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Comparison of C_{18}-Carboxypropylbetaine and Glass Bead DNA Extraction Methods for Detection of *Mycobacterium bovis* in Bovine Milk Samples and Analysis of Samples by PCR

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The purpose of this prospective study was to compare two different milk preparation methods to assay for the presence of *Mycobacterium bovis* by PCR. Detection by a C_{18}-carboxypropylbetaine (CB-18)-based sample processing method was compared to extraction of DNA from milk with glass beads. Samples from 17 skin test-positive cattle were analyzed. Following CB-18 processing and glass bead extraction, the sensitivity of IS*6110*-based PCR was 94.1 and 58.8%, respectively ($P < 0.025$). Because CB-18 processing will permit the proficient use of PCR for diagnosis and surveillance of bovine tuberculosis, it will contribute to the more efficient detection and control of tuberculosis.

Mycobacterium bovis is the etiological agent of bovine tuberculosis (TB). This pathogen is classified as a member of the TB complex, the cause of TB in humans, and is one of the primary reasons that milk is pasteurized (11). In general, there has been a resurgence of TB in the United States over the past 10 years (13). Similarly, the number of *M. bovis*-positive cattle has been shown to be on the rise in the past 10 years (7). In 1996, of those tested by the tuberculin skin test or detected at slaughter and traced back to the herd of origin, 50% of *M. bovis*-infected cattle in the United States resided in Texas (9). The epidemiological causes of the increased incidence include importation of infected animals, incomplete removal of infected individuals, and movement of TB-exposed animals between herds. Although pasteurization has drastically reduced the transmission of *M. bovis* from cattle to humans, the increasing incidence makes exposure of human populations to *M. bovis* TB more likely.

The presence of *M. bovis* also poses an economic threat to both Mexico and the United States. This disease has contributed to nontariff trade barriers by impeding the safe free trade of cattle and cattle products implemented by the North American Free Trade Agreement. Efforts to control this problem have resulted in production losses and reduced sales. The potential health risk and economic impact of bovine TB necessitate a fast and accurate method to identify infected cattle. The reduction of *M. bovis* incidence will also increase the movement and marketability of cattle in South, Central, and North America.

Unfortunately, the sensitivities of the present methods for detecting *M. bovis* in milk are deficient. The current method of detection of *M. bovis* infection in cattle, the tuberculin skin test, has been shown to display both false-positive and false-negative results. PCR-based methods have the potential to be faster, more accurate, and the most efficient means of detecting *M. bovis*; however, PCR sensitivity has been shown to be hindered by the method used to isolate the nucleic acid target (e.g., RNA and DNA). For example, the solutions (e.g., NaOH) used to process mycobacterial specimens inhibit the PCR (14) and can also affect the sensitivity of the PCR (2). In addition, methods involving centrifugation that are used for preparing clinical specimens suspected of harboring mycobacteria are deficient because of the waxy cell wall (i.e., surface tension) and the buoyant nature of the mycobacteria (5, 6, 10, 12, 14). The difficulty associated with lysing these organisms further complicates detection. Overall, the net effect is a very limited isolation of tubercle bacilli. This is an extremely important consideration when working with samples that initially present with low numbers of bacilli. The purpose of the present study was to compare two methods of preparing milk specimens for analysis by PCR. Specifically, the specimen processing method of Thornton et al. (14, 15), which uses *N*,*N*-dimethyl-*N*-(*n*-octadecyl)-*N*-(3-carboxypropyl) ammonium inner salt (Chemical Abstract no. 78195-27-4), also known as C_{18}-carboxypropylbetaine (CB-18), was compared to a glass bead-based DNA isolation procedure.

All heifers in this study originated from four different herds in Mexico and were positive for TB by the bovine tuberculin skin test. Skin testing was conducted by personnel from the National Campaign for Tuberculosis and Brucellosis Control and Eradication (México, D.F., México). Milk samples were collected from each cow prior to slaughter and were stored at −20°C until processing. Lung, liver, lymph node, spleen, and kidney samples were collected following slaughter. Tissues for bacteriologic culture were stored at 4°C in saturated sodium borate solution until processing (8). Gross anatomical and histological examinations of formalin-fixed tissues were performed in the Departamento de Patología Veterinaria at the Universidad Nacional Autónoma de México. Analysis of milk and organ samples by smear and culture was performed according to recommended procedures (5, 8).

Isolation of *M. bovis* DNA from milk by the glass bead

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TABLE 1. Comparative *M. bovis* IS*6110* PCR results from CB-18- versus glass bead-processed milk samples collected from tuberculin skin test-positive tuberculous cattle[a]

Identification no.[b]	Result						
	Skin test	Bacteriology[c]		Pathology		PCR following processing with:	
		Milk	Tissue	Gross[d]	Acid-fast histology[e]	CB-18	Glass beads
T-6	+	*M. bovis*	*M. bovis*	ND	ND	+	Neg
T-17	+	*M. bovis*	ND	ND	ND	+	+
T-19	+	Neg	ND	ND	ND	+	+
T-20	+	Neg	ND	ND	ND	+	Neg
T-23	+	*M. bovis*	ND	ND	ND	+	Neg
T-25	+	*M. bovis*	ND	ND	ND	+	Neg
S^1-80	+	ZN^+	*M. bovis*	Liver and LN	Liver and LN	+	+
S^1-84	+	Neg	Neg	LN	LN	+	Neg
S^1-85	+	Neg	Neg	LN	LN	+	+
S^1-88	+	Neg	*M. bovis*	Lung and LN	Neg	+	+
S^1-89	+	Neg	Neg	Lung	Neg	+	Neg
S^2-92	+	Neg	*M. bovis*	Neg	Neg	+	+
S^2-95	+	Neg	ZN^+	LN	LN	+	+
S^2-96	+	Neg	*M. bovis*	LN	Neg	+	+
S^2-97	+	Neg	*M. bovis*	Lung and LN	Lung and LN	+	+
S^2-98	+	Neg	ZN^+	Lung	Lung	+	+
S^3-102	+	*M. terrae*	Neg	Neg	Neg	Neg	Neg

[a] Abbreviations: Neg, negative; ND, not done; LN, lymph node.
[b] Cattle were derived either from Tizayuca, Hidalgo, México (T prefix on identification numbers) or from three ranches in Ciudad Obregon, Sonora, México: Los Morenos (S^1 prefix), Progreso (S^2 prefix), and Santa Cecilia (S^3 prefix).
[c] Those cultures that yielded mycobacteria are indicated by the species identified. Other specimens were either culture positive with acid-fast bacilli present that could not be recovered by subculture (ZN^+) or culture negative.
[d] Only those tissues that presented with suspicious lesions on gross examination are indicated. Other tissues either were negative or did not have postmortem examination done.
[e] Only those tissues presenting with acid-fast bacilli in the lesions are indicated. Other tissues either were negative for acid-fast bacilli or did not have postmortem examination done.

method used a modified version of the method of Boom et al. (1). Briefly, samples were first subjected to centrifugation at 10,000 × *g* for 15 min. The supernatant was discarded, and the resulting cellular pellet was resuspended in 50 μl of Tris-EDTA (TE). Pellets were then mixed with 4 M guanidine isothiocyanate (Life Technologies, Inc., Gaithersburg, Md.) and acid-washed glass beads (425 to 600 μm; Sigma Chemical Company, St. Louis, Mo. [catalog no. G 8772]). Bacilli were lysed by bead beating. Briefly, tubes were sonicated (Gen-Probe, San Diego, Calif.) at 35 MHz for 15 min at room temperature, and then the beads were allowed to settle. The aqueous phase was discarded, and the beads were washed twice with 70% ethanol. DNA was released by adding 50 μl of TE at room temperature and then subjecting the glass beads to centrifugation at 10,000 × *g* for 4 min. The supernatant was then transferred to a new tube, and the process was repeated two more times. Supernatants were pooled to yield a working supernatant volume of 150 μl. Samples were placed at 4°C until amplification.

The CB-18 protocol was adapted from the work of Thornton et al. (15). In a 15-ml conical tube, 1 ml of milk was mixed by inversion with 8 ml of sterile filtered water. One milliliter of 10× CB-18 buffer (1× CB-18 buffer is 50 mM Tris-HCl [pH 8.0], 0.1 mM NaCl, 1.0 mM CB-18, and 5 mM *N*-acetyl-L-cysteine) was added to the sample. The samples were shaken in an orbital shaker (140 rpm) at 37°C for 90 min prior to centrifugation at 4,000 × *g* for 20 min at 30°C. Samples were then carefully decanted, and the pellets were resuspended in 500 μl of TE and boiled for 30 min. Samples were stored at 4°C until amplification.

The PCR was optimized (3) and carried out in a Model PTC100 thermal cycler (MJ Research, Watertown, Mass.) with 25-μl reaction volumes. Each amplification contained 3 μl of sample in 1× Amplificasa reaction buffer (Biotecnologias Universitarias, México, D.F., México) supplemented with 1.25 mM $MgCl_2$ (Life Technologies, Inc.), 50 μM deoxynucleoside triphosphates (Boehringer, Mannheim, Germany), 0.2 μM (each) IS*6110* primer as described by Eisenach et al. (4), and 1.25 U of Amplificasa *Taq* polymerase (Biotecnologias Universitarias). The amplification protocol entailed denaturation, annealing, and extension steps at 96, 65, and 72°C, respectively, for 1 min each. Samples were subjected to 32 cycles before a final 15-min extension at 72°C. Amplified products were visualized with ethidium bromide (EtBr) staining and UV illumination.

In this prospective study, all samples were collected from cattle that were bovine tuberculin skin test positive (Table 1). *M. bovis* was cultivated from the milk of four (23.5%) cows and from the organs of six (35.3%). Gross anatomic analysis identified nine (52.9%) cows as having granulomatous lesions consistent with miliary disease, whereas histologic analysis of these tissues confirmed the presence of acid-fast bacilli in only six (35.3%) cows. The bacteriology and pathology data being combined, 14 (82.4%) of the 17 cows evaluated presented with results consistent with bovine TB. Assuming all cows as positive, the sensitivity of PCR among milk samples processed with CB-18 was 94.1%. In contrast, the sensitivity of PCR was 58.8% when the glass bead method was used for target preparation from milk. Surprisingly, glass bead-processed specimens missed three milk samples that were culture positive for *M. bovis*. The sensitivity of PCR was increased by approximately 60% ($P < 0.025$) when milk specimens were processed with CB-18. The one milk sample that was PCR negative by both processing methods was culture positive only for *Mycobacterium terrae*. While this cow was skin test positive, it may

not have been actively shedding *M. bovis* at the time of specimen collection. Alternatively, the skin test may have been a false-positive result due to cross-reactivity shared by mycobacterial antigens. Both of these possibilities might be related to the *M. terrae* infection and further support the specificity of the IS*6110* primers for organisms of the *M. tuberculosis* complex. If the skin test result from this cow was a false positive, the sensitivity of PCR among CB-18-processed specimens would have been 100%.

One goal of the joint collaboration between the U.S. and Mexican agricultural authorities, of which the present study was a part, is the development of improved diagnostic testing methods for *M. bovis*-infected cattle. The CB-18 processing method combined with detection by PCR will help ensure more efficient diagnosis of active TB in cattle. The ability to use milk, a specimen that can be collected easily and noninvasively, makes this diagnostic model even more attractive. The accurate and rapid isolation of mycobacteria through CB-18 processing, although a small part, may contribute to both human and animal well-being, as well as the economic viability of cattle producers, by laying the groundwork for effective surveillance programs.

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