Recovery of *Salmonella*, *Listeria monocytogenes*, and *Mycobacterium bovis* from Cheese Entering the United States through a Noncommercial Land Port of Entry

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ABSTRACT

A joint multiagency project was initiated in response to a *Salmonella* outbreak in San Diego County, California, in 2004. Samples of cheese were collected during four 1-day operations at the San Ysidro port of entry, along the United States–Mexico border. Surveyed participants were persons crossing the border as pedestrians or in vehicles who had a minimum of 2.27 kg of cheese, which may suggest a potential diversion to illegal marketing. In addition, data were collected about the cheese to identify risk factors for cheese contamination. Two hundred four cheese samples were submitted to the California Animal Health and Food Safety Laboratory System–San Bernardino Branch and analyzed for potential food pathogens. Ninety-four percent (190 of 203) of the samples tested positive for alkaline phosphatase. *Salmonella* was detected from 13% (27 of 204) of the samples comprising 11 serogroups and 28 serotypes. Pulsed-field gel electrophoresis DNA fingerprinting analysis, performed following standardized methods, determined that an isolate obtained from this study had an indistinguishable pattern from a recent *Salmonella enterica* serovar Typhimurium var. Copenhagen epidemic in the San Diego County that was linked to 14 illnesses. *Listeria* spp. were detected from 4% (8 of 204) of the samples, and of these, half were identified as *L. monocytogenes*. *Escherichia coli* O157:H7 was not detected from any of the samples. *Mycobacterium bovis* was detected from one panela-style cheese sample. Nine additional samples yielded *Mycobacterium* spp.

Unpasteurized milk and other dairy products can harbor a variety of microorganisms and can be important sources of foodborne pathogens. Milk can be contaminated as a result of direct contact of the product with contaminated sources at the dairy farm environment or processing facility and/or as the result of systemic infection or local inflammation of the udder (mastitis) of the cow (8). The California Department of Agriculture, Milk and Dairy Food Safety Branch, has made several attempts to control street vending of illegally manufactured Mexican-style soft cheeses and in some instances has succeeded taking legal actions against the vendors. To reduce the risk of human infection associated with the consumption of raw milk and raw dairy products, the U.S. Food and Drug Administration requires pasteurization of all dairy products that are sold across state lines except cheese made from raw milk that has been aged for a minimum of 60 days (5). Pasteurization combined with good management practices at the farm and strict hygienic practices at the processing plant can markedly reduce the numbers of human illness that result from the consumption of contaminated dairy products. At the same time, raw milk cheese is frequently an important traditional product of small family-owned dairy farms in both developing and developed countries. The epidemiology of outbreaks of foodborne pathogens related to cheese in the United States, Canada, and Europe demonstrates that soft cheeses pose a greater risk for the transmission of pathogens than do other cheeses (10).

During 2004, two separate disease outbreaks (comprising 14 and 49 cases, respectively) caused by *Salmonella enterica* serovar Typhimurium were reported in San Diego County, California. A case-control investigation found a strong association between the cases and consumption of unpasteurized Mexican-style soft cheese that originated from Mexico or was purchased from unregulated street vendors (6). In response to these outbreaks, a federal, state, and local multiagency workgroup was created to develop...
strategies to address the health risks associated with those cheeses.

The objectives of this study are: 1) to estimate the volume and characteristics of cheese transported across the United States—Mexico border through a noncommercial land port of entry in California; 2) to document the presence in those cheeses of potential pathogens, specifically *Listeria monocytogenes*, *Salmonella*, *E. coli O157:H7*, and *Mycobacterium* sp.; 3) to identify risk factors for the presence of these pathogens in cheese; and 4) to provide science-based information to regulatory and public health agencies in order to formulate sound importation policies and mitigate public health risks.

**MATERIALS AND METHODS**

**Sampling of cheese and conducting of interviews.** The interview was conducted at the United States—Mexico San Ysidro port of entry, San Diego, California, during 4 days (21 and 25 July, and 26 and 30 August 2005), and at different 8-h periods each day. Luggage from all pedestrians crossing the border into the United States was screened for cheese by Customs and Border Protection (CBP) agents with an x-ray machine, then visually inspected when cheese was identified on the x-ray view screen. Vehicles whose occupants declared transporting cheese or any agricultural product and vehicles selected randomly by a CBP computer program (14 to 20 vehicles per day) were sent to a secondary inspection area, where CBP officers checked the vehicles for cheese. All cheese pieces transported by a given person were weighed. All border crossers (both pedestrians and selected vehicles) transporting 2.27 kg of cheese or more were asked to voluntarily provide a cheese sample for laboratory testing and respond in English or Spanish to a one-page questionnaire. Cheese samples were taken until a total of approximately 50 samples had been collected each day (*n* = 50, 52, 50, and 53). Some cheeses were not sampled because the cheese was a single piece and could not be aseptically cut or, rarely, because the owner refused. All participants who provided cheese samples were offered, by mail or phone, the laboratory results for their cheese.

Information was gathered about the type of cheese and store, place, or state where it was acquired and about its intended use. Respondents received written information about health risks associated with unpasteurized cheese. CBP officers recorded the total number of pedestrians and vehicles crossing the border during the study period. Logistic regression (EGRET; EGRET Software Division, Statistics & Epidemiology Research Corp., Seattle, Wash.) was used to assess risk factors for cheese containing *Salmonella* or *Listeria* spp., or for pasteurized cheese. Samples were submitted to the San Bernardino Branch laboratory of the California Animal Health and Food Safety Laboratory System for microbial and phosphatase testing.

**Enrichment and primary isolation of enterohemorrhagic *E. coli*.** Aseptically, 225 ml of enrichment broth (Vet Med Biological Media Services, School of Veterinary Medicine, Davis, Calif.) was added to a 250-ml sterile bottle containing a freshly prepared solution of 0.023 ml cefixime (Dynal Inc., Lake Success, N.Y.), 0.1 ml cefsulodin (Sigma, St. Louis, Mo.), and 0.1 ml vancomycin (Sigma). The mixture was then poured into a sterile 500-ml flask, and 25 g of soft cheese was added. The broth was allowed to incubate for 6 h at 35 to 37°C on a rotary shaker at 120 rpm. After 6 h of incubation, 0.01 ml of the enrichment broth homogenate was spread onto two tellurite and cefixime sorbitol MacConkey agar plates (Hardy Diagnostics, Santa Maria, Calif.) and streaked with a sterile inoculating loop. The agar plates were incubated aerobically at 37°C for 18 to 24 h. The enrichment broth homogenate was reincubated for a total of 24 h and plated as described above. After incubation, the plates were examined, and up to five typical colonies were picked from the tellurite and cefixime sorbitol MacConkey agar plates and transferred onto Tryptic soy agar with yeast extract slants (Vet Med Biological Media Services) and incubated at 35 to 37°C for 18 to 24 h. When reisolation was indicated, MacConkey agar plate with sorbitol (Remel, Lenexa, Kans.) was used. Suspect isolates were initially screened by spotting growth from slants to a filter paper wetted with Kovac’s reagent. Further identification was carried out according to established protocols (15).

**Enrichment and primary isolation of *Listeria* spp.** Aseptically, 25 g of cheese was placed in a sterile 680-ml plastic bag and gently broken with manual pressure. Between 25 to 50 ml of *Listeria* enrichment broth (LEB-FDA) (Remel) was added to the sample, and the mixture was emulsified by gently kneading. Additional LEB was added to the sample to make a total volume of 250 ml, and it was mixed well and incubated aerobically at 29 to 31°C for 4 h. At 4 h, a mixture of supplement containing 0.445 ml 0.5% acriflavine HCl, 1.8 ml 0.5% nalidixic acid, and 1.15 ml 1% cycloheximide (all from Remel) was added. The culture broth was further incubated for 44 h at 29 to 31°C. At 24 and 48 h, a sterile cotton-tipped swab was used to inoculate the LEB-FDA, which was then streaked onto Oxford plates (Remel). The Oxford plates were aerobically incubated at 35 to 37°C for 48 h. After incubation, the plates were examined for typical black colonies with a halo (esculin positive). Up to five suspect colonies were selected, then streaked onto sheep’s blood agar plates (Remel) to check for purity. The sheep’s blood agar plates were incubated aerobically at 35 to 37°C for 18 to 24 h, and colonies were screened to rule out *Listeria* spp. This was accomplished by inoculating a blood agar plate with a single typical colony and allowing the plates to incubate for 18 to 24 h at 35 to 37°C. Multiple picks of suspect isolates with short, gram-positive rods, strongly catalase (3% H<sub>2</sub>O<sub>2</sub>) positive, and typically exhibiting tumbling motility on wet mounts (when grown at 25°C) were selected and inoculated into a string of carbohydrate tubes for biochemical identification or API *Listeria* commercial test strips (bioMérieux, Inc., Marcy l’Etoile, France) according to procedures described previously (6). The hemolytic characteristic of *L. monocytogenes* was determined by stabbing the sheep’s blood agar plate with the suspected isolate to enhance hemolysis. Strains of *L. ivanovii*, *L. monocytogenes*, *L. innocua*, and *L. seeligeri* were used in parallel when identifying *Listeria* spp. from the cheese samples.

**Pree enrichment and isolation of *Salmonella*.** Aseptically, two 25-g samples of cheese were placed into large, sterile 680-ml plastic bags. The samples were broken by massaging them manually. Twenty-five milliliters of lactose broth was added to one 25-g sample, and the mixture was emulsified by gently kneading. The remaining 200 ml of lactose broth (Vet Med Biological Media Services) was added to the bag and mixed well. The second 25-g sample was processed in a similar manner except instead of lactose broth, tetrathionate broth (TT) (Remel) was used. The lactose broth sample was allowed to stand for 60 min at room temperature and mixed well, after which the pH of the broth was adjusted to 6.8 ± 0.2 with test paper. Both the lactose and TT broths samples were incubated aerobically at 35 to 37°C for 22 to 26 h. After incubation, the lactose broth was gently mixed, and a 0.1-ml aliquot was transferred into 10 ml of Rappaport-Vassiliadis broth (RV) (Remel) and 1 ml into 10 ml of TT broth. The TT broth was supplemented with 200 μg of iodine solution and
and no further testing was performed. A reading of 350 mU/kg or less was considered presumptive positive. Confirmation steps were performed according to previously described procedures (11, 17).

Mycobacterium culture. Fifty grams of each of the 200 cheese samples were formed into aliquots and sent to the Mycobacterial Laboratory, National Veterinary Services Laboratories, Ames, Iowa, for Mycobacterium culture.

Pulsed-field gel electrophoresis. One Salmonella enterica serovar Typhimurium var. Copenhagen isolate from this study and other similar isolates obtained from Mexican-style soft-fresh cheese and human cases from San Diego County were subjected to pulsed-field gel electrophoresis (PFGE) following standard PulseNet procedures (9). Isolates were considered indistinguishable from one another if they visually appeared to be similar and if they had 100% similarity.

Determination of pasteurization/ALP test. Aseptically, 30 ml of working solution was prepared 1:10 (wt/vol) of cheese in cheese extraction buffer (Fluorophos Cheese Extraction Buffer, Advanced Instruments, Norwood, Mass.) into a sterile bag. The mixture was macerated and homogenized with a glass rod, poured into a sterile 50 ml conical centrifuge tube (Corning, Fisher Scientific, Tustin, Calif.), and centrifuged for 10 min at 1000 × g in a refrigerated centrifuge. A pipette was used to transfer the supernatant to a separate sterile tube, which was kept on ice. With an Eppendorf fixed-volume pipettor (Fisher Scientific), 75 µl of the supernatant of the cheese extract was dispensed into 2 ml of preheated substrate and mixed. The external surface of the cuvette was wiped with lens paper, then placed into a fluorometer and tested in a precalibrated channel for cheese. After 1 min of temperature stabilization, the rate of increase fluorescence was measured over 1 to 2 min. The test result was displayed and printed (mU/kg). Fluorescence readings were made with a filter fluorometer (FLM200 Fluorophos Test System, Advanced Instruments) thermocuvetted at 38 ± 1.0°C. Excitation and emission readings were 440 and 560 nm, respectively. Fluorescence output was monitored by the filter fluorometer and printed with a built-in 40-column thermal printer. A reading of 349 mU/kg or less was considered negative for the presence of alkaline phosphatase (ALP), and no further testing was performed. A reading of 350 mU/kg or more was considered presumptive positive. Confirmation steps were performed according to previously described procedures (11, 14).

RESULTS

On average, 19,190 pedestrians (range, 15,476 to 25,507) and 12,639 vehicles (range, 9,544 to 16,006) crossed the San Ysidro port of entry into the United States during each of the four sampling periods. Overall, 0.26% of pedestrians and 0.14% of vehicles going through secondary inspection were transporting 2.27 kg or more of cheese. A total of 276 personal interviews were conducted (mostly in Spanish), 74% at the pedestrian crossing and 26% at the vehicular secondary inspection area. All of the pedestrian luggage was screened for cheese by X ray and confirmed by visual inspection; vehicles with cheese were identified by agricultural inspection officers. Most persons (64%) interviewed reported being residents of California, followed by Baja California (10.9%) and Sinaloa (6.5%). Some of the border crossers resided in other U.S. states, including Washington (1.8%), Oregon (1.1%), Colorado, Indiana, Maryland, and North Carolina (with 0.4% each). Median weight of all cheeses was 5.4 kg. Cheese collected at the pedestrian crossing had a median weight of 4.5 kg, with a maximum weight of 46.8 kg. The median weight of cheeses sampled at the vehicular secondary inspection was 10.5 kg, with a maximum weight of 92.7 kg.

The most common types of cheese reported were fresco, seco, ranchero, cotija, panela, Oaxaca, quesadilla, and asadero; however, respondents frequently used different names to refer to the same type of cheese or were unsure of the answer. Ninety-three percent of the cheeses were unlabeled, and of the 23 with labels, only one had a label indicating pasteurization. Nineteen percent of the cheeses were acquired in Baja California, followed by 18% from Michoacan, 16% from Sinaloa, 12% from Jalisco, 10% from Zacatecas, 6% from Nayarit, and the rest from other Mexican states. The source from which the cheeses had been obtained were supermarkets (28%), ranches (21%), homemade by the respondent's family (18%), small stores (12%), and other sources (21%). Intended use for the cheese was self (53%), family in the household (84%), and other relatives/friends (43%). (Respondents could select more than one intended use for their cheese, and thus reported percentages do not add up to 100%.) Five percent of cheeses were intended to be served at parties, and only 1% admitted an intent to sell the cheese. If we extrapolate the estimates obtained in this study, in terms of the percentage of pedestrian border crossers transporting more than 2.27 kg of cheese and the median amount of cheese being imported, multiplied by the annual number of border crossings at San Ysidro (13), an estimate of 12.6 million kilograms of cheese may be imported annually through that port of entry. The same calculations performed for the vehicular crossings estimates an additional 26.1 million kilograms of cheese for the San Ysidro port. Extrapolating these calculations to all California noncommercial land ports of entry, an estimated total of 74.6 million kilograms of cheese was imported.

ALP was detected in 94% (190 of 203) of the samples tested, indicating that unpasteurized, raw milk was used in the production of the Mexican-style soft-fresh cheese. The
positive fluorometer readings ranged from 350 to 31,669 mU/kg. A total of 11 serogroups and 28 serovars of Salmonella were detected from 13% (27 of 204) of the total cheese samples. These Salmonella serovars were Anatum \((n = 4)\), Give \((n = 3)\), Newport \((n = 3)\), Anatum var. 15+ \((n = 2)\), and one each of the following serovars: Agona; Brandenburg; Senftenberg; IV 50:z4, z23; IV 45:g, Z51; M. leagrisid; Michigan; Montevideo; Muenchen; Newington; Poona; Rubislaw; Soahanina; Typhimurium; Urbana; and Rough "H" untypeable. Of these, 93% (25 of 27) were detected by TT enrichment in contrast to the lactose broth, which yielded only 7% (2 of 27). PFGE DNA fingerprinting analysis determined that the isolate obtained from this study had indistinguishable patterns from those of a recent Salmonella enterica serovar Typhimurium var. Copenhagen epidemic in the San Diego County. The Salmonella enterica serovar Typhimurium var. Copenhagen isolate possessed a unique aspects of the outbreak with an uncommon PFGE pattern, with a frequency of 0.15% (43 of 28,939 as of March 2006) in the national database. Listeria spp. were detected from 4% (8 of 204) of the samples. This consisted of four L. monocytogenes, two L. innocua, four L. seeligeri, and one L. welshimeri. L. monocytogenes was detected alone twice and on two occasions with L. seeligeri and L. innocua. E. coli O157:H7 was not detected in any of the cheese samples. Ten of the 200 samples sent to the National Veterinary Services Laboratories yielded one Mycobacterium bovis isolate and nine non—Tuberculous mycobacteria isolates (five M. fortuitum and one of each of the following: M. fortuitum complex, M. moriokaense, and Mycobacterium sp.).

Univariate logistic regression was used to screen variables for association with pasteurization and with isolation of Salmonella and any Listeria spp. Variables significantly associated with \( P < 0.10 \) were admitted to a multivariate logistic regression model. In the univariate model, variables associated with Salmonella isolated from the cheese were cheese acquired in Nayarit state (OR = 5.21, \( P = 0.026 \)) and cheese made at home by the family or from a ranch (OR = 3.37, \( P = 0.0006 \)). Cheese having been purchased from a supermarket was a significant protective factor (OR = 0.35, \( P = 0.042 \)). The best multivariate model from Salmonella retained cheese made at home by the family or from a ranch (OR = 3.207, \( P = 0.009 \)) and being from Nayarit (OR = 4.174, \( P = 0.063 \)). In the univariate model, variables associated with any Listeria spp. isolated from the cheese were cheese from Guanajuato state (OR = 11.24, \( P = 0.047 \)), cheese from Baja California (OR = 3.62, \( P = 0.088 \)), and cheese made at home by the family or from a ranch (OR = 6.69, \( P = 0.022 \)). The best multivariate model from Listeria retained all three of these variables with the following multivariate results: cheese made at home by the family or from a ranch (OR = 14.573.21, \( P = 0.0059 \)), cheese being from Guanajuato or Nayarit (OR = 37.204, 17, \( P = 0.01863 \)), and cheese being from Baja California (OR = 11.09, \( P = 0.008 \)). In the univariate model, variables associated with pasteurization were cheese from Oaxaca state (OR = 28.87, \( P = 0.001 \)), cheese acquired from a factory (OR = 7.77, \( P = 0.087 \)), and type of cheese reported (negative association with “queso fresco,” which was a composite variable that includes fresco, ranchero, and cecito; OR = 0.23, \( P = 0.011 \)). The best multivariate model for pasteurization retained two of the above: cheese from a factory (OR = 10.86, \( P = 0.05 \)) and being from Oaxaca (OR = 24.43, \( P < 0.001 \)).

DISCUSSION

This study was the result of a multiagency collaboration to respond to the salmonellosis outbreaks associated with consumption of unpasteurized Mexican-style soft cheese in San Diego County. Despite the existence of regulations that prevent the commercial importation of unpasteurized cheeses from Mexico for sale in the United States, there are currently no clear regulations about the amount of cheese people are allowed to import for “personal use.” Although importation of cheese for sale can only legally take place through commercial ports of entry, numerous cases of illegal sales of cheese imported as for “personal use” have been prosecuted in California. Even though a relatively small proportion of border-crossing pedestrians and vehicles were identified as transporting cheeses, if we apply that estimate to the total number of border crossings at California land ports in a year, the result is an estimate of 75 million kilograms of cheese being imported annually through a noncommercial port of entry. This represents a serious health risk because most of the cheese is likely to be unpasteurized. These figures are most likely underestimated because in this study, samples were not collected on weekends, holidays, or early mornings, when larger amounts of cheese may be imported. In addition, only cheese weighing 2.27 kg or more were weighed and recorded, and many people carry smaller amounts. Also, vehicles selected for this study cannot be considered a representative sample of all vehicles crossing the border because mostly only those who declared transporting cheese or another agricultural product were inspected for cheese, and this relied on the CBP booth inspector asking and the answer reported. The number of pedestrians and vehicles crossing into the United States from Mexico varies greatly throughout the year. Similarly, the volume of cheese being imported may also vary.

Cheeses in this study were reportedly acquired in many different Mexican states, as far away as Oaxaca. In most cases, the cheeses were observed to be transported unrefrigerated and under unhygienic conditions, thus increasing the risk for contamination and microbial growth. The distribution of states where the cheeses were acquired may have been affected by the airline or bus schedules of people crossing the border on the particular sampling days. Very few cheeses had labels of any kind, and relatively high proportions were obtained at ranches or were homemade by family members, and thus were likely unregulated at the source. Only 1% of interviewees admitted to bringing cheese across the border for selling, but this is probably an underestimate because there were several disincentives to admit intent to sell, including fear of having cheese confiscated or not wanting to be sent to the commercial port of entry. Even if the cheese was not intended for sale, fre-
quently the cheese was to be shared with family members and friends or at a party, and thus the potential existed for many people to be at risk of exposure to contaminated cheese. It was not surprising that cheese made at home by the family or at a ranch had higher risk for microbial contamination, that supermarket cheese had a lower risk for contamination, or that factory cheese was more likely to be pasteurized.

The study found that 94% of the cheese samples that were tested by fluorometry were positive for phosphatase, a natural enzyme found in raw milk or unpasteurized dairy products.

Despite the large numbers of samples examined, E. coli O157:H7 was not detected. The reason for this is not known. However, it was observed that most cheeses were being transported unrefrigerated before sample collection at the border. This may have resulted in overgrowth of coliforms, thus perhaps outnumbering the few E. coli O157:H7 that could have been present. The poor performance of the lactose enrichment broth for the Salmonella isolation was not unexpected because the samples were contaminated. The isolation of Mycobacterium bovis in one of the 200 Mexican-style soft cheese samples is not surprising because M. bovis infection is a problem in the dairy industry in most states of Mexico (7). M. bovis causes disease in humans, cattle, and other mammals. Fresh cheese (e.g., queso fresco) brought to New York City from Mexico was incriminated as a likely source of infection (3), and in previous studies fresh cheese brought from Mexico was suspected as the source of confirmed M. bovis infection in children younger than 15 years of age; approximately 90% of these children were U.S. born and of Hispanic ethnicity (2). This study also revealed that nearly 2% of the cheeses was positive for L. monocytogenes.

Traditional cheeses made with unpasteurized milk are highly desirable in many developing countries and some developed countries, and they play an economically important role in the subsistence of small dairy farms. Under California Food and Agricultural Code 35283(c), it is a felony to provide milk or milk products for resale unless the person is licensed (1). Mexican regulations only allow pasteurized milk to manufacture cheese (12), but this is difficult to enforce with thousands of small dairy producers and cheese manufacturers in the country. Thus, a sustained and culturally appropriate educational campaign will be necessary to change long-held attitudes and beliefs about health risks associated with unpasteurized soft cheeses. In addition, economic incentives need to be provided to small producers so they will consider alternatives. Additionally, enforcing laws that regulate the sale of dairy products made by unlicensed manufacturers and regulations limiting the amount of cheese that can be imported for personal use would help prevent foodborne disease cases and outbreaks associated with unpasteurized dairy products. Finally, because this is a health issue affecting both U.S. and Mexican communities, collaboration between the two countries is essential to find appropriate strategies to protect the public health on both sides of the border.

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