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Monitoring *Babesia bovis* Infections in Cattle by Using PCR-Based Tests†

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The sensitivity and specificity of PCR tests based on the small-subunit rRNA gene sequence of *Babesia bovis* were compared in a blind study of experimentally infected cattle with the corresponding parameters of the complement fixation (CF) test currently used in the United States to screen for bovine babesiosis. Cattle were experimentally infected with a single inoculum of a cloned laboratory strain of *B. bovis*. Blood samples were collected and tested over a period covering from the day of infection to 10 months postinfection. The level of parasitemia (percent infected erythrocytes) present in each sample was estimated from test results and was plotted as a function of time postinfection. These data are the first describing the course of infection by methods capable of detecting parasitemias in the range of 10^{-7}%, which frequently occur in the carrier state. Parasitemias in the samples tested strongly influenced the sensitivity and negative predictive value of the PCR-based tests which varied with time postinfection. The average sensitivities of the three PCR-based tests for *B. bovis* ranged from 58 to 70% for a single determination, while the sensitivity of the CF test was only 6%. Both PCR-based and CF tests for *B. bovis* had high specificity values ranging from 96 to 100%.

Bovine babesiosis is a tick-borne disease found worldwide which is caused by several species of protozoal hemoparasites of the genus *Babesia*, phylum *Apicomplexa* (17). After acute or primary infections, recovered animals frequently sustain subclinical infections which are microscopically undetectable. This carrier state serves as reservoir for infection in the herd, since animals which are not clinically ill may continue to infect the tick vector. The goal for development of new diagnostic tests is to be able to reliably detect this carrier state. Current tests which depend on serology lack sensitivity and specificity for this infection status (24); thus, nucleic acid probes have been developed by numerous investigators to specifically detect small numbers of parasites in the blood (3, 5, 6, 10, 14, 25–28). Probes of genomic DNA targets generally detect a level of parasitemia (percent infected erythrocytes [RBCs]) lower than that detected by light microscopy, but they are not sufficiently sensitive to reliably detect the carrier state. The present limitations of direct detection are $\sim 10^4$ parasitized cells for a DNA probe and $\sim 2 \times 10^3$ parasitized cells for probes detecting rRNA (30). This is sufficient to detect the primary infection, but the parasitemia is not detected on some days after the acute infection (30).

The fluctuations in parasitemia during the carrier state are poorly understood because of lack of sufficiently sensitive methods to monitor them. Thus, in an effort to improve detection, recent approaches have employed in vitro target amplification. Reports from Figueroa et al. (9) and Fahrimal et al. (8) indicate that the limits of detection may be reduced to approximately 10 infected cells per ml of blood by PCR amplification. However, neither of these diagnostic methods has been formulated into a diagnostic test and evaluated for test sensitivity and specificity as a function of time postinfection. In the present study, we have developed PCR-based diagnostic tests which target the small-subunit rRNA (SSrRNA) genes of *Babesia bovis* and also have similar lower limits of detection. Here, the test procedures are defined, and test sensitivity and specificity are measured in experimentally infected animals. These results are compared with those of the complement fixation (CF) test, the official test used to screen cattle imported into the United States. In addition to evaluating these critical test parameters, experimental conditions have been controlled sufficiently to make possible estimating for the first time the parasitemias of *B. bovis*-infected cattle over a time course of as long as 10 months postinfection.

MATERIALS AND METHODS

In vitro cultivation of *B. bovis*. Clone MO7 of the Mexican isolate of *B. bovis* was continuously cultured in fresh bovine RBCs by using a modified microaerophilous stationary-phase culturing system (34). Parasitemia was monitored by light-microscopic examination of stained blood smears (LeukoStat; Fisher Scientific). Cultures were harvested when the parasitemia was equal to approximately 5%.

Cloning, sequencing, and analysis of an SSrRNA gene from *B. bovis*. DNA for cloning and Southern blot analysis was extracted from *B. bovis* by the sodium dodecyl sulfate (SDS)-proteinase K method essentially as described elsewhere (31). The DNA encoding the SSrRNA of *B. bovis* (Mexican isolate [MO7]) was amplified with *Taq* DNA polymerase from genomic DNA with primers derived from consensus sequences of SSrRNA, UP1 and UP22, which were previously described (33). The amplified SSrRNA gene fragment was cloned into the pCR1000 vector from the TA Cloning system (Invitrogen Corp., San Diego, Calif.). Sequencing in both strands was accomplished by the dideoxynucleotide chain termination method described by Sanger et al. (32) with the set of oligo-

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TABLE 1. Oligonucleotide primers and probes used

Name	Sequence	Position (nt)[a]	Length (bp)
JD111[b]	5′-CCAAAGTCAACCAACGGTACGACAGGGTCA-3′	699–670	30
JD127[b]	5′-TTGGCATGGGGGCGACCTTCACCCTCGCCC-3′	428–457	30
JD120[b]	5′-ACGAGGCGTCCCGGCGAGGAAAGGAC-3′	601–576	26
JD311[b]	5′-GCAGGTTTCGCCTGTATAATTGAGC-3′	629–653	25
JD256[c]	5′-GGGCGAGGGTGAAGGTCGCCCCCATGCCAAGATAGAGTTCGTGTC-′3	NA[d]	45
JD257[c]	5′-TGACCCTGTCGTACCGTTGGTTGACTTTGGGGTTATCGAAATCAG-′3	NA	45

[a] Positions are for the *B. bovis* SSrRNA gene (GenBank accession no. L31922).
[b] *B. bovis*-specific oligonucleotide.
[c] Primer for amplification of the IC fragment prepared from lambda bacteriophage DNA in the test for *B. bovis.*
[d] NA, not applicable.

nucleotide primers described elsewhere (33). Computer-aided analysis of gene sequences was performed with the Genetics Computer Group suite of programs, version 7 (Genetics Computer Group, Inc., Madison, Wis.).

Sample preparation. RBC pellets were prepared from 20 ml of blood by centrifugation and removal of the plasma and buffy coat and were washed once in phosphate-buffered saline (PBS). RBC pellets equivalent to 2 ml of blood were frozen until used. The RBCs were lysed by two rapid freeze-thaw cycles, and the parasites and/or cell debris was washed twice with 1 ml of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (pH 8.0) (TE buffer) and then centrifuged at 10,500 × *g* for 5 min. DNA was extracted from the final pellet essentially as described by Boom et al. (2) and was eluted in 110 μl of TE buffer. The eluant containing the DNA was transferred to a clean 1.5-ml centrifuge tube and stored at −70°C until used.

PCR amplification for diagnosis. PCR tests for diagnosis were performed on 10 μl of DNA samples prepared as described above (the equivalent of ~0.2 ml of blood). The DNA sample was placed in a mixture containing 50 pmol each of species-specific upper (JD127) and lower (JD111) PCR primers (Table 1), 40 nmol of deoxynucleoside triphosphate (dNTP) mix (10 nmol each), 1 μl internal control DNA (IC), 1.5 U of *Taq* polymerase, and PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.5], 1.5 mM $MgCl_2$, 0.001% gelatin) in a final reaction volume of 50 μl. The IC was prepared as a 1:1 dilution in TE buffer of the PCR products from a 100-μl reaction mixture containing 10 ng of lambda bacteriophage DNA, 100 pmol of primers (JD256 and JD257 [Table 1]), and 40 nmol of dNTPs in the PCR buffer. The parameters for thermocycling following an initial 10 min at 92°C were 92°C for 1 min, 60°C for 3 min, and 72°C for 3 min, which was repeated 35 times; after the last cycle, the mix was held at 72°C for 10 min. Ten microliters of the PCR products was analyzed in duplicate on a 1.5% agarose gel (31) in buffer containing ethidium bromide (1 μg/ml), electrophoresed for 1 h at 250 V, and then photographed under UV light. The DNA was transferred by blotting (31) onto a nylon membrane and was UV light cross-linked (1,200 μJ).

Probe preparation and blot hybridization. DNA fragments were labeled with [α-^{32}P]dATP by random-primer labeling (31). Oligonucleotide JD120 (Table 1) was end labeled with [γ-^{32}P]ATP by using T4 kinase as described elsewhere (31). Nonradioactive (NR) probes were prepared by conjugating alkaline phosphatase to oligonucleotide JD120 with the LightSmith I kit (Promega Corp., Madison, Wis.) according to the manufacturer's instructions (4) (NR-PCR tests).

Blots prepared for use with radiolabeled probe were prehybridized and hybridized essentially as described elsewhere (30), except that 1 μg of nonspecific oligonucleotides per ml was added to reduce background. Probe was added at 4×10^6 cpm/ml and was hybridized at 60°C for 2 h. The wash conditions were as follows: two 10-min washes and one 30-min wash in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0])–0.1% SDS at 55°C and a final 1-min wash at 60°C in 2× SSC and 0.1% SDS. Autoradiograms were prepared by exposing the blot to X-ray (XR) film MP (Amersham Life Sciences, Arlington Heights, Ill.) for 1 h at −70°C in a cassette containing an intensifying screen (XR-PCR test). The blot was subsequently placed into a PhosphorImager (PI) cassette overnight to generate an image of the blot (PI-PCR test) and to quantify the radioactivity in each band essentially as described by the manufacturer (Molecular Dynamics, Sunnyvale, Calif.).

NR detection was performed according to the protocol of the manufacturer (Promega) with the following minor modifications. The blot was blocked by incubation for 1 h at 50°C in 10 ml of the blocking buffer. The blocking buffer was replaced with 10 ml of preheated Quantum Yield low-stringency hybridization buffer containing 500 fmol of probe per ml and this was hybridized at 50°C for 45 min. Washing and detection were performed as described by the manufacturer, except that the blots were washed one additional time to reduce background, and the chemiluminescent substrate was used at 0.125 mM. Blots were used to expose XR film MP for 30 min at 37°C.

Standards and contamination controls. Cultured parasites were counted by determining the percent infected erythrocytes and the number of erythrocytes per ml of in vitro culture. The culture was then serially diluted in whole bovine blood to yield sets of samples containing a range from 20 to 200,000 parasitized cells in 2 ml. The blood was processed, and DNA was extracted as for diagnostic assays to establish the limits of detection for the tests. To control for cross-contamination during extraction, 2 ml of known uninfected bovine blood was also extracted with each set. As a positive control for the extraction solutions, 1 ml each of culture containing ~5×10^7 parasites was also extracted with the test samples. A PCR-negative control was included to control for the PCR reagents, which contained all components of the PCR mixture except template. Reaction tubes were closed in the room in which the reaction mixtures were prepared and were only opened after amplification. When the amplified IC band was not visible after electrophoresis, the test was considered invalid. Contamination by cloned SSrRNA or amplicons was controlled by performing reaction setup, sample extraction, and amplification-analysis in three separate laboratories. Screw-cap tubes were used to prevent splashes or aerosolization from occurring when the tubes were opened. Solutions used for PCRs and DNA extraction were aliquoted into small volumes, used only once, and then discarded. When disposal of aliquots was impractical because of costs, they were used a maximum of three times. Aerosol-resistant pipette tips were used in all stages of the test, except during the post-PCR stages.

CF tests. Serum samples were analyzed by the CF tests performed at the National Veterinary Services Laboratories, Animal and Plant Health Inspection Services, U.S. Department of Agriculture, Ames, Iowa, essentially as described by Mahoney (18–20).

Experimental infection of cattle. Six Holstein-Friesian steers ranging from 4 to 6 months of age were purchased and kept in a tick-free environment at the National Veterinary Services Laboratories, Animal and Plant Health Inspection Services, U.S. Department of Agriculture. The steers were divided into two groups. Group 1 consisted of three steers (nos. 226, 233, and 234), which were infected intravenously with 5×10^7 *B. bovis*-infected RBCs obtained from a splenectomized calf infected with the MO7 clone of the Mexico isolate (13). Group 2 consisted of three steers (nos. 230, 231, and 232) maintained as uninfected controls. Blood was taken from each steer in group 1, starting 113 days postinfection (dpi) and on a schedule of 5 days per week from days 130 to 302. Twenty milliliters of blood was collected into evacuated tubes containing ethylene diamine tetraacetic acid and was centrifuged at 9,000 × *g* for 15 min, the plasma and buffy coat were removed, and the blood was washed in PBS. The cell pellet was transferred to 2-ml screw-cap tubes, frozen (−20°C), and shipped weekly to the University of Florida for analysis. In addition, 10 ml of blood was collected in siliconized red-top tubes for serum used in the CF test. Rectal temperatures and packed-cell volumes of whole blood were recorded daily. To prevent detection bias, samples were labeled with a set of randomly generated numbers. The carrier state was confirmed in each steer of group 1 by using 200 ml of packed RBCs which was resuspended in 400 ml of PBS and subinoculated intravenously into splenectomized calves 11 months postinfection. The splenectomized calves were monitored daily for signs of infection by noting rectal temperatures and examining Giemsa-stained blood smears until parasites were identified microscopically.

Three additional four-month-old Holstein-Friesian steers were added to the study, forming group 3. Steers in group 3 (nos. 1105, 1108, and 1116) were infected with 5×10^7 *B. bovis*-infected RBCs obtained from a splenectomized calf infected with clone MO7 as described for group 1. Each was observed for signs of infection, and blood samples were taken according to the routine described above, except that sampling was begun 2 dpi and continued for 5 months. The carrier states of all steers in groups 1 and 3 were confirmed by subinoculation. At 129 dpi, 200 ml of packed RBCs from each steer was subinoculated into splenectomized calves to verify infection status. Steers in groups 3 were treated by subcutaneous injection of imidocarb dipropionate (imizol; 3 mg/kg of body weight) 145 dpi to effect a cure. Treated animals were observed for an additional month, and the success of treatment was verified by subinoculating 200 ml of packed RBCs from each steer in group 3 into splenectomized calves which remained asymptomatic for at least 30 days.

Schedule of testing and controls. Tests were performed on sample sets from at least 2 days of each week over the time courses indicated. Samples from all animals in all groups gave negative test results on all PCR and CF tests prior to experimental infection. Two samples from known uninfected controls were processed with each group of test samples to control for cross-contamination during DNA extraction and to control for test specificity. Samples from in vitro culture

with parasitemias of ~5% were processed as positive controls for DNA extraction.

Estimation of parasitemia in carrier animals. DNA extracted from a set of standards containing 10 to 100,000 parasites per ml of blood was amplified with every test run, blotted, and hybridized with a ^{32}P-labeled probe along with unknown samples. After exposure to XR film (XR-PCR results), the blots were placed in a PI cassette overnight. The PI was used to quantitate the radioactive signal from each band. Standard curves were generated from the relative counts for the *B. bovis* standards by using a combined log-linear and spline regression model in the SAS System (12, 15). Standard curves were generated separately for each experiment to control for variability between experiments. Parasitemias in experimentally infected animals were estimated with the coefficients generated from the standard curves and were plotted against time postinfection.

Determining the sensitivity and specificity of the diagnostic tests. The study was unblinded, and each steer was matched with its respective PCR and CF test results and with packed-cell-volume and rectal-temperature readings. The results were analyzed to estimate the sensitivity and specificity of each test (22) and to calculate the kappa coefficients (16). Specificity values were calculated relative to uninfected controls. Confidence limits (95%) were calculated by the Bonferroni method as described elsewhere (23). Tests were examined for independence by Fisher's exact test (11).

Nucleotide sequence accession number. The sequence reported here was deposited in GenBank under accession no. L31922.

RESULTS

Detection of *B. bovis* DNA by PCR. A 1.65-kb fragment of the SSrRNA gene(s) was amplified from *B. bovis* DNA by using consensus SSrRNA primers (33). This fragment was subsequently cloned, sequenced, and compared with the sequence of *Babesia bigemina* SSrRNA gene A (29). Species-specific oligonucleotide probes and PCR primers suitable for specifically amplifying *B. bovis* were identified from the sequence alignment (Fig. 1; Table 1). DNA extracted from parasites serially diluted in bovine blood were amplified with the species-specific primers to establish the limits of detection by PCR amplification of the SSrRNA genes. PCR products were analyzed by agarose gel electrophoresis as shown in Fig. 2, in which a 560-bp fragment from the IC and a 275-bp fragment amplified from *B. bovis* were present. Southern blot analysis of the amplified DNA from blood samples containing as few as 10 *B. bovis* parasitized erythrocytes per ml (10^{-7}% parasitemia) gave detectable signals with the respective species-specific probes by any one of three detection methods (Fig. 2B through D). Hybridization signals from ^{32}P-labeled probes were detected by using XR film and a PI (Fig. 2B and C). Signals from NR probes were detected with a chemiluminescent substrate and XR film (Fig. 2D).

The reliability of detection at various parasitemias was evaluated by a large number of test results from serially diluted cultured parasites. DNAs from sets of serially diluted samples containing from 10 to 100,000 infected RBCs per ml were extracted on 12 separate occasions and PCR amplified in 31 different experiments (nearly 200 independent PCRs). At lower parasitemias, the reliability of detection decreased, as shown in Fig. 3. The frequency of a positive test result was plotted as a function of the percent parasitemia, where results were scored as either positive or negative. At parasitemias of less than 10^{-4}%, the frequency of detection decreased, such that at a parasitemia of 10^{-6}% (100 parasites per ml), the current tests detect the parasitemia ~50% of the time, whereas at 10^{-7}% (10 parasites per ml), the value is ~15%. Since the DNA from the equivalent of only 0.2 ml of blood is used in the PCR, only an average of 2 infected cell equivalents of DNA could be expected in the PCR assay from a sample with a 10^{-7}% parasitemia. Testing by the silica particle extraction method indicates that the recovery is reproducibly approximately 50% that of the SDS-proteinase K method (31) (data not shown). Thus, the number of equivalents of parasitized erythrocytes placed in the PCR must on average be 1 or fewer from a sample at 10^{-7}% parasitemia.

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B. bovis
          428
L31922    TTGGcATGggGGcGAccTtCAcCCTCgCCcGAGTACCcATTGGAGGGCAA
L19077    ..................................................
L19078    ..................................................
M87566    .......-..........................................

          478
L31922    GTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAA
L19077    ..................................................
L19078    ..................................................
M87566    ..................................................

          528
L31922    CTTGTTGCAGTTAAAAAGCTCGTAGTTGTACTTCA---------cgTccC
L19077    ............................a.Tc...---------.....g
L19078    ............................a.Tc...---------.....g
M87566    ................................t.---------T.....

          569
L31922    CCgcTTGgTcctTtccTcgcCgGGacgccTcgTTACTTTGAGAAAATTAG
L19077    ..................................................
L19078    g.-..a.Tgtt..C.acTa.gc............................
M87566    g.cT.a.Tg.t.cCa..TtG....C.........................

          619
L31922    AGTGTTTCAAGCAG--gTTTcgCcTGtATAaTTgAGCATGGAATAAcctt
L19077    ..............--..................................
L19078    ..............--..................................
M87566    ..............--..................................

          667
L31922    GTAtGA-CCcTGtcgtaccgTTG-----gTTGActtTgG
L19077    ......-.............-----....g......
L19078    ......-.............-----....g......
M87566    ......C.............-----....g......
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FIG. 1. Alignment of SSrRNA genes over the segment amplified for the *B. bovis* test. Sequences L19077 and L19078 are derived from the S vaccine isolate, M87566 is from the Samford isolate, and L31922 is the sequence reported here from the cloned Mexico isolate, MO7. Multiple sequence alignments, including *B. bigemina* SSrRNA sequence X59604 (not shown), were performed. Capital letters, the base at the position is present in both *B. bovis* and *B. bigemina* SSrRNAs; dashes, gaps introduced to preserve alignment with the *B. bigemina* sequence; periods, positions conserved between L31922 and the other *B. bovis* sequences (sequence differences are presented as the base present at that position). Sequence numbering is from L31922. Italics, PCR primers; underlining, the detection probes JD120 and JD311.

Applicability of the test to diagnosis. The PCR primer pairs and probes described also detected parasite populations from other areas of North America, including the U.S. Virgin Islands (our unpublished data). The PCR primers and probes were specific, and the tests did not detect nanogram amounts of purified DNA from other hemoparasites of cattle, namely, *B. bigemina*, *Anaplasma marginale*, *Trypanosoma brucei brucei*, and *Cowdria ruminantium* (data not shown). In addition, comparison with the SSrRNA sequences of *Theileria annulata* (GenBank accession nos. M34845 and M64243), *Theileria parva* (GenBank accession no. L02366), and *Theileria taurotragi* (GenBank accession no. L19082) indicate that these species would neither be amplified by the PCR primers nor hybridize with either probe JD120 or JD311 (see below). The test as designed is highly specific for detecting only *B. bovis*; thus, it is unlikely to give false-positive results because of the presence of a commensal parasite.

While this research was under way, three other *B. bovis* SSrRNA sequences were published. One was from the Samford isolate obtained in Australia (GenBank accession no. M87566) (7), and two sequences were from the S stock, a vaccine strain of South Africa (GenBank accession nos. L19077 and L19078) (1). A comparison of these three *B. bovis* sequences with the sequence reported here from the Mexico isolate indicated that the sequence of the Mexico isolate differs

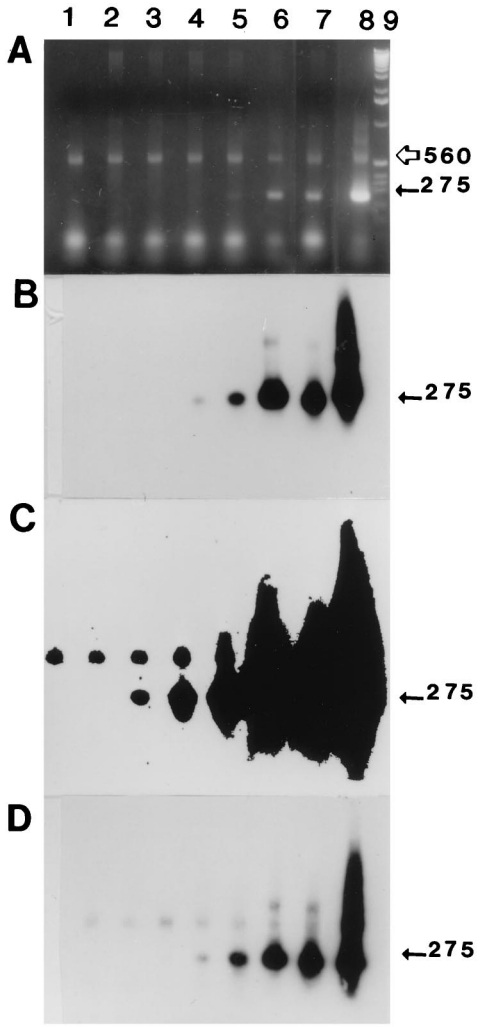

FIG. 2. PCR-amplified serial dilutions of cultured *B. bovis* in bovine blood. (A) Ethidium bromide-stained gel. Lanes: 1, PCR-negative control; 2, uninfected bovine blood; 3, 10 parasites per ml; 4, 100 parasites per ml; 5, 1,000 parasites per ml; 6, 10,000 parasites per ml; 7, 100,000 parasites per ml; 8, positive control; and 9, size standards. Open arrow, PCR IC; filled arrows, PCR-amplified *B. bovis* SSrRNA gene fragment. (B) Autoradiogram of a Southern blot of panel A hybridized with ^{32}P-labeled probe; (C) PI display of the blot from panel B; (D) Film exposed by chemiluminescence from Southern blot of panel A probed with NR probes.

from that of the Samford isolate at 66 positions, from one S stock sequence (L19077) at 14 positions, and from the other S stock sequence (L19078) at 26 positions. Some of the differences among the four *B. bovis* sequences occur in the variable region selected for designing the species-specific probe, JD120, in this study. The variability among SSrRNA genes within the species may be due either to strain differences or to the presence of sequence heterogeneity among the different SSrRNA genes in the *B. bovis* genome. In our previous studies (29), we have demonstrated the microheterogeneity in the SSrRNA coding regions of the three SSrRNA genes of *B. bigemina.*

The detection probe for *B. bovis*, JD120, spans a portion of the region in which the sequence differences were concentrated (Table 1; Fig. 1). As a result, the data indicate that JD120 hybridizes to the Mexico isolate and is expected to hybridize to one of the S stock sequences (L19077), but not to the other (L19078) or to the Samford isolate. If these differing SSrRNA gene sequences are due to differences among gene copies in a single genome, the probe JD120 would be expected to hybridize to only a subset of those genes. To explore this, JD120 was hybridized to blots of *Eco*RI-digested genomic DNA from the *B. bovis* MO7 clone of the Mexico isolate, and all three gene copies were equally hybridized, indicating that the sequence differences between genes of the same cloned isolate are minor or nonexistent, at least in this variable region (data not shown). This suggests that the observed differences are more likely due to strain variation.

To overcome this potential problem, a second detection probe, JD311, that differs from *B. bigemina* but is conserved among all four reported *B. bovis* sequences (Table 1; Fig. 1) was selected. This probe detects all three SSrRNA genes from strain MO7 and is equivalent to JD120 in detecting the SSrRNA gene fragment amplified from small numbers of parasites for the test. Although the four *B. bovis* SSrRNA sequences reported come from diverse parts of the world, it remains to be determined whether the SSrRNA sequences of all *B. bovis* strains are constant in the regions that have been employed in developing this test. As with any new test, some caution must be exercised in interpreting negative test results until *B. bovis* isolates from the geographical region of interest have been tested.

Sensitivity and specificity of the diagnostic tests. Test results were judged positive when a 275-bp fragment which hybridized to probe JD120 was produced. Negative test results were considered valid only when the amplified 560-bp fragment from the IC was visible (Fig. 2A). CF tests were performed at the National Veterinary Services Laboratories with serum samples paired with samples tested by PCR. Results from all of the tests were obtained for each sample, were interpreted as positive or negative, and were recorded by sample number and date of sample collection. The identity of each sample was revealed, and the sensitivity and specificity of each of the PCR-based and CF tests over the full set of samples were analyzed. These results are summarized in Table 2.

The PCR-based tests had sensitivities ranging from 58 to 70% and specificities of 96 to 98%. In contrast, the sensitivity of the CF test was only 6%, although the specificity as judged by analyzing uninfected animals was 100%. These data indicate

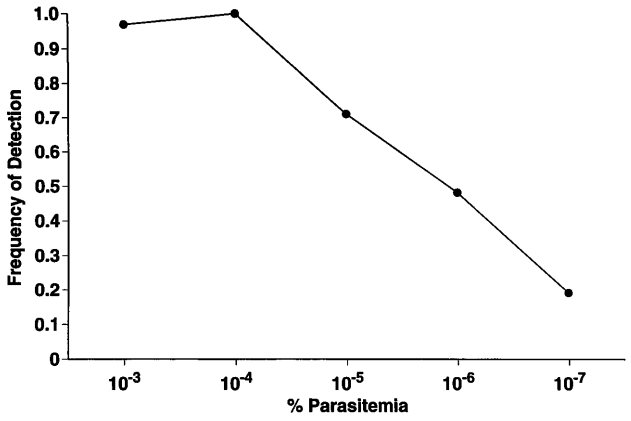

FIG. 3. Reliability of detection at various parasitemias. Sets of five serially diluted blood samples containing decreasing numbers of parasitized RBCs ranging on average from 10 to 100,000 cells per ml (parasitemia, 10^{-7} to 10^{-3}%) were tested to determine the lower limits of detection with the radioactive probes and autoradiography. The results were scored as positive or negative relative to uninfected blood, and the frequencies with which each parasitemia was detected are plotted.

TABLE 2. Sensitivity and specificity of diagnostic tests for *B. bovis*

Test (range of dpi)	Sensitivity (%) (95% CL[b])	Specificity (%) (95% CL[b])	PPV[a]	NPV[a]
NR-PCR (1–300)	58 (53–64)	97 (93–99)	95	70
XR-PCR (1–300)	65 (60–71)	98 (95–99)	97	74
PI-PCR (1–300)	70 (65–75)	96 (91–98)	95	76
CF (1–300)	6 (4–10)	100 (98–100)	100	52

[a] The positive predictive value (PPV) and negative predictive value (NPV) apply only to experiments in which the prevalence of infection was 50%. They will vary depending on the prevalence of the disease in the sample population.
[b] CL, confidence limits.

that the CF test rarely detected infection with *B. bovis* under these experimental conditions. Complement-fixing antibodies were first observed in steer 1105 of group 3 on dpi 46. Steer 1105 had 18 additional positive CF test results, whereas steer 1108 had only 3 positive results and steer 1116 had only 1 positive result (Fig. 4). Positive CF test results were also rare from group 1 samples, and none were detected at time points of greater than 6 months postinfection (Fig. 5). None of the CF

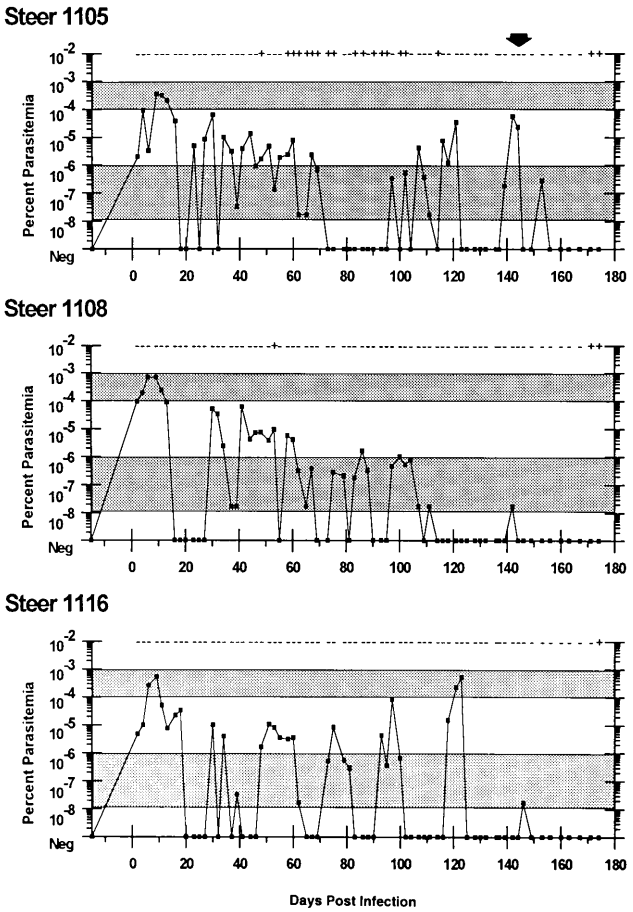

FIG. 4. Plot of percent parasitemia and CF test results over time for *B. bovis*-infected steers of group 3 during early infection; narrow and wide grey bands, lower limits of light microscopy and the PCR-based tests, respectively. Parasitemias of less than 10^{-6}% have less than a 50% probability of being detected by the PCR-based tests. + and −, CF test results for each sample analyzed (above the plot of the PCR results). CF values of 2 or higher were considered positive and reflected a 2+ or higher reaction at a 1:5 dilution of serum. Arrow, the day on which the steers were treated with imizol; ■, percent parasitemia by PCR.

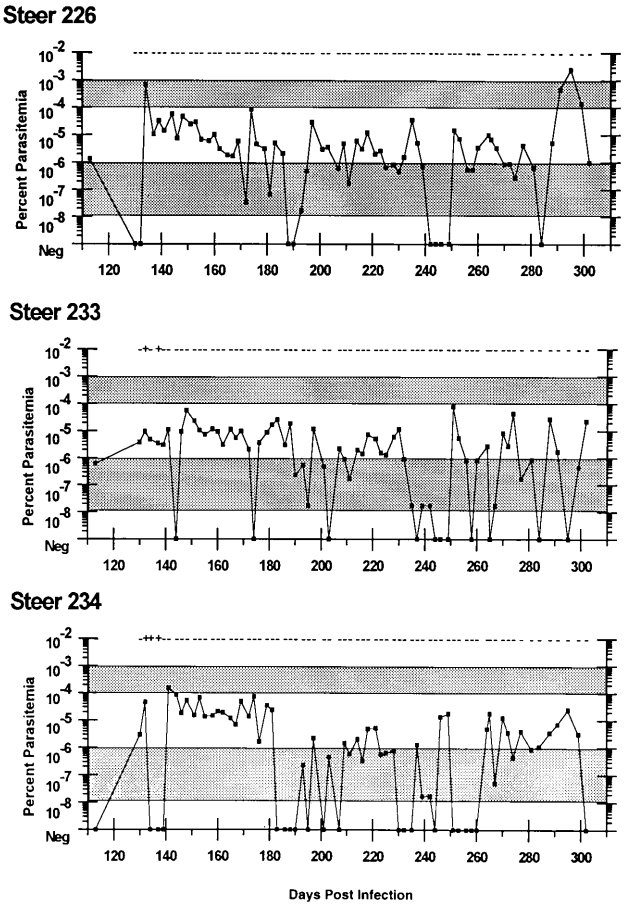

FIG. 5. Plot of percent parasitemia and CF test results over time for *B. bovis*-infected steers of group 1 during chronic infection. See legend to Fig. 4 for symbols.

tests performed on steer 226 were positive, and positive test results were obtained only twice for steer 233 and three times for steer 234, with all three of these animals being tested more than 60 times each.

The distribution of false-positive results appeared to be random. Uninfected group 2 controls tested 70 times each during the 6-month time period (a total of 210 times) with the most sensitive PCR-based test for *B. bovis* infection resulted in nine false-positive reactions (two for steer 230, six for steer 231, and one for steer 232). The results for the other two methods of detection were similar.

Fisher's exact tests indicated that the three PCR-based tests were not independent of each other for *B. bovis* infections (data not shown). Thus, an animal that is test positive by one test is likely to be test positive by the other two tests. The kappa coefficients for the PCR tests were high, ranging from 76 to 88% (Table 3). The kappa coefficients for the PCR tests and the CF test ranged from 1 to 3% (Table 3). Thus, the results from the CF test for *B. bovis* were independent of results from the PCR tests.

Testing was conducted for 30 days post-drug treatment of animals in group 3 to follow for the loss of parasites from circulation and to compare the specificities of the PCR-based tests with that of the CF test under this experimental situation. Positive test results by PI-PCR were obtained from two group 3 animals on day 1 post-drug treatment. Only one other ap-

TABLE 3. Kappa coefficients for tests of *B. bovis*

Tests compared[a]	Kappa coefficient (%)
NR-PCR vs XR-PCR	80
NR-PCR vs PI-PCR	76
NR-PCR vs CF	2
XR-PCR vs PI-PCR	88
XR-PCR vs CF	3
PI-PCR vs CF	1

[a] Range of dpi for all tests, 1 to 300.

parently false-positive result was obtained in the 27 runs of each of the PCR-based tests scattered over the remainder of the 30-day period after drug treatment. In contrast, 19% of the CF tests were positive during this period.

Test performance as a function of the stage of infection. The natural history of the infection process and the apparent decrease in sensitivity of the PCR-based tests during the period following the acute phase prompted examination of test sensitivity as a function of time postinfection. This parameter is presented in Fig. 6 as a function of time postinfection for each of the PCR-based tests and the CF test for each group of animals. The experimentally determined sensitivity values and the upper 95% confidence limits are plotted in 30-day periods postinfection for each test. Acute infection began 2 days after subinoculation in the steers of group 3 and lasted for 8 to 14 days, manifesting with an increase in rectal temperature (up to 2°C) and a modest decrease in packed-cell volume (up to 12 points). During this acute stage of infection in group 3 animals, the sensitivity was 100% among the PCR-based tests. However, when averaged over the first 30 days of infection, the values dropped to 67 to 69%. During the period following the acute phase, the sensitivity increased during the second 30-day period and then decreased steadily, resulting in sensitivity values for 1 to 144 dpi ranging from 45 to 55%. In group 1 animals, the sensitivity of the tests in detecting the asymptomatic carrier phase was higher during the period 130 to 302 dpi, with values of 70 to 84%. Furthermore, the sensitivity values for each 30-day period fluctuated little; thus, sensitivity was relatively constant over this period. Specificity was also examined as a function of time postinfection and was consistently high for all *B. bovis* tests over the full range of samples analyzed (data not shown).

Estimation of parasitemia in carrier animals by PCR. Parasitemias of *B. bovis* infections in the animals of groups 3 and 1 were estimated over periods of from 2 to 144 and from 130 to 302 dpi, respectively. Parasites were detected in samples from group 3 animals on day 2 following infection at parasitemias averaging $\sim 10^{-5}$% among the three animals (Fig. 4). The initial parasitemia in each animal peaked between days 6 and 10 at $\sim 10^{-3}$% and by day 20 had decreased to undetectable levels. In each case, parasitemias returned again to detectable levels a few days later in undulating waves of parasitemia. Some waves appeared to be symmetrical peaks, spiking with a periodicity of approximately every 20 to 30 days, as for steer 1116. For others, some periodicity, but with multiple smaller peaks overlapping to give broader, less-defined peaks, was evident. Recrudescence in only one case among group 3 animals (steer 1116 at 122 dpi) resulted in a parasitemia as high as that of the initial parasitemia (Fig. 4). The maximum parasitemia observed among all infected animals (3×10^{-3}%) was seen in chronically infected steer 226 during recrudescence between 280 and 302 dpi (Fig. 5). During the 4 months following the acute phase of infection (Fig. 4), parasitemia levels decreased below PCR limits of detection more often and for longer periods of time than during a period from 5 to 10 months postinfection (Fig. 5). The lengths of these periods ranged to as long as 30 days (steer 1108 between 111 and 142 dpi with 12 samples tested) in early infection (also, steer 1105 between 69 and 97 dpi with 12 samples tested). Among group 1 animals examined between 130 and 302 dpi, the maximum period during which the parasitemia was not detected was only 14 days (steer 234 on 249 to 264 dpi with five samples tested [Fig. 5]).

DISCUSSION

Infections with *Babesia* spp. in bovines have only rarely been examined over a long time course because of the difficulty in detecting the low parasitemias of the postacute, carrier-stage animal by traditional methods (21, 35). PCR-based methods described here allow parasitemias of as low as 10^{-6} to 10^{-7}% to be identified with a defined frequency. These limits of detection are similar to reports from Figueroa et al. (9) and Fahrimal et al. (8) for *B. bigemina* and *B. bovis*, respectively, using other target DNA sequences. At very low parasitemias, the reliability of our test decreased in a predictable fashion which has not previously been reported in descriptions of these PCR-based tests for *Babesia* infections. Analyses of test reliability at these low parasitemias has allowed us to better in-

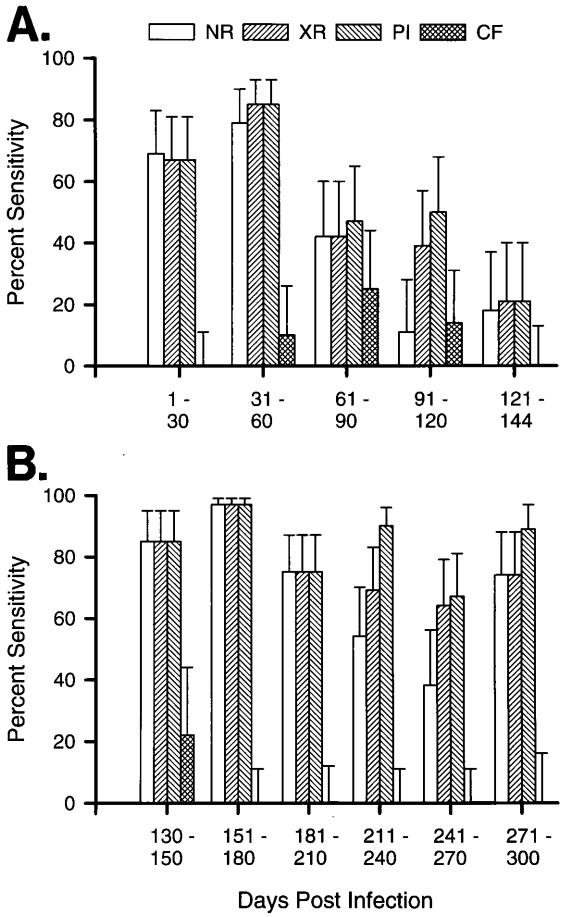

FIG. 6. Test sensitivity as a function of days postinfection. (A) *B. bovis*-infected steers of group 3; (B) *B. bovis*-infected steers of group 1.

terpret the fluctuations in parasitemias that we observed over the time course of infection. The results described here provide a first approximation of the fluctuations in parasitemia which occur during the chronic phase of a *B. bovis* infection and approach defining the limits of detection required for any reliable direct test for this infection.

The acute parasitemias in the *B. bovis* infections examined here lasted only 1 to 2 weeks and peaked at 10^{-3}%. Only the occasional recrudescent parasitemia approached or exceeded 10^{-4}%. Fluctuations in parasitemia varied over the time course, differing between early (<150 dpi) and late (>150 dpi) infection, and detection of the carrier animal infected with *B. bovis* was not uniform over the time course as a result of low parasitemias. During early infection, parasitemia levels fell below PCR limits of detection for periods ranging to as long as 30 days. In late infection, these periods were shorter, only as long as 14 days. Thus, it appears that during the early postacute phase, the host is efficient at suppressing the circulating *B. bovis* parasitemia to levels undetected by the PCR tests. Failure to detect the parasitemia is due either to parasitemias of less than 10^{-7}% or to test failure at a higher parasitemia in the range of 10^{-5} to 10^{-7}%. The probability of detecting parasitemias in this range decreases in a log-linear fashion to 0.15 at 10^{-7}%. The significant probability of a negative test when the parasitemia is very low is likely responsible for many of the single-point negative test results scattered through the curves presented in Fig. 3, rather than the actual parasitemia decreasing to less than 10^{-7}%.

Late in infection, the parasites appear in less-defined waves of parasitemia at a higher minimal parasitemia. This may be due to the host immune response being able to partially block the binding of the parasite to the endothelial cells, resulting in a larger proportion of the parasites appearing in circulation. These results may also be due to differences among experimental groups, since the infections were initiated at different times and the parasites used to infect the two groups were passaged through different splenectomized donor calves immediately prior to infection. A small difference in the average parasitemia could affect the sensitivity of the test, since much of the time the parasitemia hovers at 10^{-6} to 10^{-7}%, near the limits of detection.

The sensitivity and specificity of PCR-based tests for *B. bovis* infections were compared with those of the official CF tests during acute and postacute carrier stages of the infection under experimental conditions which may be more challenging than detecting naturally infected cattle. This is due to use of a cloned isolate which has a genetic potential smaller than that of a mixed strain infection and to the fact that the animals were challenged with the parasite only once. The PCR-based tests are superior in sensitivity to the official CF test under these conditions. Our data indicate that the sensitivity of the PCR-based tests is also a function of the parasitemia in the infection. For these reasons, the sensitivity values must be viewed as minimal values when examining samples from naturally infected cattle. Thus, the tests should also be evaluated with samples from naturally infected cattle over a time course of infection to appreciate the full potential of these new tests.

The PCR-based tests compared here differ only in the method of detecting the amplified rRNA gene (rDNA) fragment. PI-PCR has the highest sensitivity, as judged by our comparative tests, but it is subject to bias introduced by observer variability. It also requires radioisotopes, a longer time for analysis, and the use of expensive equipment. XR-PCR was second in sensitivity, but NR-PCR, which employs chemiluminescence detection on XR film, was the simplest and fastest method of detection. The small loss in sensitivity from using the NR-PCR test may be overcome by redefining the test to require testing at two or more time points. Given the nature of the test, it is possible to significantly increase test sensitivity by retesting animals giving negative results with a blood sample drawn 10 to 14 days later. The sensitivity of the NR-PCR test for *B. bovis* would increase to >80% over the entire time course of the infection and to >90% for late-stage infections.

The frequency of false-positive PCR test results was only 4% but was higher than expected. Possible reasons are recombinant DNA and amplicon DNA contamination of the laboratory environment prior to and during development of the PCR test and human error in sample handling. Stringent contamination control (e.g., isopsoralen sterilization or use of uracil-*n*-glycosylase) should be implemented when assays are established in a veterinary diagnostic laboratory.

The CF test for *B. bovis* had a sensitivity of only 6%; thus, it did not reliably detect *B. bovis* infections. Furthermore, samples from only one of the six infected steers provided two-thirds of the positive samples. Our CF results differ from the observations made by Mahoney (19, 20), in which after a single infection with *B. bovis*, animals produced complement-fixing antibodies that were still detectable in 50% of the animals 12 months postinfection for *B. bovis.*

The United States currently imports cattle to the mainland from its possessions and from other countries where infections with *Babesia* spp. are common. Testing procedures with high sensitivity are needed to prevent introduction of infected cattle. Negative predictive value (NPV) is the test parameter which most accurately defines the suitability of a diagnostic test for preventing introduction of infected cattle into the United States. The CF test is currently used, which for *B. bovis* has a NPV of only 52% under the conditions of our experiment with the prevalence of infection at 50%. The PCR-based tests have NPVs of 70 to 76% for a single determination, and this would increase to 84% by testing a second time after a 10- to 14-day interval. The PCR-based tests evaluated here have suitable sensitivity and specificity for discriminating between infected and uninfected animals during importation and exportation.

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