra-pulmonary [10]. A study performed at the Siglo XXI Hospital in Mexico City in samples from 2000 to 2007, reported 22.3% of cases due to *M. bovis* [11].

In humans, *M. bovis* is an opportunistic pathogen that takes advantage of vulnerable populations, such as those with acquired immunodeficiency syndrome (AIDS) [12-14], diabetes mellitus, the presence of lymphomas, leukemia, chronic renal insufficiency, immunosuppressant treatments, disorders associated to gastrectomy and jejunoileal surgery, malnutrition, overpopulation, smoking and drug addiction [15-21]. The presence of *M. bovis* in human cases is relevant since it is resistant to pyrazinamide, a first-line antibiotic used in the treatment of tuberculosis.

In Mexico, the prevalence of tuberculosis in cattle has been reported to be less than 0.5% in 25 regions or states, classified by APHIS-USDA as low prevalence [22]. Twelve states are in the eradication phase, with prevalence lower than 2%, and 21 states are in the control phase,with prevalence higher than 2% or unknown. In general, it has been estimated that the prevalence of tuberculosis in dairy cattle in Mexico is ≈16%. Actual federal regulation does not make pasteurization of milk mandatory, which is why there is still risk of infection with *M. bovis* in humans through the consumption of non-pasteurized dairy products. Thus, the objective of the present study was to determine the proportion of cases of tuberculosis caused by *M. bovis* in patients from the Mexican Social Security Institute (IMSS) in the City of Queretaro; as well as to compare the genotypes from people and those obtained from cattle.

Materials and Methods

Biological samples

Samples from sputum, urine and other tissues were collected from TB suspicious patients, three in consecutive days at the Mexican Social Security Institute (IMSS) of the City of Queretaro, Mexico from October 2013 to July 2014. Isolation of *Mycobacterium* was performed by culturing in Stonebrink and Lowenstein-Jensen mediums [23]. Isolates were genotyped by spoligotyping [24] and whole genome sequencing for the identification of single nucleotide polymorphisms (SNP).

Samples from cattle

Suspicious lesions were obtained from cattle at slaughterhouses in different parts of Mexico. These samples were decontaminated by the Petroff method and inoculated onto Stonebrink medium for the isolation of *M. bovis*. All of the resulting isolates were genotyped by spoligotyping and 200 of them were whole genome sequenced.

Whole genome sequencing and phylogenetic analysis

Whole genome sequencing was performed at the National Veterinary Services Laboratory of APHIS-USDA (NVSL-APHIS-USDA) in Ames, Iowa, USA, according to their own protocols [25]. Isolates were sequenced using a MiSeq (Illumina, San Diego, CA) and the Nextera XT library preparation kit (Illumina, San Diego, CA). Using the NVSL in-house pipeline, the sequences were aligned and compared to the reference genome AF2122/97 (NCBI accession number NC_0002945) for SNP calling. Only informative and validated SNPs were used to construct maximum likelihood phylogenetic trees using RAxML.

Results

A total of 2,736 samples were analyzed from 1,154 tuberculosis suspicious patients. Two-thousand two-hundred thirty six (83%) of the samples were sputum, 434 (16%) urine and 37 (1%) from other tissues/fluids: gastric juice, cerebrospinal fluid, pleural fluid and ascitic fluid, among others. According to the sputum quality, some were classified as saliva, 329 (14.5%), and 1936 (85.5%) as phlegm. Most patients were from out-patient consulting (55%), 22% from hospitalization and 23% had no information, 46% were women and 43% men. Eleven percent had no information about gender. Fifty percent were younger than 30 years, 23% were between 31 and 60 years, and 27% were older than 60 years.

From the 1,154 patients in the study, a total of 21 (1.8%) isolates were obtained by culture and identified as *Mycobacterium*, all of them from sputum. As sputum is obtained from the lungs, this confirms this organ as the site of tuberculosis infection, which indicates pulmonary disease. From these, 17 were also positive to AFB stain (Table 1). Six of the positive patients were women (29%) and seven men (33%). It was not possible to obtain information for the other 8 (38%) patients. According to age, 5 (24%) were between 31 and 60 years old, and 4 (19%) were older than 60 years old; no information could be obtained from the rest. Positive cases corresponded mainly to hospitalized patients (57%).

	CULTURE			
		Positive	**Negative**	**Total**
AFB	Positive	16	1	17
	Negative	4	0	4
	Total	20	1	21
		SENSITIVITY	80%	

Table 1: Diagnostic results for AFB and isolation tests of tuberculosis suspicious samples of people in Queretaro, Mexico from October 2013 to July 2014.

Real-time PCR was performed for the 21 isolates, 2 (13%) were identified as *M. bovis*. These isolates were also whole genome sequenced, and 13 (87%) were identified as *M. tuberculosis*. Considering the total number of isolates (n=21), 9.5% were *M. bovis*. Suprisingly, one isolate, 13-1206497FM, was identified as *Mycobacterium terrae*, a species that does not belong to the MTBC complex (Figure 1).

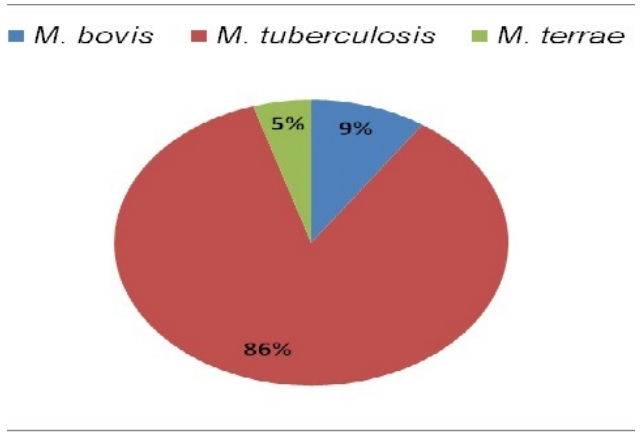

Figure 1: Species of *Mycobacterium* involved in cases of human tuberculosis in suspicious patients from Queretaro, Mexico, October 2013 to July 2014.

The spoligotype pattern of one of the *M. bovis* isolates was identified as SB0673, which is one of the most frequent spoligotypes in cattle in Mexico. This spoligotype has been identified previously in other cases of human tuberculosis, in Mexico [26], as well as in the United States in patients of Mexican origin [27,28]. The spoligotype from the second isolate was SB0971, which is the fifth most common spoligotype in Mexico, and it has also been identified in humans from previous studies in Mexico, as well as in the United States in patients of Mexican origin.

Using the SNP patterns obtained for the *M. bovis* isolates, both from human origin (n=2) and cattle origin (n=200), alignments were performed and phylogenetic trees were constructed in order to find genetic relationships between them. The same was performed using only isolates from cattle in Mexico. The SNP patterns for the two isolates of human origin, 13-1212317FM and 14-805568FM, were almost identical to those from cattle of the region of Queretaro (97-2166, 97-2398, 97-2167; 97-1525 and 97-2453, respectively) (Figure 2). These results confirm that *M. bovis* plays an important role in human tuberculosis in Mexico.

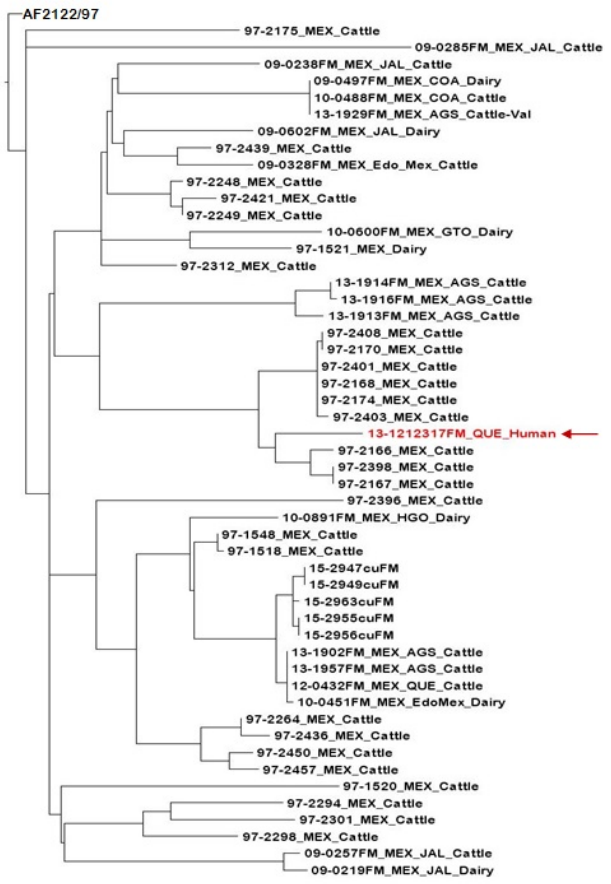

Figure 2A: Maximum likelihood SNP-based phylogenetic trees presenting genetic relationships between SNP-genotypes of *M. bovis* isolates from cattle and humans-13-1212317FM. Red arrows show how the human isolates lies within a clade of isolates of bovine origin.

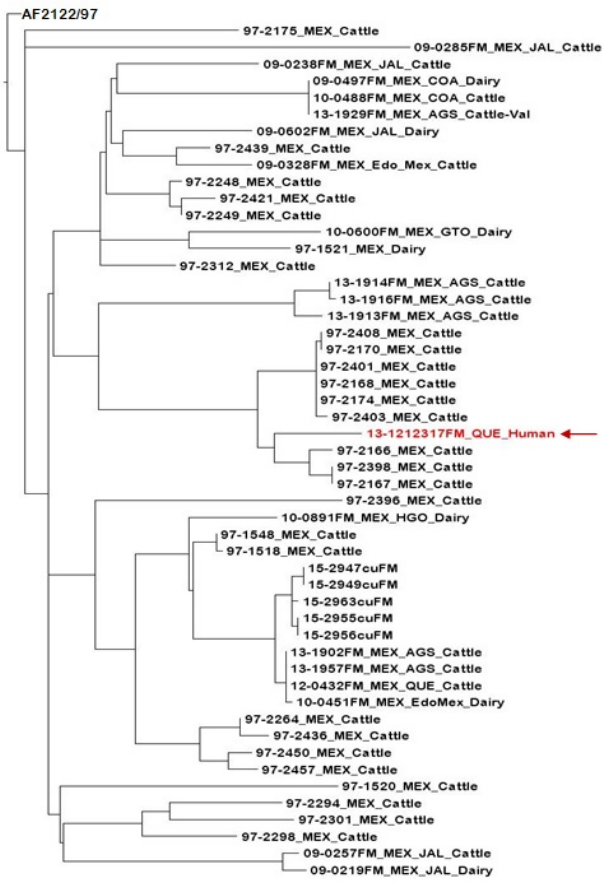

Figure 2B: Maximum likelihood SNP-based phylogenetic trees presenting genetic relationships between SNP-genotypes of *M. bovis* isolates from cattle and humans-14-805568FM. Red arrows show how the human isolates lies within a clade of isolates of bovine origin.

Discussion

The objective of this study was to estimate the proportion of human cases of tuberculosis that is caused by *M. bovis* in patients suspected of having the disease in the City of Queretaro, Mexico, and to compare the *M. bovis* spoligotypes and SNP-types from humans to those found in cattle. The proportion of cases of human tuberculosis due to *M. bovis* was 9%, which is close to the 4.5% reported by Laniado-Laborin et al. [29], but lower than the 28% reported by Portillo-Gomez and Sosa-Iglesias [10], and the 31.6% by Toledo-Orduña et al. [30]. All of these figures, however, are higher than the 3.1% reported by WHO worldwide [31]. Recent studies in other parts of the world have reported numbers such as 6.9% in Uganda [32], 5% in Nigeria [33], 0.5% in Taiwan [34] and between 0 and 2.5% in ten Latin American countries [35]. The difference across these proportions may be due to the fact that cases reported are mainly from urban areas or industrialized countries, whilst undeveloped countries rarely or sporadically have access to the resources needed to perform differential analysis of the causal agent.

It is surprising that from 2,736 samples, only 21 were positive to AFB and isolation. This suggests that the quality of the samples turned into the laboratories is not useful for diagnosis; training courses for medical staff dealing with patients could be appropriate. Better quality of samples reduces the amount of resources used in diagnosis. This is important given the low sensitivity of the AFB stain, which overlooks about 20% of the cases (Table 1); which is confirmed in our study.

Through molecular and phylogenetic analysis the genetic and epidemiological relationship between *M. bovis* strains obtained from humans and cattle (Figure 2) in the same geographic region could be established. Isolates 13-1212317FM and 14-805568FM obtained from humans had the same molecular patterns as those obtained from cattle, which confirms that transmission of *M. bovis* between these populations is occurring. Interestingly, both isolates were obtained from sputum, which suggests respiratory as the most probable route of transmission, making the lungs the site of infection; challenging the common belief that *M. bovis* causes only extra-pulmonary infections. This is also important because *M. bovis* has been mostly associated with the digestive route of transmission, with lesions in mesenteric lymph nodes. From our results, it can be inferred that transmission occurs directly from cattle to human by inhalation of infected aerosols, causing pulmonary tuberculosis. Unfortunately, it was not possible to obtain information about patients´ occupation.

A weakness of this study is the lack of epidemiological information of patients. The reason for this is because we were not successful in obtaining this information from IMSS authorities in spite of our constant requests for it.

Conclusion

The proportion of cases of tuberculosis by *M. bovis* in suspected patients in Queretaro, Mexico was 9%. Spoligotypes SB0673 and SB0971 from the human isolates are identical to spoligotypes obtained from cattle across Mexico in general, but particularly from Queretaro. SNP patterns from the whole genome analysis of the *M. bovis* strains from humans were very similar to those identified in cattle from various states of Mexico, including Queretaro. *M. bovis*, the causal agent of tuberculosis in cattle has a very important role in human cases of TB.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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