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Tracing the origins of *Mycobacterium bovis* tuberculosis in humans in the USA to cattle in Mexico using spoligotyping☆

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Abstract

Objectives—To compare genotypes of *Mycobacterium bovis* strains from humans in Southern California with genotypes of *M. bovis* strains in cattle in Mexico and the USA to explore the possible origins of human infections.

Methods—We conducted a descriptive analysis of *M. bovis* genotypes from a binational population of humans and cattle using spacer oligonucleotide typing (spoligotyping).

Results—One hundred six human *M. bovis* spoligotypes were compared to spoligotypes from 496 Mexican cattle and 219 US cattle. Twelve spoligotype patterns were identified among human cases and 126 spoligotype patterns were detected in cattle. Over 91% (97/106) of the human *M. bovis* isolates had spoligotypes that were identical to those found in Mexican cattle. Four human cases had spoligotypes that matched both cattle born in Mexico and in the USA. Nine human cases had spoligotypes that did not match cattle born in Mexico or the USA.

Conclusions—Our data indicate that the population of *M. bovis* strains causing human TB disease in Southern California is closely related to the *M. bovis* strain population found in Mexican cattle and supports existing epidemiological evidence that human *M. bovis* disease in San Diego likely originated from Mexican cattle.

Keywords

Molecular epidemiology; *Mycobacterium bovis*; Tuberculosis; Spoligotyping

☆The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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1. Introduction

Tuberculosis (TB) is currently one of the leading causes of death due to infectious disease globally, with 9.3 million incident cases and over 1.7 million deaths reported in 2007.[1] TB in humans is caused mostly by *Mycobacterium tuberculosis*, but this was not always the case. In the early 1900 s an estimated 30% of TB cases in Europe were caused by the cattle TB pathogen, *Mycobacterium bovis*,[2] a closely related Mycobacterium species largely transmitted to humans via inhalation of infectious droplets from infected cattle and consumption of contaminated, unpasteurized dairy products.[3]

The introduction of milk pasteurization and 'test and slaughter' cattle control programs in the early 1900 s all but eradicated *M. bovis* from cattle and humans in most of the USA and other developed nations.[2,4] A recent study of all human TB cases in the USA from 1995 through 2005 estimated that only 1.4% of cases were still being caused by *M. bovis*, most of which were among individuals born outside of the USA.[5]

However, in certain regions of the USA along the border with Mexico, and in Mexican-born individuals in New York City, *M. bovis* TB prevalence has been shown to be significantly higher than the national prevalence.[6–12] In San Diego, California in particular, over 45% of all culture-confirmed TB cases in children and 8% of all TB cases were recently found to be due to *M. bovis*.[13]

Epidemiological evidence suggests that *M. bovis* TB in Southern California is largely the result of the consumption of unpasteurized dairy products, such as unpasteurized cheese commonly referred to as queso fresco, from infected cattle in Mexico,[10,13–15] but evidence linking the *M. bovis* pathogen populations in cattle and humans is lacking. The aim of this study was to utilize molecular genotyping to examine the relationships between the population of *M. bovis* strains collected from cattle in Mexico and the USA, and the population of *M. bovis* strains isolated from human TB cases in San Diego.

2. Methods

2.1. Study design

We conducted a retrospective analysis of all known *M. bovis* genotypes in humans from San Diego County, and a convenience sample of genotypes from cattle in Mexico and the USA, represented by spacer oligonucleotide types (spoligotypes). The study protocol was approved by an Institutional Review Board at the University of California, San Diego.

2.2. Data source

We obtained human *M. bovis* isolates from the San Diego County Tuberculosis Surveillance Program for the years 2004 through 2007, which included all known *M. bovis* TB cases. *M. bovis* TB cases represented approximately 10% of all TB cases during that period. All TB isolates from patient specimens were initially identified as *M. tuberculosis* complex based on the AccuProbe hybridization protection assay (GenProbe, San Diego, CA, USA). We identified specimens as either *M. bovis* or *M. tuberculosis* based on culture morphology, the results of the niacin strip test, the nitrate reduction test and their susceptibility to pyrazinamide,[16] and confirmed species designations with genotyping[17] conducted by the California Department of Public Health, Microbial Diseases Laboratory.

M. bovis genotypes from cattle born in Mexico were collected as convenience samples as part of cattle TB surveillance efforts in Mexico from 1997 through 2007. Mexican isolates were genotyped by the Programa Nacional de Epidemiología (CENIDFA-INIFAP) in Mexico. We also obtained Mexican cattle *M. bovis* spoligotypes from peer-reviewed, published studies[14,

[18–24] listed in PubMed from 1997 through 2008. To our knowledge these genotypes represent all known reported *M. bovis* genotypes by spoligotyping from cattle in Mexico.

For this study, we also examined all available spoligotypes in the US national surveillance database of *M. bovis* isolates sent to the National Veterinary Services Laboratories (NVSL), Ames, Iowa (US Department of Agriculture, Animal and Plant Health Inspection Services) from 1997 through 2008. The NVSL surveillance database represents a convenience sample of all cattle suspected of having TB in the USA from 1997 to 2008.

2.3. Spoligotyping

Spoligotyping was performed independently in Mexico and in the USA. Spoligotyping was performed by all laboratories using the Spoligo Kit (Isogen Bioscience BV, Maarsen, the Netherlands) using standard methods described in detail in Kamerbeek et al.[25] Briefly, the direct repeat (DR) region of the *M. bovis* genome was amplified using a polymerase chain reaction (PCR), then probed for the presence or absence of 43 different spacer sequences using a dot blot method. Primers were labeled with biotin, and amplified products were detected using a streptavidin–POD conjugate (Boehringer Mannheim, Mannheim, Germany) or a chemiluminescent detection system (Amersham ECL, Rockford, IL, USA) with X-ray film.

2.4. Mycobacterial interspersed repeat units (MIRU)

Spoligotypes of *M. bovis* isolates from humans in San Diego County were further subtyped by MIRU typing as described elsewhere.[26–28] Briefly, loci consisting of multiple copies of tandem repeats distributed around the *M. bovis* chromosome were amplified by PCR before reactions were multiplexed and fragment lengths were evaluated using a capillary sequencer. The standard 20-locus set was tested. Cattle spoligotypes were not further subdivided by MIRU genotyping as MIRU data were not available for the cattle isolates and isolates are no longer available for testing.

2.5. Data management and analysis

We converted all spoligotype data to a 15-digit number using an octal coding system and compiled the data into a database using MS Excel software (Microsoft, Seattle, WA, USA). Each spoligotype was cataloged with a unique identifier along with information on its source (human or cattle), cattle breeding purpose (dairy, beef, unknown), and state within Mexico or USA from which the case originated. For each cohort, cattle and human, we classified *M. bovis* isolates with identical spoligotype patterns as clusters of related strains. Isolates with spoligotypes that did not match any other human or cattle spoligotypes were considered unrelated or ‘orphan strains’.[24,29–32] All spoligotype patterns were submitted to the public *M. bovis* spoligotype database (www.mbovis.org), and given a unique identifier number (SB number) to identify them in this publication.

3. Results

We obtained a total of 106 *M. bovis* genotypes from 109 human *M. bovis* TB cases in San Diego County from 2004 through 2007 (spoligotypes for three cases could not be resolved). These human *M. bovis* spoligotypes were compared to spoligotypes from 496 *M. bovis* isolates from Mexican cattle and 219 *M. bovis* isolates from US cattle.

3.1. Mycobacterium bovis isolates from humans

Twelve different spoligotype patterns were identified among the 106 human cases in San Diego County from 2004 through 2007 (Table 1). Overall 98% (104/106) of cases were Hispanic;

two cases occurred in non-Hispanic whites. Sixty-two percent of cases (66/106) reported being born in Mexico while the remainder reported being born in the USA.

Eighty-five percent (90/106) of the human *M. bovis* cases were clustered in two spoligotypes, SB0145 and SB1040. Eleven of the remaining 16 human *M. bovis* cases were in clusters of two or three isolates, and four cases had orphan spoligotypes that did not match any other human or cattle cases. Isolates were further subdivided by MIRU subtype into 22 different strain types. The MIRU pattern for six out of the 106 human isolates could not be resolved.

3.2. Mycobacterium bovis isolates from cattle

M. bovis genotypes were obtained from Mexican cattle populations in 13 of 31 Mexican states (Table 2). The majority were from Chihuahua (n = 92) and Jalisco (n = 61), and the northern and eastern Mexican states. Baja California contributed 10 isolates. We could not determine the state of origin for 117 Mexican cattle genotypes.

Of 496 *M. bovis* cases in Mexican cattle, we identified 126 different spoligotype patterns. Ninety-three Mexican cattle spoligotypes were orphan strains (Table 3). Approximately one third (146/496) of Mexican cattle tested were dairy cattle and one third were beef cattle (167/496). The breeding purpose for 37% (183/496) of cattle tested was unknown.

3.3. Human/cattle spoligotype matches

Over 91% (97/106) of the human *M. bovis* TB isolates from San Diego County had spoligotypes that were identical to those found in Mexican cattle (Table 1). Four human cases had *M. bovis* spoligotypes that matched cattle originating from both Mexico and the USA, and nine human cases had spoligotypes that did not match cattle in Mexico or the USA. Eleven of the 13 Mexican states sampled yielded cattle *M. bovis* isolates with spoligotypes that matched human *M. bovis* spoligotypes in San Diego (Table 2).

Human *M. bovis* spoligotypes SB0145, SB1040, SB0152, and SB0971, which accounted for 88% (93/106) of all the human cases, were found in only 11.5% of the Mexican cattle sampled (Table 3), but were found widely distributed in all but two of the Mexican states sampled (Figure 1). Human *M. bovis* spoligotypes SB0673 and SB0140, found in both Mexican and US cattle, accounted for only 3.8% of the human cases, but were found in 31% (152/496) of *M. bovis* isolates obtained from Mexican cattle (Table 3), and in 45% (99/219) of US cattle tested (Table 4).

4. Discussion

Our analyses indicate that the majority of human *M. bovis* pathogen strains identified in San Diego from 2004 through 2007 had the same spoligotype as strains found only in cattle in Mexico, supporting existing epidemiological evidence[7–14] that the population of *M. bovis* strains causing human *M. bovis* disease in this region most likely originated in Mexican cattle. Most of the human *M. bovis* cases with spoligotypes matching Mexican cattle spoligotypes were clustered into two closely-related patterns (SB0145 and SB1040). These spoligotypes were rare among the sampled Mexican cattle, but widely distributed across 11 of the 13 Mexican states sampled, and were not detected among the US cattle surveillance cohort. This lack of concordance in the frequency of *M. bovis* strains circulating in the suspected animal source population relative to the strains circulating in humans has been documented in other countries where *M. bovis* is a zoonotic disease.[33–35] It is possible that the discordance observed is a sampling artifact as the Mexican cattle spoligotypes represent only a small proportion of the approximately 23 million head of cattle in Mexico, but it might also reflect changes in the Mexican cattle population that occurred between the time the human infections were acquired

and the time the infections reactivated as TB disease, as has been documented in the UK.[36] It is also possible that human *M. bovis* strains SB0145 and SB1040, which differ by only a single spoligotype spacer, have been spread from person-to-person, which would increase their frequency.

While person-to-person transmission of *M. bovis* has recently been demonstrated in France[37] and the UK[38] and has been hypothesized to be occurring in San Diego,[11,12] there is considerable epidemiological evidence in San Diego that indicates person-to-person transmission is probably rare, despite the fact that approximately 54% of *M. bovis* cases in this region have pulmonary disease.[13] This evidence includes the absence of *M. bovis* TB cases in children not yet exposed to dairy products (these children do get *M. tuberculosis* TB which is only transmitted person-to-person);[10] and the evidence that *M. bovis* incidence in San Diego, which increased significantly from 1994 through 2005, has not followed the same trends as *M. tuberculosis* incidence, which has steadily decreased since 1994. This suggests that *M. bovis* transmission mechanisms are different from the person-to-person transmission of *M. tuberculosis*.[13]

The two spoligotypes from four human cases that matched strains from Mexican and US cattle (SB0673 and SB0140), were common in Mexican cattle, and were also found in cattle from six different states in the USA, including California. It is not surprising that some of the spoligotypes examined were present in cattle from both sides of the border. Restrictions on cattle trade between Mexico and the USA have only been in place for 10 years, and only prevent the importation of dairy cattle into the USA.[20] Beef cattle can still be exported from Mexico into the USA if they are demonstrated to be tuberculin skin test (TST)-negative at the time of export.

Since the US bovine TB eradication program began in 1917, *M. bovis* infection in US cattle has been extremely rare.[4] Less than 0.02% of US cattle tested positive for TB by TST in 2002,[39] and less than 0.002% of 377 000 cattle tested in a 2008 California *M. bovis* investigation were positive for TB.[40] Localized *M. bovis* outbreaks, low prevalence of *M. bovis* infection in US cattle herds, and strict national pasteurization requirements in the USA make it unlikely that the San Diego human cases with SB0673 and SB0140 spoligotypes are associated with US-born cattle with these spoligotypes.

In contrast, there have been several reports of widespread *M. bovis* infection in Mexican cattle.[18–21,23,32,41] In some regions of Mexico, up to 13% of dairy herds are reported to be infected with *M. bovis*[42] and up to 30% of milk produced in Mexico is not pasteurized.[19] Additionally, unpasteurized soft fresh cheeses, such as queso fresco, which is very popular within the Mexican community, are brought into the USA from Mexico for personal use and have been shown to be contaminated with *M. bovis*,[14,15] making it much more likely that human cases with SB0673 and SB0140 spoligotypes are associated with Mexican cattle with those spoligotypes.

4.1. Limitations

While spoligotyping is a practical approach for genotyping in large scale, population-level *M. bovis* studies such as this one, it is not a complete tool for molecular epidemiological analyses.[43] Spoligotyping requires less DNA than other methods, is easily replicated in low-resource settings, and results can be digitally expressed,[25,30,44,45] but, it can have limited discriminatory power if used in isolation.[46]

While we were able to obtain MIRU data for all of the human *M. bovis* cases, which demonstrated some heterogeneity amongst the human spoligotype clusters, it was not possible, due to the retrospective nature of the study and the lack of access to the cattle isolates, to obtain

MIRU data for any of the cattle cases. However, we do not believe that the lack of MIRU data limited our conclusion that the population of human *M. bovis* strains from San Diego likely originated from the population of *M. bovis* strains found in Mexican cattle, as that is the most parsimonious conclusion from all of the available molecular and epidemiological data. We could not exclude the possibility of homoplasy, where similar mutations in the human and cattle *M. bovis* strains could have arisen in phylogenetically unrelated strains. But given the clonal nature of *M. bovis*, the approximately 10–20 year stability of spoligotypes and the unidirectionality of mutations in the DR region (spacers can only be lost not re-acquired),[36] the likelihood of significant homoplasy in our binational pathogen populations is remote in the time-period under study. Additional MIRU and restriction fragment length polymorphism sub-typing, based on a systematic cattle sample, to further distinguish strains, are needed to determine the contribution of specific dairy sources to specific human cases in the USA.

Little can be inferred from the nine human *M. bovis* cases that did not match any of the cattle cases from Mexico or the USA. It is very unlikely that these cases match undetected cattle cases in the USA as the US cattle sample represents all known cattle TB suspects from 1997 through 2008. The same cannot be said for the Mexican sample. It represents only a small fraction of incident *M. bovis* cases among cattle in Mexico and additional matching strains may have gone undetected. Furthermore, the use of a convenience sample of cattle isolates from Mexico may have introduced a selection bias.

5. Conclusions

Our genotyping data indicate that the population of *M. bovis* strains causing human *M. bovis* TB disease in Southern California is closely related to the *M. bovis* strain population found in Mexican cattle and supports existing epidemiological evidence[10,13] that human *M. bovis* TB in San Diego likely originates in Mexican cattle. This suggests that elimination of *M. bovis* TB in San Diego will require a reduction in *M. bovis* prevalence in Mexican cattle, increasing pasteurization of milk products in Mexico, and reducing the flow of unpasteurized milk products between Mexico and the USA.

The California State and San Diego County Health Departments have long standing enforcement programs that target the illegal sale of dairy products, in addition to providing educational resources regarding the risks of unpasteurized dairy products. Furthermore, in 2008, the San Diego Health Department initiated a media campaign to educate the Hispanic community on both sides of the border about prevention of TB from *M. bovis*; however, studies to evaluate the effectiveness of these efforts have not been completed.

Restriction of importation of unpasteurized cheeses for personal use is also being tested at the region's USA–Mexico border,[47] and may assist in reducing exposures in the USA if found feasible to implement. However, since exposure will likely continue via consumption of dairy products in Mexico, it is critical that binational cooperation and resources for collaboration be made available in the USA and Mexico in order to eradicate *M. bovis* from Mexican dairy herds. Our analysis indicates that most *M. bovis* strain clusters are found throughout Mexico, suggesting that unrestricted cattle movement in the past has played a role in *M. bovis* transmission in Mexico. In order to fully address *M. bovis* TB infections in Mexican cattle, prevention measures that incorporate a nationwide approach will be required. Increased emphasis on pasteurization of most dairy products could also assist in prevention efforts.

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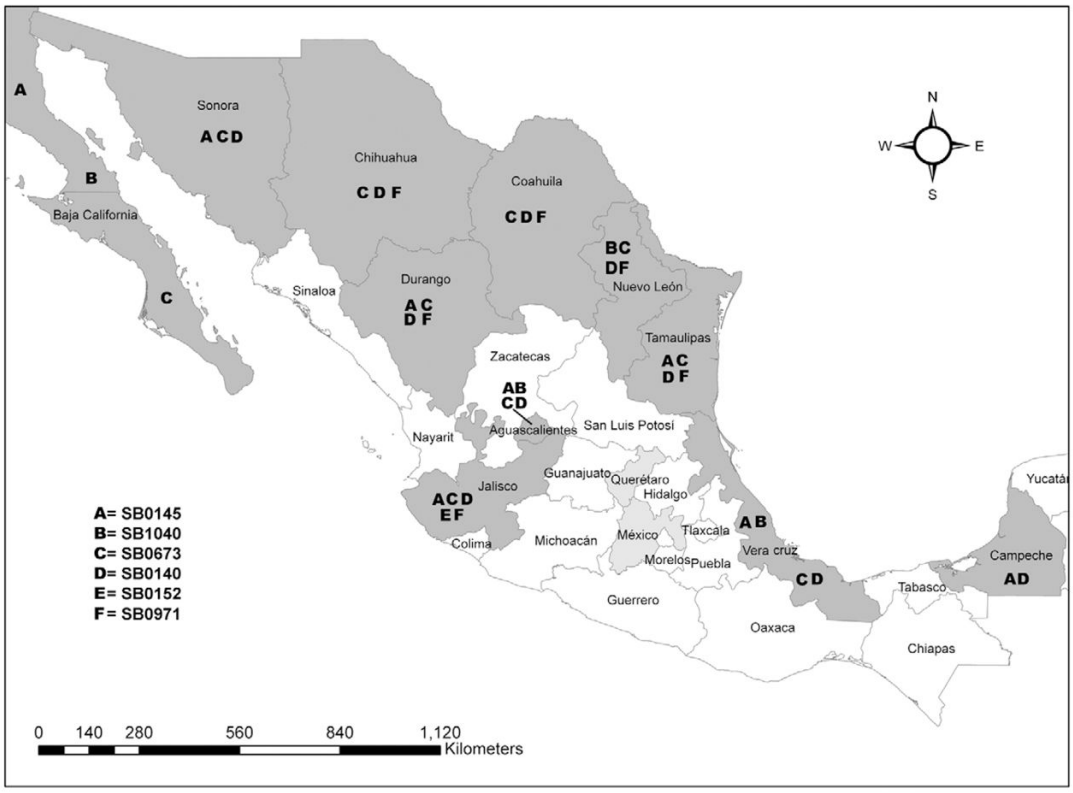

Figure 1.

Mexican states where cattle were sampled for *Mycobacterium bovis* (gray shading). Letters represent the San Diego human *M. bovis* spoligotypes that matched cattle in each state sampled. States shaded in light gray indicate Mexican states in which *M. bovis* was found in cattle but their spoligotypes did not match San Diego human cases.

Table 1

Frequencies of spoligotype patterns for *Mycobacterium bovis* cases reported in San Diego County from 2004 to 2007 showing matches to cattle originating in Mexico and the USA

Spoligotype name	Spoligotype (octal)	No. of MIRU subtypes[a]	Number of cases, *n* (% of total)	Match to Mexican cattle	Match to US cattle
SB0145	640013777777600	6	51 (48.1)	Yes	No
SB1040	640013377777600	4	39 (36.8)	Yes	No
SB1758	676573777776600	1	3 (2.8)	No	No
SB0673	264073777777600	2	2 (1.9)	Yes	Yes
SB0140	664073777777600	2	2 (1.9)	Yes	Yes
SB0152	400000077777600	1	2 (1.9)	Yes	No
SB1759	640013776377600	1	2 (1.9)	No	No
SB0971	664073777700600	1	1 (0.9)	Yes	No
SB1760	640012377777600	1	1 (0.9)	No	No
SB1761	640013357777600	1	1 (0.9)	No	No
SB1762	672573777777600	1	1 (0.9)	No	No
SB1345	676713677777600	1	1 (0.9)	No	No
		Total	106		

[a]Isolates were subtyped by mycobacterial interspersed repetitive units (MIRU) analysis. Six out of the 106 cases could not be resolved by MIRU analysis.

Table 2

Total number of *Mycobacterium bovis* strains and spoligotypes reported from cattle originating in Mexico. Table ordered by number of cattle spoligotypes from each Mexican state that matched human cases, 1997–2008.

State	Number of isolates by cattle type				Number of unique spoligotypes (% per state)	Number of unique cattle spoligotypes matching human spoligotypes in San Diego (% matching per state)
	Dairy	Beef	Unknown	Total (%)		
Jalisco	39	5	17	61 (12.3)	48 (78.7)	5 (10.4)
Tamaulipas	1	23	5	29 (5.8)	11 (35.5)	4 (30.1)
Veracruz	0	5	18	23 (4.6)	12 (54.5)	4 (26.7)
Durango	0	25	5	30 (6.0)	13 (44.8)	4 (30.1)
Aguascalientes	13	15	2	30 (6.0)	18 (60.0)	4 (21.1)
Nueva Leon	0	22	7	29 (5.8)	17 (58.6)	4 (21.1)
Sonora	7	11	13	31 (6.3)	10 (32.3)	3 (25.0)
Chihuahua	65	17	10	92 (18.5)	17 (18.5)	3 (18.8)
Coahuila	0	9	3	12 (2.4)	6 (50.0)	3 (50.0)
Baja California[a]	2	4	4	10 (2.0)	8 (80.0)	3 (37.5)
Campeche	0	3	0	3 (0.6)	2 (66.7)	2 (100)
Querétaro	8	0	0	8 (1.6)	7 (88.9)	0 (0)
Mexico State	11	0	10	21 (4.2)	18 (85.7)	0 (0)
Unknown[b]	0	28	89	117 (23.6)	24 (21.2)	5 (26.3)
Total	146	167	183	496		

[a] Baja California includes both Baja California and Baja California Sur.

[b] Cattle isolates obtained from animals for which state of origin was not available or untraceable.

Table 3

Mycobacterium bovis spoligotypes reported among isolates from cattle originating from Mexico, 1997–2008

		No. of isolates, *n*				
Spoligotype ID	**Spoligotype (octal)**	**Dairy**	**Beef**	**Unknown**	**Total (% of all)**	**Match to human cases**
SB0673	264073777777600	9	51	58	118 (23.8)	Yes (SB0673)
SB0121	676773677777600	29	15	4	48 (9.7)	No
SB0140	664073777777600	10	18	6	34 (6.9)	Yes (SB0140)
SB0669	264063777777600	5	8	18	31 (6.3)	No
SB0145	640013777777600	4	16	10	30 (6.0)	Yes (SB0145)
SB0971	664073777700600	0	9	9	18 (3.6)	Yes (SB0971)
SB0269	664063777777600	2	2	10	14 (2.8)	No
SB0327	676573777077600	3	9	2	14 (2.8)	No
SB1165	640033777777600	0	3	6	9 (1.8)	No
SB0986	264071777777600	4	2	2	8 (1.6)	No
SB0663	640003777777600	4	0	4	8 (1.6)	No
SB1040	640013377777600	1	5	2	8 (1.6)	Yes (SB1040)
SB1116	664063777700600	2	2	4	8 (1.6)	No
SB0119	676763677777600	5	0	0	5 (1.0)	No
SB1044	064063777777600	4	0	0	4 (0.8)	No
SB1496	640023777777600	0	1	3	4 (0.8)	No
SB0272	664073777757600	3	1	0	4 (0.8)	No
SB0130	676573777777600	0	1	3	4 (0.8)	No
SB1111	064061777777600	3	0	0	3 (0.6)	No
SB1114	440003777777600	3	0	0	3 (0.6)	No
SB0987	624073777700600	3	0	0	3 (0.6)	No
SB1118	640003777776600	0	0	3	3 (0.6)	No
SB1216	076573777077600	0	3	0	3 (0.6)	No
SB1110	064263777777600	2	0	0	2 (0.4)	No
SB1696	444063777746600	1	1	0	2 (0.4)	No
SB1125	464063735546600	0	0	2	2 (0.4)	No
SB1113	464063777756600	0	0	2	2 (0.4)	No
SB1129	674563777077600	0	0	2	2 (0.4)	No

		No. of isolates, *n*				
Spoligotype ID	**Spoligotype (octal)**	**Dairy**	**Beef**	**Unknown**	**Total (% of all)**	**Match to human cases**
SB1503	674573777077600	0	1	1	2 (0.4)	No
SB0807	676773675777600	2	0	0	2 (0.4)	No
SB1757	644073777700600	0	2	0	2 (0.4)	No
SB1755	666073777777600	0	1	1	2 (0.4)	No
SB0152	400000077777600	0	1	0	1 (0.2)	Yes (SB0152)
Mutliple[a]	Multiple	47	15	31	93	No
	Total	146	167	183	496	

[a]There are 94 isolates each represented by a single cow. Ninety-three did not match the human isolates and were grouped together in this table as ‘Multiple’. SB0152 was listed separately in this table since it was a match to human spoligotype SB0152.

Table 4

Mycobacterium bovis spoligotypes collected from US cattle, 1997–2008, that matched human *M. bovis* spoligotypes in San Diego

Spoligotype ID	Spoligotype/state	Number of isolates (%)
SB0673	264073777777600	
	New Mexico	78 (78.8)
	California	6 (6.1)
	Texas	5 (5.1)
	North Dakota	3 (3.0)
	Arizona	2 (2.0)
SB0140	664073777777600	
	Texas	2 (2.0)
	Colorado	2 (2.0)
	California	1 (1.0)
	Total	99 (100)