Recovery of *Mycobacterium bovis* from soft fresh cheese originating from Mexico.

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Running title: *Mycobacterium bovis* isolated from Mexican cheese.

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Abstract

Recent outbreaks of human tuberculosis in the U.S. caused by *Mycobacterium bovis* have implicated Mexican-origin cheese as a source of these infections. Two hundred and three Mexican-origin cheese samples were cultured, and *M. bovis* was recovered from one specimen. Therefore, *M. bovis* can be recovered from cheese, and may be a source for human infections.
Bovine tuberculosis, caused by *Mycobacterium bovis*, is a zoonotic disease that also affects humans. Although people are generally infected through inhalation of droplet nuclei, a significant proportion of human cases involve extrapulmonary tuberculosis, presumably caused by the consumption of non-pasteurized milk or dairy products (23). Indeed, milk pasteurization requirements in the United States were developed to prevent many foodborne infections, including tuberculosis, botulism and scarlet fever caused by consuming contaminated milk or dairy products (2). With the implementation of strict pasteurization requirements and a mandatory control program in live animals for bovine tuberculosis, the incidence of *M. bovis* infections in cattle in the United States has decreased to an all-time low of less than 0.001% (3). Consequently, human cases of *M. bovis* infections in the United States have also declined (23). However, several reports have shown an elevated incidence of human tuberculosis due to *M. bovis* in certain regions of the United States (4, 7, 14). For example, a study in San Diego County, California found that 129/1931 (6.7%) of all culture-positive tuberculosis cases in the County was due to *M. bovis* (14). A similar epidemiologic investigation in New York City also reported that 1% of culture-positive tuberculosis cases in this area were due to *M. bovis* (1). In both reports, patients of Hispanic ethnicity were especially at risk, and approximately 1/3 of the cases occurred in children. In both instances, epidemiologic investigations indicated that the consumption of unpasteurized dairy products, including soft fresh cheese originating from Mexico may have accounted for these cases (1). Therefore, to investigate this possibility, a survey of fresh cheese products entering the United States from Mexico for the presence of *M. bovis* was initiated as a collaborative
project between USDA-National Veterinary Services Laboratories (NVSL) and the California Animal Health and Food Safety Laboratories (CAHFS).

Two hundred and three cheese samples were collected from travelers entering California at the United States Customs and Border Protection Port in San Ysidro, CA from March through August, 2005. All cheese samples had been purchased by individuals for private consumption and were being imported through non-commercial channels. Thus, it is unknown if these products were derived from pasteurized or unpasteurized milk. These samples were shipped to USDA National Veterinary Services Laboratories (NVSL) for mycobacterial culture. For this, 5 g portions of cheese were weighed, aseptically transferred into a sterile stomacher bag containing 45 ml of sterile 2% sodium citrate, and homogenized in a stomacher (Model 80 Lab Blender, Seward Laboratory, England) for 2 minutes. The bag was then heat sealed and submerged in a 37°C water bath for 1 hour to liquefy the specimen. This suspension was then aseptically transferred into a sterile 50-ml centrifuge tube for ease of handling. The cheese suspension was decontaminated using the N-acetyl-L-cysteine (NALC)-NaOH method as previously described (20). For this, 10 ml of the homogenized sample was mixed with 10 ml of digestant consisting of sterile 0.05 M trisodium-citrate, 2% (wt/vol) sodium hydroxide, and 0.5% (wt/vol) NALC. The mixture was vigorously shaken for 20 s and allowed to stand at room temperature for 15 min. This mixture was then neutralized with 30 ml of 0.067 M phosphate buffer and centrifuged at 5,000 x g for 15 min at 10°C. After removal of the supernatant, 0.5 ml aliquots of the remaining pellet were inoculated into both BACTEC 12B and BBL™ MGIT™ 960 liquid media (Becton Dickinson Diagnostic Systems, Sparks, MD). Each BACTEC 12B bottle was supplemented with 0.2
ml of BACTEC™ PANTA™ PLUS. A total of 6.3 mg/ml of erythromycin was also added to help eliminate overgrowth by contaminants. Similarly, each BBL™ MGIT™ Mycobacteria Growth Indicator Tube was supplemented with 0.8 ml of BBL™ MGIT™ Growth Supplement/BBL™ MGIT™ PANTA antibiotic mixture (Becton Dickinson) and 7.0 mg/ml of erythromycin. Specimens were incubated at 37° C and monitored for growth for a total of six weeks according to the manufacturer’s protocols. Positive identification of M. bovis was performed using the AccuProbe Mycobacterium tuberculosis Complex Culture Identification Test Kit (Gen-Probe, San Diego, CA) and negative biochemical reactions for niacin and nitrate (8). Genetic confirmation of M. tuberculosis complex isolates as M. bovis was accomplished using a PCR-based typing method targeting the M. tuberculosis complex chromosomal region-of-difference deletion loci, as described previously(9).

Of the 203 cheese samples cultured, ten (4.9%) were positive for bacteria belonging to the genus Mycobacterium, with one isolate being identified as M. bovis. The ability to recover M. bovis from raw milk is well documented, especially from milk obtained from cattle residing in areas with a high regional prevalence of bovine tuberculosis (11, 13). In Mexico, the incidence of bovine tuberculosis varies by region, with beef cattle in the northernmost states having the lowest prevalence at less than 2% (21). However, the prevalence of M. bovis in dairy cattle in Mexico is significantly higher, with an estimated infection rate in this population of 16-17% (15, 17). Using partial 16s rDNA sequencing (12) and standard biochemical tests (8), seven mycobacterial strains were identified as M. fortuitum or M. fortuitum complex, one as M. moriokaense, and one as Mycobacterium species resembling M. moriokaense (Table 1).
The presence or absence of mycobacteria could not be confirmed on six of the cheese cultures due to overgrowth by contaminants, and the remaining 187 cultures were negative for acid-fast bacteria. The ten samples from which *Mycobacterium* species was recovered comprised several cheese varieties, including a hard-grating type, a semi-hard and several types of soft cheese (Table 1). The recovery of nontuberculous mycobacteria from cheese samples in this study is consistent with other reports that describe the recovery of various *Mycobacterium* species, including *M. fortuitum*, from raw milk obtained from dairy cattle (11, 13). Although not as severe of a public health concern as *M. bovis*, *M. fortuitum* complex bacteria are opportunistic pathogens and are implicated in a variety of clinical disease, especially in humans with immunocompromised immune systems (5). Because no history is available regarding the production of the cheese obtained during this survey, environmental sources of these mycobacteria cannot be ruled out due to contamination of the milk during either handling or processing.

Drug susceptibility testing was performed on the sole *M. bovis* isolate using BACTEC 12B medium and the radiometric modified proportion method (BACTEC 460; Becton-Dickinson) (8) at the following concentrations; streptomycin (2 µg/ml), isoniazid (0.1 µg/ml), rifampin (2 µg/ml), ethambutol (2.5 µg/ml) and pyrazinamide (100 µg/ml). This isolate was susceptible to all antibiotics tested except pyrazinamide, to which *M. bovis* is intrinsically resistant (22). Because the transmission of drug-resistant bacterial pathogens from animals to humans is a significant public health concern, an additional 11 random *M. bovis* isolates from the NVSL culture collection, obtained from cattle with epidemiological links to Mexico were also tested for antimicrobial susceptibility (data not shown). All strains of *M. bovis* were pan-susceptible to the antibiotics tested, with the
exception of pyrazinamide. Although a comprehensive survey of antibiotic resistance in 
*M. bovis* field isolates was beyond the scope of this survey, it appears that resistance to 
anti-tuberculosis drugs occurs infrequently in cattle from Mexico. This lack of antibiotic 
resistance is consistent with federal bovine tuberculosis control programs in both the 
United States and Mexico, which require that all infected animals be depopulated rather 
than treated for infection. However, an antibiotic-susceptible phenotype may be 
associated with diverse genotypes and thus be unrelated, requiring caution in the 
interpretation of these antibiograms.

To determine if the *M. bovis* strain recovered from the cheese sample was related 
to other *M. bovis* strains seen in cattle from North America, this isolate was genotyped 
using the standard NVSL protocol of spoligotyping, IS6110 RFLP and PGRS-RFLP, as 
described elsewhere (10, 18), with the following modifications for the IS6110 RFLP. For 
this technique, *M. bovis* genomic DNA was digested with 10 U of *Pvu*II, and a 445 bp 
IS6110 probe, spanning the *Pvu*II restriction site and thus producing two bands for each 
copy of IS6110 present, was utilized. To generate this probe, a portion of the IS6110 
element was PCR amplified using the primers 445R (5'- CGG ACA GGC CGA GTT 
GGT CAT C-3') and 445L (5'- GAC CAC GAC CGA AGA ATC CGC TG-3').

As seen in Figure 1, the *M. bovis* isolate recovered from the Mexican-origin 
cheese is highly similar to three other *M. bovis* isolates recovered from cattle entering the 
United States from Mexico. Although this spoligotype pattern is identical for all isolates 
reported here, it does not match any other spoligotypes reported previously in cattle from 
Mexico (6, 16). However, it should be noted that these previous studies focused on 
discrete regions of Mexico, and thus may not represent a comprehensive survey of *M.*
*bovis* strains present in this country. Analysis of the IS6110 RFLP patterns indicate that all of these isolates contain a single copy of this transposable element, as evidenced by two fragments of approximately 3.6 and 1.9 kb in size. This is similar to approximately 85% of all *M. bovis* isolates genotyped at NVSL over the last six years (N. B. Harris, unpublished data). These data are also consistent with previous studies looking at *M. bovis* from cattle in Texas and Mexico, in that the majority of animal strains in these studies also carried a single copy of IS6110 and demonstrated a hybridization band of 1.9 kb in size (19, 24). Because spoligotyping and IS6110 RFLP typing is less discriminatory for *M. tuberculosis* complex isolates with few copies of IS6110, PGRS typing was used to further discriminate among isolates. The PGRS RFLP profile of the *M. bovis* cheese isolate was also highly similar to the three bovine isolates. However, no direct epidemiological link among any of these isolates is available to support any of these strains having a common origin.

In summary, the recovery of *M. bovis* from fresh cheese suggests that human infection through the consumption of unpasteurized dairy products is possible. It also supports the epidemiological conclusions in recent outbreaks that milk products may serve as a reservoir for *M. bovis* transmission to at-risk human populations residing in the United States. However, it should be noted that this survey was not intended to be a systematic study of the recovery of *M. bovis* or other foodborne pathogens in Mexican-origin fresh cheese, and thus it is difficult to accurately assess the true impact on public health from this data. Therefore, it is recommended that a more structured study be undertaken, in which the prevalence of *M. bovis* in the animal population within a specific geographic location is examined in conjunction with the recovery of this
pathogen from dairy products manufactured within the same region. Nonetheless, this appears to be an important emerging public health concern, and will be best addressed by a collaborative effort between federal and state agencies in both the United States and Mexico.
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Table 1. Recovery of *Mycobacterium* species from Mexican-origin fresh cheese.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Origin</th>
<th>Epidemiology</th>
<th><em>Mycobacterium</em> species recovered</th>
<th>Type of cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>05-9001</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>M. fortuitum</em> complex</td>
<td>Hard grating</td>
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<tr>
<td>05-9019</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>M. fortuitum</em></td>
<td>Soft fresh</td>
</tr>
<tr>
<td>05-9040</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>Mycobacterium sp.</em></td>
<td>Soft fresh</td>
</tr>
<tr>
<td>05-9363</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>M. fortuitum</em></td>
<td>Soft fresh</td>
</tr>
<tr>
<td>05-9389</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>M. fortuitum</em></td>
<td>Soft fresh</td>
</tr>
<tr>
<td>05-9392</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>M. moriokaense</em></td>
<td>Soft fresh</td>
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<tr>
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<td>Cheese</td>
<td>Baja California</td>
<td><em>M. bovis</em></td>
<td>Soft fresh</td>
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<td><em>M. fortuitum</em></td>
<td>Soft fresh</td>
</tr>
<tr>
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<td>Cheese</td>
<td>Baja California</td>
<td><em>M. fortuitum</em></td>
<td>Soft fresh</td>
</tr>
<tr>
<td>05-10248</td>
<td>Cheese</td>
<td>Baja California</td>
<td><em>M. fortuitum</em></td>
<td>Semi-hard</td>
</tr>
</tbody>
</table>

*a* Original diagnostic source of mycobacterial isolate.

*b* State in Mexico that the diagnostic specimen originated from, based on concurrent epidemiological information.

*c* Classification of cheese recovered from travelers entering the United States from Mexico, based on visual appearance.

*d* Identified as a *Mycobacterium species* most closely resembling *M. moriokaense*. 
Figure 1. Genotyping results of *M. bovis* isolates recovered from Mexican-origin fresh cheese and cattle. Strain designation (Strain), origin of the *M. bovis* isolate (Source) and the state in Mexico that the diagnostic specimen or animal originated from (Epidemiology) is listed for each isolate. (A) Spoligotype patterns of cheese and cattle *M. bovis* isolates. (B) PGRS and IS6110 RFLP patterns of cheese and cattle *M. bovis* isolates. Approximate molecular weight sizes (Kbp) are given above each panel.