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A Multiplex Approach to Molecular Detection of *Brucella abortus* and/or *Mycobacterium bovis* Infection in Cattle

SRINAND SREEVATSAN,[1] JACK B. BOOKOUT,[1]* FIDEL RINGPIS,[1] VEERA S. PERUMAALLA,[2] THOMAS A. FICHT,[2] L. GARRY ADAMS,[2] SUE D. HAGIUS,[3] PHILIP H. ELZER,[3] BETSY J. BRICKER,[4] GIRISH K. KUMAR,[5] M. RAJASEKHAR,[6] SRIKRISHNA ISLOOR,[6] AND RAJ R. BARATHUR[1]

ClinCyte, LLC, San Diego, California 92121[1]; Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843-4467[2]; Department of Veterinary Science, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803[3]; Division of Zoonotic Disease Research, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa 50010[4]; Department of Genetics and Plant Breeding, University of Agricultural Sciences, Bangalore, India 560065[5]; and Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore, India 560024[6]

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A multiplex amplification and detection platform for the diagnosis of *Mycobacterium bovis* and *Brucella abortus* infection simultaneously in bovine milk and nasal secretions was developed. This system (designated the bovine pathogen detection assay [BPDA]-PCR) consists of duplex amplification of species-specific targets (a region of the BCSP31K gene of *B. abortus* and a repeat-sequence region in the *hsp65* gene of *M. bovis*, respectively). This is followed by a solid-phase probe capture hybridization of amplicons for detection. On the basis of spiking experiments with normal milk, the analytical sensitivity of the assay was 800 CFU equivalents/ml of milk for *B. abortus* and as low as 4 CFU equivalents per ml of milk for *M. bovis*. BPDA-PCR was validated with 45 liver samples from lemmings experimentally infected with *B. abortus*. The assay sensitivity, based on culture status as a "gold standard," was 93.9%. In this experiment, BPDA-PCR also identified five culture-negative liver samples as positive (41.7%). Field studies for the evaluation of BPDA-PCR were performed with samples from dairy animals from geographically distinct regions (India, Mexico, and Argentina). A high prevalence of shedding of *B. abortus* (samples from India) and *M. bovis* (samples from Mexico) was identified by BPDA-PCR. In samples from India, *B. abortus* shedding was identified in 86% of milk ring test-positive animals ($n = 15$) and 80% of milk ring test-negative cows ($n = 5$). In samples from Mexico, *M. bovis* was identified by PCR in 32.6% of pools ($n = 46$) of milk that each contained milk from 10 animals and in 56.2% of nasal swabs ($n = 121$) from cattle from tuberculin test-positive herds. In contrast, the Argentine cattle ($n = 70$) had a modest prevalence of *M. bovis* shedding in nasal swabs (2.9%) and milk (1.4%) and of *B. abortus* in milk (11.4%). On the basis of these analyses, we identify BPDA-PCR as an optimal tool for both screening of herds and testing of individual animals in a disease eradication program. A combination of the duplex assay, screening of milk samples in pools, and the proposed algorithm provides a highly sensitive, cost-effective, and economically viable alternative to serological testing.

Animal health and human health are inextricably linked. For millennia, humans have depended on animals for nutrition, socioeconomic development, and companionship (1). Yet animals can transmit many different but potentially devastating diseases to humans.

Brucellosis is a zoonosis of both public health and economic importance in many developing countries (5, 24). Six species of *Brucella* are presently known: *B. abortus*, *B. suis*, *B. melitensis*, *B. ovis*, *B. neotomae*, and *B. canis* (1, 24). Brucellosis has been effectively controlled in many developed or industrialized nations, thereby reducing the number of cases of brucellosis among humans (5). Despite the preventive and control measures that exist in developed nations, there is still a high potential for transmission and spread of brucellae via animal products imported from developing nations (1). Thus, animal brucellosis poses a barrier for trade of animals and animal products and could seriously impair socioeconomic progress in the developing world (19, 20, 37).

Official estimates put losses due to brucellosis in Latin America equivalent to about US$600 million annually, which explains the priority given by animal health services to reducing the incidence of the disease (5). The World Health Organization reports an annual incidence of brucellosis in people of less than 1 to 78 cases per 100,000 population in the Middle East, with six countries reporting an annual total incidence of over 90,000 cases. Among the Latin American countries, Argentina reports the largest incidence, followed by Mexico and Peru (5, 37). Although brucellosis is a notifiable disease in many countries, official figures do not fully reflect the number of cases reported annually, and the true incidence has been estimated to be between 10 and 25 times higher than what the reported figures indicate (5).

Currently, identification of brucella-infected animals is based on either the milk ring test, enzyme-linked immunosorbent assay (ELISA), or some PCR-based tests (on an experimental basis only) (2, 32, 33, 36). In addition, reports of brucellosis in free-ranging bison in Yellowstone National Forest and the need to diagnose such infections rapidly and unambiguously (to prevent potential spillover of infection to domestic animal populations) reemphasize the necessity for the development of a molecular biology-based assay.

One-third of the world's human population is infected with *Mycobacterium tuberculosis*, and 3 million human deaths annu-

* Corresponding author. Mailing address: ClinCyte, LLC, 11055 Flintkote Ave., STE H, San Diego, CA 92121. Phone: (858) 457-9669. Fax: (858) 457-1827. E-mail: bookout8@earthlink.net.

ally are attributable to the organism (1, 6). The pulmonary form of zoonotic tuberculosis (TB) caused by *Mycobacterium bovis* in humans is indistinguishable from the TB caused by *M. tuberculosis* (strict sense) (7, 8, 31). Both species belong to the *M. tuberculosis* complex group, which includes *Mycobacterium microti* and *Mycobacterium africanum* (31). The four species are closely related at the genetic level (with 95% DNA-DNA homology) and are considered host specialists (31) on the basis of their ability to cause disease in limited host populations. However, *M. bovis* (considered the ancestral variant of the other three species) has the widest host range including animals and humans (1, 31). Hence, animal TB poses a potential source of TB in humans. In countries where bovine TB is uncontrolled, most human cases occur in young persons and result from the drinking or handling of contaminated milk or milk products (1, 7, 8). Little is known about the frequency of involvement of *M. bovis* in nonpulmonary TB in the developing nations because of limited laboratory facilities for the culture and identification (or typing) of tubercle bacilli (7; F. Miolan-Suazo, M. Salman, J. Payeur, C. Ramirez, and J. Rhyan, Proc. U.S. Anim. Health Assoc. Meeting, Tuberculosis session, p. 51–61, 1997).

Information on the incidence of human disease due to *M. bovis* in developed and developing countries is scarce. A review of zoonotic TB from 1954 to 1970 estimated that the proportion of cases due to *M. bovis* accounted for 3.1% of all forms of TB: 2.1% of pulmonary forms and 9.4% of extrapulmonary forms (7). The use of direct smear microscopy as the only method for diagnosis of suspected TB, a requirement of any national TB program, may partly explain the relatively low rate of notification of disease due to *M. bovis* in developing countries (7). Direct smear microscopy (in addition to its low sensitivity) does not permit differentiation between species of the *M. tuberculosis* complex; in addition, culture and species assignment, even when carried out, are time-consuming and not confirmatory for the accurate identification of *M. bovis* (7, 16). Currently, animal TB is diagnosed by a skin test that includes a caudal fold, comparative cervical, single cervical, or double-strength cervical test (1, 14; Miolan-Suazo et al., Proc. USAHA Meeting). The skin test has a low sensitivity (65.6 to 70%) (25; Miolan-Suazo, Proc. USAHA Meeting). Although some reports suggest that skin tests have a high specificity (98.8%) (25), false-positive results due to exposure to atypical mycobacteria, corynebacteria, *Fasciola hepatica* (liver fluke), and/or nocardia species are problematic in some countries. Alternate tests including ELISA, lymphocyte stimulation tests, gamma interferon assay (35), and blood tuberculosis battery tests are useful but are complementary to skin tests rather than real alternatives (25; Miolan-Suazo et al., Proc. USAHA Meeting).

In the absence of a highly sensitive and specific diagnostic assay, prevention and control of these zoonoses is dependent on the control and eradication of infection in animals, because the transmission of these diseases is primarily from animals to humans and is seldom between humans. This in turn is dependent upon a rapid, yet sensitive and specific means of detection of brucellae or *M. bovis* organisms in potentially infective materials such as milk or nasal swabs. In this study we describe a duplex amplification assay that can identify one or both organisms in a single reaction. The assay described herein is designed for milk samples and nasal swabs but can easily be expanded to test other samples such as lymph node aspirates, aborted fetal tissues, placental fluids or placenta from aborted cows, lungs or lung lesions collected at necropsy, and nasal aspirates.

MATERIALS AND METHODS

Bacterial strains. (i) *B. abortus*. Purified genomic DNAs from two bovine isolates (isolates NADC 8-1070 and NADC 8-0840), two vaccine strains (strains RB51 and S19), and one type strain (strain 544) of *B. abortus* (3) were obtained from the collection of the National Animal Disease Center (NADC), Ames, Iowa, for development of the assay.

An aliquot of milk spiked with 10^9 CFU of strain 2308 (killed by pasteurization) per ml was obtained from the brucellosis laboratory of the Department of Veterinary Science, Louisiana State University, Baton Rouge. Liver samples from 46 experimentally infected lemmings were also provided for the evaluation of the assay. The lemmings had been infected intraperitoneally with 10^3 CFU of either strain 2308 or strain RB51 (10). Clearance of infection was monitored by culturing the lemming liver samples collected aseptically after euthanasia.

(ii) *M. tuberculosis* complex. Whole-cell lysates enriched with genomic DNA from 15 *M. bovis* strains and prepared with a mini-bead beater (15) were a generous gift from the Tuberculosis Laboratory of the Centers for Disease Control and Prevention, Atlanta, Ga. This set included 11 field isolates of *M. bovis*, characterized on the basis of *oxyR285* polymorphism (30), and four strains of *M. bovis* BCG from the Trudeau Medical Collection (strains TMC1010, TMC1002, TMC1029, and TMC1012). Six *M. tuberculosis* (strict sense) isolates that had been characterized on the basis of IS*6110* fingerprints and for mutations in certain drug resistance-associated genes (*rpoB*, *katG*, and *pncA*) were obtained from the Public Health Research Institute, New York, N.Y., and were analyzed in this study.

A freeze-dried vaccine strain of BCG was used in spiking and serial dilution experiments to define assay parameters.

Negative milk samples. A total of 10 raw milk samples were obtained from a local herd in Colorado with no history of brucellosis or tuberculosis. The 10 samples were PCR tested for true-negative status. These samples were used in all spiking experiments and in the entire study as negative controls for PCR with milk.

The optical densities (ODs) of 63 negative samples (including negative milk samples from a local disease-free herd [$n = 10$], samples from a disease-free herd from Argentina [$n = 16$], PCR-negative controls [$n = 27$], and amplicons generated from *Ehrlichia equi* [$n = 2$], and *Babesia microti* [$n = 2$], and drug resistance-associated gene-sequence regions from *M. tuberculosis* [two amplicons each; *katG*, *rpoB*, and *pncA*]) were used to define a negative cutoff value (negative mean $\pm$ 3 standard deviations).

DNA extraction (milk). In this study, DNA was extracted by using a 1-ml aliquot of milk with a Boom extraction modification of a QIAamp Blood and Tissue DNA Extraction protocol (34). Briefly, 1 ml of milk was centrifuged at $6{,}000 \times g$ for 10 min. The clear whey portion was suctioned out with a transfer pipette and discarded. The remaining milk solids and butterfat were used for further processing and DNA extraction. Preheated sterile, double-distilled, deionized water was used to bring the volume of the samples to 200 μl, and the mixture was vigorously vortexed to release the pellet from the bottom of the tube. A total of 25 μl of proteinase K (a 20-mg/ml stock) was added, and the mixture was vortexed to mix. Subsequently, 200 μl of preheated lysis buffer was added to each tube, and the contents were vortexed again until the mixture was homogeneous. The mixture was then incubated at 70°C for 30 min. A second aliquot of proteinase K was added, and the mixture was incubated at 70°C for an additional 30 min. After incubation, 210 μl of ethanol was added, the mixture was vortexed, and the samples were processed to DNA through QIAamp columns (catalog no. 29106; Qiagen, Valencia, Calif.) as described in the product insert (QIAamp Blood and Body Fluids protocol; Qiagen). DNA was eluted in 200 μl of sterile, double-distilled, deionized water in all cases except where specified.

***B. abortus* (31-kDa protein or BCSP31K gene region).** The gene that encodes the 31-kDa protein is well conserved within the genus *Brucella*, with stretches of nucleotide sequences that are species specific. A 311-bp region of the BCSP31K gene (GenBank accession no. M20404) with four *B. abortus*-specific regions was identified. The primers located at nucleotides 844 to 864 (5′-ACGCAGTCAG ACGTTGCCTAT-3′) and nucleotides 1154 to 1131 (5′-TCCAGCGCACCATC TTTCAGCCTC 3′) amplify a *B. abortus*-specific region (311 bp). A *B. abortus*-specific probe was designed by using this amplicon sequence and a series of GenBank database searches. The design involved combination of two regions within the amplicon so as to be able to capture both sense and antisense strands of the amplicon. The probe used was 5′-CGACGATGGTGCCAGAAGATTT GCGCCTTCTG-3′ (nucleotides 1035 to 1048 and 910 to 927). Since database searches indicated that both the amplification primers and the probe were unique to *B. abortus*, this region was used for assay development.

***M. tuberculosis* complex (putative host cell receptor binding protein [a segment of *hsp65* gene with a region of direct-repeat sequences]).** The *hsp65* gene is well conserved within the *M. tuberculosis* complex group and has stretches of specific nucleotide sequences that can be used as probes. A direct-repeat segment in *hsp65* gene (GenBank accession no. S46909) that contains four regions specific for the *M. tuberculosis* complex was identified. The primers for one such region located at nucleotides 272 to 293 (5′-GGCTGGTTACACCTTCGATG CG-3′) and nucleotides 618 to 601 (5′-AGCCGCCGAAACCCATCT-3′) amplify an *M. tuberculosis* complex-specific region (347 bp). From this amplicon sequence two *M. tuberculosis* complex-specific regions were identified by database searches. These regions could serve as a probe for the duplex assay. An *M.*

TABLE 1. Source of field samples used to validate BPDA

Geographic locality	Animal	Specimen type	No. of samples	Comment
United States	Laboratory animals[a]	Liver	45	Experimentally infected (*B. abortus*)
Mexico	Bovine	Milk	46[b]	Bovine TB suspect[c]
Mexico	Bovine	Nasal swabs	121	Bovine TB suspect[c]
India	Bovine	Milk	20	*B. abortus* suspect[d]
Argentina	Bovine	Milk, nasal swabs, lymph node aspirates	128	Infected and disease-free herds[e]

[a] Operators were blinded as to the infection status of the lemming liver samples for this part of the study.
[b] Pools of 10 samples each.
[c] Suspicion of bovine TB was based on caudal fold tuberculin test or comparative cervical test positivity.
[d] A subset of Indian samples ($n = 15$) was positive by the milk ring test. The other five samples were negative.
[e] Argentine samples came from TB-positive herds (three herds) or herds with brucellosis (two herds). One disease-free herd was sampled as a control.

tuberculosis complex-specific probe was designed to capture both sense and antisense strands of the amplicon. The probe sequence identified was 5′-ACAT CGGCAACAACAACATCGGC-TGCCGGTGTTGCCGCT-3′ (nucleotides 406 to 426 and 506 to 488). The probes have more than one homologous site within the 347-bp amplicon and may enhance the probability of successful hybridization even under very high stringency conditions. Since BLAST database searches indicated that both the amplification primers and probes were unique to *M. tuberculosis* complex, this region was used for the assay development.

Internal standard (bovine β_2-microglobulin). An internal amplification control was designed and used with all samples to assess the integrity of DNA (during transport at room temperature) and the absence (or presence) of PCR inhibitors in the sample. A 226-bp stretch of the bovine β_2-microglobulin sequence (GenBank accession no. X69804) was used as an internal standard. Specific forward and reverse primer sequences at nucleotides 135 to 155 (5′-ACCTGAACTGCTATGTGTATG-3′) and nucleotides 360 to 340 (5′-TCT CGATCCCACTTAACCTATC-3′), respectively, were identified by database searches, as described above for the other two targets. A β_2-microglobulin probe (nucleotides 207 to 240; 5′-TTAAATCGGAGCAGTCAGACCTGTCTTTCAG CAA-3′) was also designed for the solid-phase detection assay.

Duplex amplification. Experiments for amplification of either *M. bovis* alone, *B. abortus* alone, or the two organisms in combination were performed with genomic DNA purified from the respective bacterial cultures. All four primers used in the amplification reactions were biotinylated. The amplification conditions and master-mixture components ($MgCl_2$ concentration and primer concentrations) were optimized to amplify all DNAs as singlets or in 15 different combinations of *B. abortus* and *M. bovis* as duplexes. The master-mixture composition used for a 50-μl reaction mixture was 10× PCR buffer (with 15 mM $MgCl_2$; Perkin-Elmer, Branchburg, N.J.), deoxynucleoside triphosphates (final concentration, 200 μM; Pharmacia), *M. tuberculosis* complex-specific sense and antisense primers (final concentration, 1 μM), *B. abortus*-specific primers (final concentration, 0.25 μM), 2.5 μl of genomic DNA from pure bacterial cultures (10 μl of DNA was used for extracts from all primary clinical samples including nasal swabs, lymph nodes, vaginal swabs, and milk), and *Taq* polymerase (2.5 U per reaction mixture). The thermal cycler parameters used for amplification were initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 62°C for 20 s, and extension at 72°C for 20 s. At the end of 35 cycles, a final extension step at 72°C for 7 min was performed.

Solid-phase hybridization and detection. The species-specific probes were designed to target a well-conserved region within the amplicon of interest. The specificities of the probes were identified by a series of GenBank database searches. Each probe was resuspended in freshly prepared 1 M ammonium acetate solution (pH 7.5). *M. tuberculosis* complex-specific probe was used at a concentration of 50 ng/100 μl, while the *B. abortus*-specific probe was used at 75 ng/100 μl. For screening for both pathogens in one well, both probes were added in one solution at the concentrations indicated above. For the detection of individual pathogens, only one specified probe was used per coating solution. Each well of the microtiter plates was coated with 100 μl of the solution that contained the probes, and the plates were incubated at 37°C overnight. After overnight incubation, the plates were washed twice with phosphate-buffered saline (PBS)–Tween 20 and were then blocked with PBS–1% bovine serum albumin for 2 h at 37°C. The plates were washed twice with PBS–Tween 20, air dried, and stored in airtight sachets with desiccant at 4°C until they were used.

On the basis of a series of optimization experiments with different salts (guanidium thiocyanate or sodium thiocyanate [NaSCN]) and concentrations (1 and 2.5 M) for hybridization, 1 M NaSCN (pH 5.0 ± 0.2) was identified as the ideal neutralization-hybridization buffer for the duplex detection protocol. Briefly, 25-μl aliquots of the denatured amplicons were loaded into precoated microtiter plate wells that contained 100 μl of hybridization buffer (1 M NaSCN). The plates were incubated at 37°C for 1 h. The plates were then washed five times with PBS–Tween 20. A 100-μl aliquot of a 1:2,500 dilution of neutravidin-peroxidase (Pierce) was added to each well, and the plate was incubated for 15 min at 37°C. The plates were washed seven times with PBS–Tween 20, and 100 μl of tetramethylbenzidine (Moss Inc., Pasadena, Md.) was added to each well. The plates were incubated in the dark for 10 min at room temperature. The reaction was stopped with 5% sulfuric acid (100 μl/well), and the plates were read at 450 nm with an automated ELISA Reader (Bartels Prima System; Baxter Scientific).

The background and nonspecific reactivities were tested by using negative controls that consisted of the amplification reaction mixture, amplicons that targeted other drug resistance-associated regions in *M. tuberculosis* (*katG*, *pncA*, *rpoB*), and 16S rRNA amplicons from other organisms such as *B. microti* and *E. equi*. These served as within-species and other extraneous negative controls.

Field studies. The sources of the field samples for these studies are described in Table 1. Animals from brucella buffered antigen plate agglutination test- or caudal fold test-positive and -negative herds from a variety of geographic localities were sampled (Table 1) and were analyzed by the bovine pathogen detection assay (BPDA)-PCR. The samples included milk ($n = 136$), nasal swabs ($n = 149$), lymph nodes aspirates ($n = 7$), and other specimens ($n = 9$).

The samples originated from Mexico, Argentina, and India. All samples were processed to DNA at local laboratories with appropriate biosafety facilities. The DNA was analyzed at the ClinCyte laboratory in San Diego, Calif.

(i) Herd sampling in Argentina. A total of 128 bovine samples were obtained from farms that were classified as either "brucellosis positive" (defined by reactivity in a buffered plate antigen assay), "TB positive" (defined by delayed hypersensitivity to an intradermal injection of purified protein derivative), or disease-free (defined by a negative skin test and a negative buffered plate antigen assay result). The samples included milk ($n = 70$), nasal swabs ($n = 28$), retropharyngeal lymph node aspirates ($m = 7$), and historical samples from a slaughterhouse (vaginal and lymph node swabs) ($n = 9$). Fourteen pools of five individual milk samples each were also analyzed. Table 2 gives the stratified sampling scheme used for this study.

(ii) Samples from TB-positive herds in Mexico. Two bovine TB-suspect herds (considered heavily infected, as indicated by caudal fold test results) were sampled. Nasal swabs ($n = 121$) and milk samples ($n = 460$) were collected. The milk samples were pooled into batches of 10 milk samples each, and the DNA was analyzed at the ClinCyte laboratory.

(iii) Bovine brucellosis-positive milk samples from India. Milk was sampled from 20 dairy cows either positive ($n = 15$) or negative ($n = 5$) for brucellosis as determined by the milk ring test. DNA was extracted in a local laboratory and was analyzed by BPDA-PCR at the ClinCyte laboratory.

Milk collection technique. All milk samples were obtained from animals during their routine milking time. This approach prevented disruption of farmers' day-to-day on-farm operations and simplified sample collection. The samples from each animal were obtained from all four quarters of the mammary gland.

TABLE 2. Summary of samples collected in two Argentine counties for BPDA-PCR

Suspected pathogen on farm[a]	No. of samples			
	Milk	Nasal swabs	Lymph node aspirates	Other (lymph node swabs)
M. bovis	16	16	7	
M. bovis	6	6		
M. bovis	10			
B. abortus	18			
B. abortus	10			
"Negative" farm	10	6		
Total samples collected	70	28	7	9

[a] Suspicion of *M. bovis* infection was based on purified protein derivative-positive status, and that for *B. abortus* infection was based on brucella BAPA test-positive status.

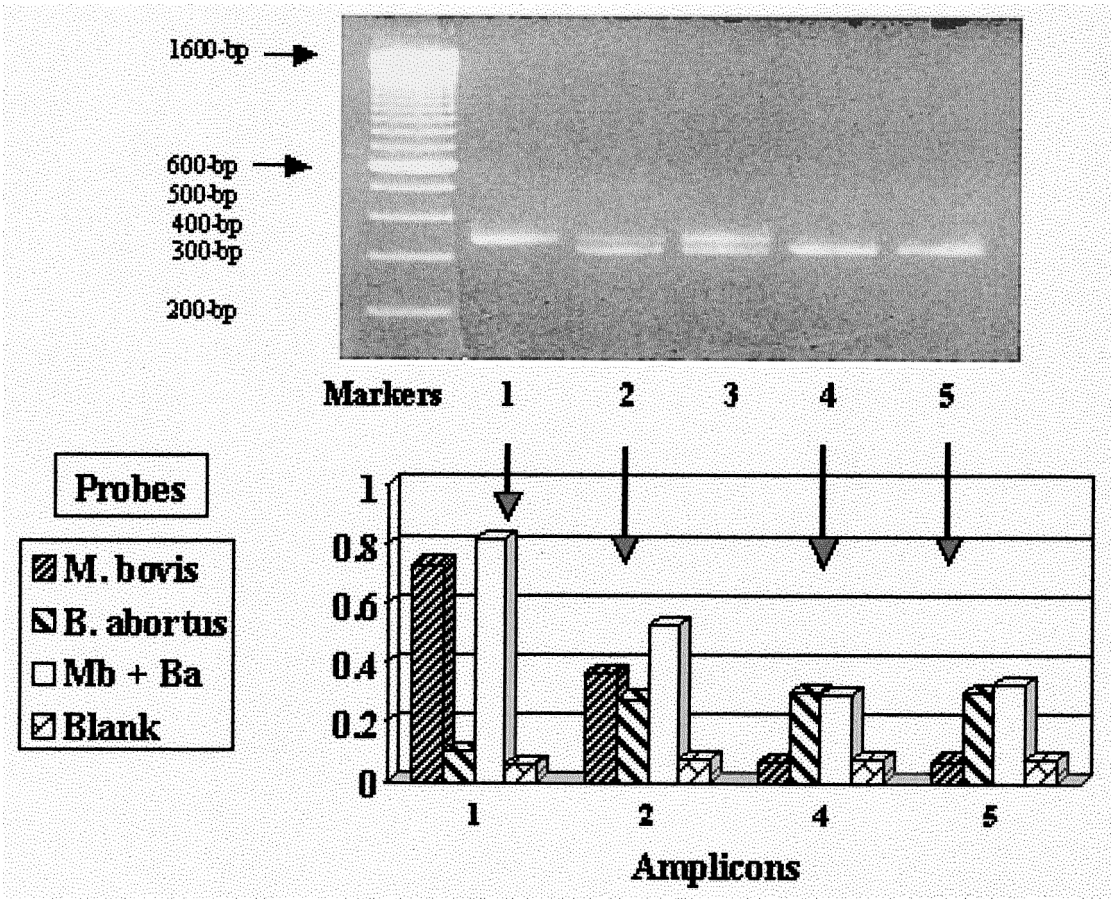

FIG. 1. (Top panel) Agarose gel electrophoretic pattern of amplicons generated from genomic DNA of *M. bovis* alone (lane 1), *M. bovis* and *B. abortus* (clinical strain 8-1070) mixed (lane 2), *M. bovis* and *B. abortus* (vaccine strain RB51) mixed (lane 3), or *B. abortus* alone (strain 8-1070 in lane 4 and RB51 in lane 5). (Bottom panel) Related ODs at 450 nm recorded for each amplicon when probed with either the *M. bovis* (Mb) probe alone (▨), the *B. abortus* (Ba) probe alone (▨), or a combination of both probes (□). Also shown (▨) are the ODs at 450 nm of blank wells (negative controls). Clear increases in ODs are noted for each individual amplicon with species-specific probes with no evidence of cross-reactivity (lanes 1, 4, and 5), while reactivity with both probes was seen when both amplicons were present (lane 2). Blank wells were negative throughout.

Collections were done after thorough disinfection of the teat area with povidone iodine. The milk sampler's (veterinarian's) gloves were also disinfected and wiped dry between samplings. Two to three strippings of milk from each teat were drawn into a sterile 50-ml tube, and the tubes were capped and stored on ice until further use. The entire collection process involved only two technicians (one who collected the milk and one who assisted) to rapidly obtain samples from the 16 to 24 animals in the entire milking shed at a time.

Nasal swab collections. Animals were individually restrained in a trevis or chute with a nose ring, which kept their necks extended. Both nasal passages of each animal were swabbed. Sterile applicators were applied deep into the nostrils, the nostril was vigorously scrubbed two to three times, and the applicator was placed in a sterile tube. The swabs were rehydrated with 500 μl of sterile water and were vortexed before an aliquot was taken for DNA extraction.

Lymph node aspirates. Retropharyngeal lymph node aspirates were collected while the animals were restrained in a chute. An 18-gauge (1.5-in) needle attached to a 10-ml syringe was inserted into the lymph node, and an aspirate was obtained. Aspirates were transferred to a sterile 15-ml tube and transported on ice. The tubes were vortexed, and a 200-μl aliquot was taken for DNA extraction.

RESULTS

Duplex amplification (initial setup and optimization). Experiments for amplification of either *M. bovis* alone, *B. abortus* alone, or the two organisms in combination were performed with the following genomic DNAs purified from the respective bacterial cultures: DNAs from 10 *M. bovis* strains (including 2 TMC strains, 1 BCG vaccine strain, and 7 field isolates), DNAs from 2 *M. tuberculosis* strains (patient isolates), and DNAs from 5 *B. abortus* strains (including 2 bovine isolates, type strain 544, and 2 vaccine strains). The amplification conditions and master mixture components were optimized to amplify all DNAs as singlets or in different combinations of *B. abortus* and *M. bovis* as duplexes (Fig. 1).

The negative cutoff value of 0.200 for the assay was based on the average ODs for all negative samples + 3 standard deviations.

Analytical sensitivity estimations. (i) Serially diluted *M. bovis* and *B. abortus* amplicons. *M. bovis* and *B. abortus* amplicons were generated and column purified (Hi-Pure; Boehringer Mannheim) to remove excess primers. The copy number in each amplicon was determined by using the estimated DNA concentration (calculations were based on the absorbance at an OD of 260 nm and amplicon size). Equal numbers of copies of each amplicon were mixed and serially diluted (2×10^5, 2×10^4, 2×10^3, 2×10^2, and 2×10^1 copies per reaction mixture), and the amplicons were then reamplified. The reamplified amplicons were analyzed by agarose gel electrophoresis and solid-phase probe-capture hybridization and detection as described above. The analytical sensitivity in this amplicon dilution experiment was 20 copies (on the basis of the lowest copy number used in the analysis) for amplicons of both pathogens.

(ii) Serial dilution of *B. abortus* genomic DNA in milk. One milliliter of normal raw cow milk was spiked with from 200 ng to 0.002 pg of *B. abortus* DNA (i.e., at equivalents of 2×10^8 to 2 genomes). The copy number (or genome equivalent) estimates were calculated on the basis of the fact that 100 fg of *B. abortus* DNA is equivalent to 20 organisms (P. H. Elzer, personal communication, 1999). One aliquot of milk was not spiked and was used as a negative control. The DNA was reextracted from the milk by the protocol described above. Forty microliters of the reextracted DNA was amplified as described above and was analyzed on a microtiter plate. By this analysis the analytical sensitivity of the assay was 40 genome equivalents per reaction mixture by microtiter plate detection.

(iii) Serial dilution of *B. abortus* organisms in milk. Strain 2308 of *B. abortus* was spiked into a sample of milk at 10^9 CFU/ml, and the mixture was pasteurized. An aliquot of this was serially diluted (10^8 to 0 CFU/ml) in raw milk from a healthy cow, and DNA was extracted in 200 μl of distilled deionized water. Ten microliters of this DNA was PCR amplified and was detected by microtiter plate analysis. The results of this analysis indicated that the assay could detect down to 1×10^2 CFU equivalents of *B. abortus* per reaction mixture (or 2×10^3 CFU equivalents/ml of milk).

Since the results obtained with whole-cell dilutions and genomic DNA dilutions are more accurate than those obtained with amplicons, the analytical sensitivity of the assay was estimated to be 40 to 100 CFU/reaction mixture (or 800 to 2,000 CFU/ml of milk).

(iv) Dilution of *M. bovis* BCG in milk. An aliquot of *M. bovis* BCG was diluted in PBS–Tween 20 (0.05% Tween 20), and the mixture was resuspended to match a McFarland 0.1 standard (bacterial count equivalent to 4×10^7 CFU/ml). A total of 100 μl of this suspension was placed in 900 μl of milk (corresponding to 4×10^7 CFU/ml of milk), and the mixture was serially diluted to 10^{-3} CFU/ml. The DNA was extracted from these dilutions as described above. Subsequently, amplification and detection were performed as described above.

Microtiter plate detection identified less than 1 CFU of *M. bovis*/ml of milk by our assay format. This indicates a high analytical sensitivity for the detection of *M. bovis*, possibly due to the presence of four to eight copies of the direct repeat of the amplification target per organism.

Assay performance with liver samples from lemmings experimentally infected with *B. abortus* 2308 or RB51. The assay performance was tested with a set of 45 liver samples from infected lemmings. In total, 33 culture-positive and 12 culture-negative liver samples were tested. Nine of 12 negative samples were from RB51 (an avirulent vaccine strain)-infected lemmings. These samples were interpreted as being from animals that had "cleared" the infection. Of the 33 culture-positive liver samples, 31 were positive by PCR. Therefore, by using culture as a "gold standard," the sensitivity of BPDA-PCR was 93.9% (31 of 33 samples). The two PCR-negative, culture-positive samples were from lemmings infected with the avirulent RB51 strain. Among the 12 culture-negative liver samples, 5 (3 from the strain 2308-inoculated group and 2 from the strain RB51-inoculated group) were identified as positive by PCR, while the other 7 (all RB51 group) culture-negative samples were classified as negative by PCR. These experiments show that the performance of BPDA-PCR may be comparable to that of culture in that BPDA-PCR identified five culture-negative samples from experimentally lemmings as positive. These animals may have incompletely cleared the infection and may thus be carrying damaged organisms that could not be revived by the conventional culturing method used. Additionally, identification of infected animals by culture is often difficult because brucellae are fastidious and slow growers.

Duplex identification of *B. abortus* and/or *M. bovis* in milk samples from brucellosis-positive animals sampled in India. DNA was extracted from the milk of 20 cows from a herd in Bangalore, India, known to be infected with *B. abortus* and was analyzed by BPDA-PCR. Of these 20 milk samples 15 were milk ring test positive (i.e., positive for *B. abortus* antibodies). The PCR analysis identified 13 of the 15 milk ring test-positive animals and 4 of the 5 milk ring test-negative animals as *B. abortus* shedders. The ODs at 450 nm ranged from 0.287 to 0.838 and 0.121 to 0.209 for positive and negative samples, respectively. The other negative milk ring test-samples and two milk ring test-positive samples were negative by PCR (Table 1). In addition, upon analysis of these samples by individual probe (*M. tuberculosis* complex- or *B. abortus*-specific) hybridization assays, two samples were found to be positive for both *B. abortus* and *M. bovis*. All other samples were positive only for *B. abortus* by BPDA-PCR. These studies further validate the high degree of sensitivity of BPDA-PCR in identifying infected, shedding animals that may be missed by the milk ring test. BPDA-PCR analysis was also able to identify two animals concurrently infected with *M. bovis* and *B. abortus*.

Duplex PCR identification of *B. abortus* and/or *M. bovis* in milk samples from brucellosis-suspect animals sampled in Argentina. (i) BPDA-PCR performance with suspected *M. bovis*-positive samples. Thirty-two animals from three purified protein derivative-positive farms were tested. The types of samples tested were milk ($n = 32$), nasal swabs ($n = 22$), and retropharyngeal lymph node biopsy specimen ($n = 7$). Only 1 of 32 milk samples and 2 nasal swabs (from two different farms) were positive for *M. bovis* (positive OD range, 0.241 to 2.842; negative OD range, 0.067 to 0.192). All lymph node biopsy specimens were negative by tests with both multiple and individual probes. All samples from one farm suspected of being positive were negative.

(ii) BPDA-PCR performance with suspected *B. abortus*-positive samples. Twenty-eight milk samples from brucellosis-positive animals (according to the results of the buffered antigen plate agglutination [BAPA] test) in two herds were tested. Seven BAPA test-positive animals tested positive (positive OD range, 0.219 to 0.518; negative OD range, 0.078 to 0.189) by BPDA-PCR and were defined as *B. abortus* shedders by analysis with individual probes (Tables 2 and 3).

(iii) BPDA-PCR performance with milk sample pools. Fourteen milk sample pools (with each pool made up of five samples) were analyzed by BPDA-PCR. From retrospective analysis of individual milk samples, it was expected that 6 of the 14 milk sample pools should be positive. BPDA-PCR analysis correctly identified four of six milk sample pools as positive. The two pools in which organisms were not detected were low-positive pools, in that each of these had a sample that was borderline positive (ODs of 0.219 and 0.295, respectively, when individual samples were tested with *B. abortus*-specific probes). The results indicate that pooling of samples from five animals for BPDA-PCR may miss some low-level shedders or low-level-positive animals. Additional studies with pools of samples from three animals or other combinations will be required to define low-level shedders adequately.

(iv) Internal standard analysis. A total of 125 samples from Argentina were tested for amplification of a 226-bp region of bovine β_2-microglobulin to determine if any samples contained PCR inhibitors or if the DNA extraction was functionally intact. All samples analyzed by agarose gel electrophoresis of the amplicon products were shown to have single, nondiffuse bands with high concentrations of amplicons for the β_2-micro-

TABLE 3. Summary of shedding status within *M. bovis*- or *B. abortus*-infected herds in two argentine counties[a]

Sample source	No. of farms positive by BPDA-PCR/no. of farms tested	Total no. of animals tested	Total no. of PCR-positive animals	% of animals shedding organism in milk	Comments
M. bovis suspect farms (PPD positive)	2/3	32	3	3.1% of suspect cows (1/32)	Nasal swabs alone from two animals tested positive, and milk only from one animal tested positive
B. abortus suspect farms (BAPA test positive)	2/2	28	8	25% of suspect cows (5/28) or 11.4% of all cows tested	
Milk pools (see milk pool summary sheet)	4/6	14 pools of 5 milk samples each from a total of 70 animals	4	42.8% of pools were positive	Included milk samples from one "negative" farm; two pools were false negative; no false-positive samples were identified

[a] All animals tested from specific-pathogen-suspect farms were positive by conventional testing. The sampling did not include any healthy animals within infected herds and therefore may not reflect the true shedding status (sampling bias). However, these data can be used as an estimate to project the sampling load (testing and retesting) and total assay load.

globulin region of interest. The amplification of the 226-bp region from all samples indicates no PCR inhibition or DNA degradation; therefore, any BPDA-PCR-negative sample was considered to be from a nonshedder or to contain organisms at a level below the detection limits of the assay.

Identification of *M. bovis* in milk samples or nasal swabs from skin test-positive herds sampled in Mexico. Two sets of samples from Mexico were analyzed. One set consisted of milk samples from animals from caudal fold tuberculin test- and comparative cervical test-positive farms. Forty-six milk sample pools were tested. Each pool was a mixture of milk from 10 individual animals. Of the 46 milk sample pools, 15 were positive (positive OD range, 0.241 to 1.124) with the BPDA-PCR multiplex probe- and *M. tuberculosis* complex-specific probe-coated microtiter plates. Retrospective analysis of all 10 individual milk samples from one positive pool identified two positive animals. The second set of samples analyzed included nasal swabs ($n = 121$) obtained from a second geographically distinct area in Mexico. Of the 121 samples, 68 (56.2%) were BPDA-PCR-positive for *M. bovis* (OD range, 0.406 to 2.882). Animals from these farms were also bovine TB suspect on the basis of intradermal test results.

DISCUSSION

Economics of disease eradication and the need for an accurate tool. The presence of infection caused by the two zoonotic pathogens, *B. abortus* and *M. bovis*, in animal populations poses an economic threat to countries around the world (1, 5, 6, 7, 24). This disease has contributed to nontariff trade barriers by impeding the safe, free trade of cattle and cattle products implemented by international and other national trade agreements (6). Losses due to reduced sales and lowered production and the trade barrier that these diseases have created reemphasize the need for a rapid, sensitive, specific, and efficient molecular biology-based diagnostic tool. This would eventually lead to reduced rates of *M. bovis* and *B. abortus* infection and therefore an increase in the trade, movement, and marketability of cattle in South, Central, and North America. Countries in these regions look to the United States for technologies and protocols that they can use to aid them with their screening for such zoonoses and to initiate profitable livestock trading across international borders. On the basis of the prevalence data from the three countries surveyed (5, 6, 36; Miolan-Suazo et al., Proc. USAHA Meeting), an algorithm for an eradication program is proposed (Fig. 2). The algorithm includes the need for a stepwise, multistage, periodic testing process for a successful eradication program. The algorithm allows the testing of milk samples in pools made up of milk from 5 to 10 animals in each pool in high-incidence areas, thus reducing the cost of screening each animal. As demonstrated in the samples from Mexico, analysis of pools made up of 5 to 10 milk samples is feasible and will help identify positive samples as a part of the proposed eradication algorithm.

Current diagnostic practices and their pitfalls. Diagnosis of brucellosis primarily uses the milk ring test or the BAPA test. The milk ring test or the BAPA test has been widely applied for identification of infected herds in the United States and other countries, followed by ELISA, the complement fixation test, or the standard tube agglutination test to confirm infection in individual animals (2, 5, 33). The milk ring test is an agglutination test with fair specificity (79.4 to 86.7%) and sensitivity (85.4 to 99.3%) (32). False-positive results by the milk ring test occur immediately after parturition and during mastitis. Additionally, batch-to-batch variations in milk ring test antigen levels lead to variability associated with the lower sensitivity of the assay (P. H. Elzer, personal communication). The results indicate that the milk ring test may be less sensitive than PCR. On the other hand, while ELISAs have proved to have higher sensitivities and specificities, like the milk ring test they measure antibodies that reflect exposure of the animal to brucellae and do not necessarily indicate present infection or the ability of the animal to spread the disease (5). Despite some of these drawbacks, the two serological tests have been used in successful eradication schemes. Additionally, these tests can be used as an initial screen to identify exposed or infected herds in an eradication program.

PCR with random or selected primers has shown promising results, but large-scale field validations and evaluations are lacking, especially for detection of chronic infections. For PCR, various targets (species-specific, biotype-specific, and genus-specific targets) have been investigated, including the genes for Omp2 (12), the 31-kDa (BCSP31K) antigen (22), the 16S rRNA, the 16S-23S spacer region, and an insertional element, namely, IS*711* (3, 4). Among these, the gene that encodes a 31-kDa protein has proved to be a promising target because of the presence of species-specific signature regions in the gene (22). In the past there has been considerable interest in the use of rapid molecular biology-based assays to identify and determine the species of brucellae (3, 4, 5). By using this strategy with species-specific signature regions on Omp2 gene

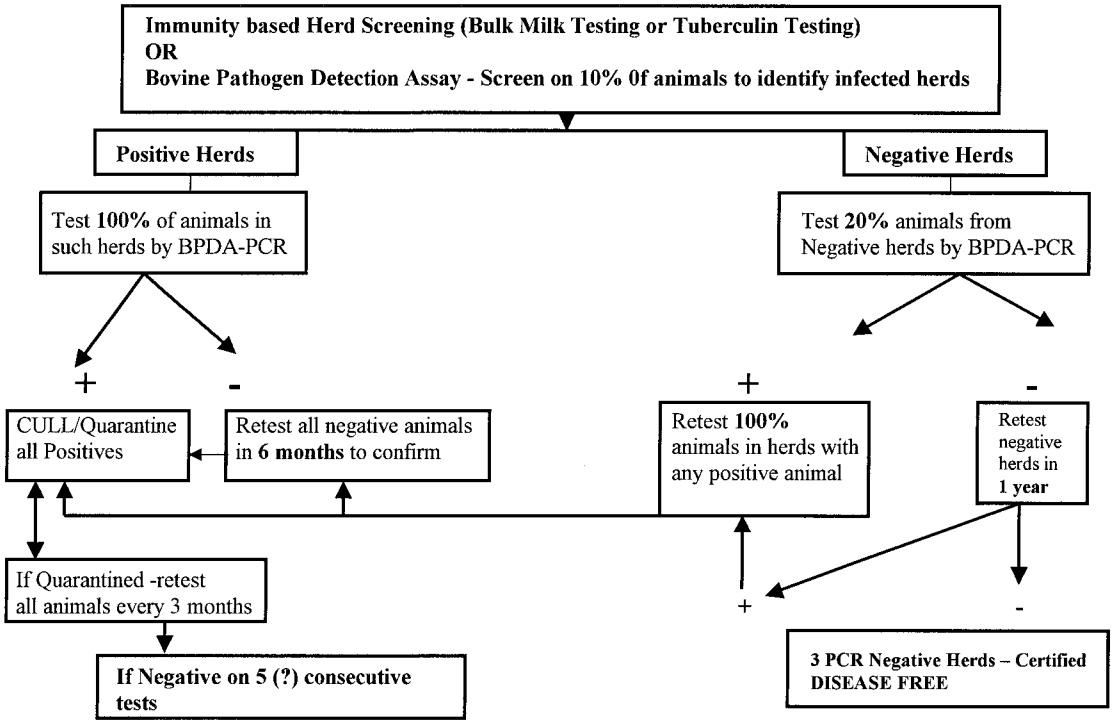

FIG. 2. Proposed algorithm for a stepwise repeat sampling and testing protocol for a disease eradication scheme. The proportion of animals to be tested will vary with the prevalence of disease in a given geographic locality (higher numbers in lower-prevalence areas; however, low-prevalence areas will not require many iterations of the same tests).

(12), a PCR assay has been developed and patented (T. A. Ficht, B. A. Sowa, and L. G. Adams, May 1994, U.S. patent 5310649). The antigen detection methods are potentially useful but have not been validated. Combinations of these with PCR, such as immuno-PCR, have considerable potential but require evaluation (5).

Recent developments in the molecular genetics of mycobacteria have identified species-specific markers in MPT40 (27), *oxyR* (30), and *pncA* (9) that enable specific identification of *M. bovis*. Among these, only the *oxyR* polymorphism at nucleotide 285 has proved to be an accurate and consistent *M. bovis*-specific marker (9). Other targets that have been investigated include the 16S rRNA gene region with a TB complex-specific signature sequence, targets identified by randomly amplified polymorphic DNA analysis (18, 26, 28, 29), and mycolic acid profiles identified by the high-performance liquid chromatography (13). The high-performance liquid chromatography method requires a considerable bacterial inoculum, thereby requiring mycobacterial culture, which takes 3 to 6 weeks, before application.

It may be argued that IS*6110* has more copies and would form a good target for the identification of *M. tuberculosis* complex organisms. However, numerous ancestral isolates from the *M. tuberculosis* complex group (especially *M. bovis* from cattle, goats, or other animal sources and some *M. tuberculosis* isolates from certain geographic localities that have now spread globally) with no copies of IS*6110* exist (31). While the use of *oxyR* for the specific identification of *M. bovis* is very attractive, *oxyR* is a pseudogene in the *M. tuberculosis* complex; therefore, it is expected under the classical evolutionary theory that this pseudogene may mutate spontaneously (and at a higher rate than other structural genes) over time and may not be detected in some isolates by the use of specific probes. Thus, the *hsp65* region with a direct-repeat sequence, which is present in multiple copies, was used in the present study.

Performance of BPDA-PCR: experimental and field studies. The experiments performed in the present study with purified genomic DNA optimized the assay for *B. abortus* identification at <2,000 CFU/ml and for *M. bovis* identification at <10 CFU/ml. Although the detection level for *M. bovis* is as low as 4 CFU/ml, we believe that this could be an overestimate due to the indirect nature of CFU equivalent estimation by the use of McFarland standards. Overall, experimental studies with either organism proved that the assay was very sensitive and specific on the basis of the data obtained for normal and spiked milk samples and liver samples from experimentally infected lemmings. The performance of BPDD-PCR in field studies with infected herds from countries that report the occurrence of the two zoonotic diseases further validates its use as an optimal tool for surveillance for bovine brucellosis and TB. One of the concerns with PCR is the occurrence of false-negative results due to the presence of substances in the sample that interfere with amplification. The extraction process used in the study efficiently eliminates PCR inhibitors. To test this, all Argentine specimens and a subset of samples from Mexico were tested for the presence of a β_2-microglobulin segment that is highly conserved and that is expected to be present in host cellular debris of any secretion or excretion. All samples tested positive for β_2-microglobulin, thus increasing the degree of confidence in the technical interpretation of a negative test result as "negative." On the other hand, false-positive results may occur as a result of the cross-reactivity of PCR primers and probes or because of contamination during any stage of collection, processing, and detection. Therefore, a series of database searches was performed to identify specific regions for primer and probe design. Additionally, the study

used a hygienic milk collection protocol and laboratory procedures to minimize cross contamination of samples. This is reflected by the fact that all negative samples (from disease-free herds) and controls yielded ODs below the cutoff value.

The surveillance and monitoring algorithm proposed in this study emphasizes a multistage identification system because PCR identifies animals actively shedding the organism and contaminating the environment. Thus, a negative PCR result should be interpreted with caution, especially with a chronic granulomatous disease such as bovine TB.

However, PCR results are similar to culture results. A positive PCR test indicates the presence of the pathogen, and this is often correlated with infectivity. In the context of infections with *M. bovis*, a positive PCR result represents a status in which the animal has been infected, is shedding mycobacteria or brucellae, and may infect other animals, but a negative result does not rule out infection. A similar argument can be made about culture, since not all attempts to culture an organism are successful, and not all samples provide sufficient material to demonstrate positivity. However, BPDA-PCR is extremely sensitive, and this sensitivity lessens the probability of false-negative results. Sample volumes have been optimized in this study to reduce sampling error, and our data show that <10 organisms (BCG serial dilution studies) may be detectable in 1 ml of a milk sample pool. Others have also shown that PCR detects as few as one organism (5 fg of DNA or less) in a specimen aliquot (13, 34).

Miller et al. (24) demonstrated that PCR is effective in diagnosing TB in cattle with various tissues collected at necropsy. They found a 93% success rate compared with the results of necropsy examination for the detection of ruminant TB with paraffin-embedded tissues (23). A recent study by Vitale et al. (34) addressed the performance of PCR versus those of necropsy examination for infection and conventional test result. Using PCR analysis of tissues from slaughtered animals as a gold standard in an evaluation of 100 cattle, they showed 100% sensitivity and specificity for milk samples and lymph node biopsy specimens (collected at necropsy). The lack of PCR-positive lymph node biopsy specimens by BPDA-PCR in our study may be associated with the fact that the representative nodes were not sampled. In most instances appropriate lymph nodes are not superficially accessible for optimal diagnosis of infection by PCR or culture. Additionally, it is suggested that multiple nodes must be sampled for *M. tuberculosis* complex-specific PCR to improve the diagnostic value (6, 25). Technical difficulties and the stress imposed on the animals, which may reduce milk production, make it somewhat impractical for the use of lymph node biopsy specimens for antemortem diagnostic assays.

Although historical data for the detection of *B. abortus* bacteremia for as long as 251 days in nonvaccinated animals and 60 days in vaccinated animals exists (21), most animals in advanced carrier states also shed organisms in their milk. Blood sampling is useful for early detection of the disease process, while BPDA-PCR analysis of milk will efficiently detect most ($>90\%$) chronically infected animals in the herd. Thus, the optimal sampling strategy may be to use milk alone for the diagnosis of brucellosis and nasal swabs in addition to milk for the diagnosis of bovine TB. Studies by Vitale et al. (34) and Cornejo et al. (6) for bovine TB and those by Leal-Klevezas et al. (17) and Fekete et al. (11) for bovine brucellosis support the idea that the use of milk and/or nasal swab specimens may be optimal for the efficient diagnosis of the diseases caused by these two zoonotic pathogens. BPDA-PCR does identify but cannot differentiate vaccine strains of *B. abortus* (S19 and RB51) when they are present. Given that only calfhood vaccination with strains S19 and RB51 is practiced and that strain RB51 is not excreted or cleared from the body in milk, the possibility of false-positive results by BPDA-PCR due to vaccination is significantly reduced.

The use of PCR for the diagnosis of bovine infections has not been evaluated in large-scale field or herd studies so that guidelines for its use in an eradication program can be recommended. Although there are limited data from studies with animal isolates (6, 34), to our knowledge, this is the first study to test the ability of a multiplex molecular biology-based assay to detect any animal disease in the field. Such an assay, when available for large-scale disease screening and surveillance, is very appealing because of its ability to identify the presence of multiple pathogens in herds as well as in individual animals. This multiplex format further translates into a lower cost per pathogen tested per herd. In conclusion, the ability of BPDA-PCR to identify either pathogen in field samples from herds from a variety of geographic regions known to be infected or exposed to the pathogens demonstrates that the test is robust and accurate. Use of a combination of the duplex PCR assay, screening of milk samples in pools, and the proposed algorithm provides a highly sensitive and economically viable alternative to immunodiagnostic methods.

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