JOURNAL OF CLINICAL MICROBIOLOGY, July 1992, p. 1811–1814
0095-1137/92/071811-04$02.00/0

Vol. 30, No. 7

rRNA-Based Method for Sensitive Detection of *Babesia bigemina* in Bovine Blood†

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Received 18 November 1991/Accepted 14 April 1992

Three synthetic oligonucleotide probes complementary to unique regions of *Babesia bigemina* small-subunit rRNA were developed for detecting the parasite in bovine blood. These probes specifically detected a parasitemia of 2×10^{-5}% by autoradiography in less than 24 h by using a 200-μl sample of bovine blood. These probes did not bind to total RNA or genomic DNA isolated from another closely related species, *Babesia bovis*, or to bovine leukocyte RNA. This method detected *B. bigemina* infections in calves inoculated with as few as 1,000 infected erythrocytes from the second day onward for 16 days.

Babesiosis is caused by tick-borne, protozoan hemoparasites of the genus *Babesia*. Various species of this genus are capable of inducing disease in a wide range of vertebrates (9, 13). *Babesia bigemina* and *Babesia bovis* are responsible for bovine babesiosis in tropical regions of the world (13). On a global basis, over 500 million cattle are estimated to be at risk of babesiosis (13).

Detection of this parasite by microscopy lacks sensitivity, since a relatively large number of parasites (>0.01% parasitemia) must be present to be reliably detected (11). After acute or primary infections, recovered animals may sustain a subclinical infection which is microscopically undetectable. These animals, known as carrier animals, serve as reservoirs for disease transmission. Currently, a carrier animal is identified by subinoculation of 500 to 1,000 ml of its blood into a splenectomized calf (24). The test calf is examined regularly for clinical symptoms of infection, and blood smears are examined for parasites by light microscopy (11). These methods are tedious, time-consuming, and too costly for routine diagnostic use. Recently, DNA probes for *B. bigemina*, *B. bovis*, and *Babesia caballi* have been developed from repetitive genomic DNA sequences (1, 14, 15). The *B. bigemina* probe detects as few as 150 infected erythrocytes in a 1-μl sample of in vitro-cultured parasites diluted in phosphate-buffered saline. Equivalence of this value to parasitemia is not clear, but it is likely comparable to *B. caballi* probes which could detect parasitemias as low as 0.0016% in washed erythrocytes. The *B. bovis* probes, on the other hand, detected only a 0.01% parasitemia. It is clear that these probes are not sufficiently sensitive to detect the carrier state, since the most sensitive probes for *B. caballi* detected the infection on only 7 of 23 days postinfection in an experimentally infected horse (15). Use of the probes described to date would require in vitro amplification of target DNA to detect carriers which appear to have parasitemias less than 0.001% most of the time.

rRNA sequences offer an alternate target for detecting parasites in a host even at very low levels of infection, because rRNA is the most abundant cellular macromolecule. This facilitates the development of sensitive detection assays, in which rRNA based probes can be about 100 times more sensitive than probes based on repetitive DNA (21). rRNA is a conserved and essential constituent of all cellular organisms, but comparative analysis of rRNA sequences reveals some stretches of highly variable, species-specific sequence (3). Species-specific rRNA probes have been developed successfully for several *Plasmodium* spp. (10, 20) and for procaryotes (5, 7, 8, 17, 22, 23).

Recently, we have reported the complete nucleotide sequence of the three small-subunit rRNA units (SSrRNA) of *B. bigemina* (16). In the present study, the utilization of synthetic oligonucleotides selected from unique regions of the SSrRNA for its sensitive detection in bovine blood was investigated.

MATERIALS AND METHODS

In vitro cultivation of *B. bigemina*. A clone of the Mexico isolate prepared by limiting dilution, JG29, was continuously cultivated in vitro in fresh bovine erythrocytes by using a modified microaerophilic stationary-phase culturing system (18). Infected bovine erythrocytes were concentrated by Percoll gradient (19) when the parasitemia reached 3 to 5%.

Extraction of total RNA and slot blot preparation. RNA was isolated by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction as described elsewhere (2). RNA concentrations were determined spectrophotometrically (12). Slot blots of DNA denatured with NaOH and RNA denatured with formaldehyde were prepared by using a manifold apparatus (Gibco-BRL, Gaithersburg, Md.) and nylon membrane as described elsewhere (12).

Synthetic oligonucleotides. Oligonucleotides prepared for this study are listed in Table 1. All oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer at the Interdisciplinary Center for Biotechnology Research DNA Synthesis Core Facility at the University of Florida.

Oligonucleotide hybridization. Oligonucleotides were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and were purified by ethanol precipitation (12). The specific activity of each probe used was in the range of 1,800 to 2,400 Ci/mmol. Hybridizations were performed as follows. Prehybridization was performed for 2 h in a solution containing 6× SSC (standard saline citrate; 1× SSC is 0.15 M NaCl plus

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† University of Florida Agricultural Experiment Stations journal series no. R-02310.

TABLE 1. Synthetic oligonucleotides used in this study and their positions in the SSrRNA sequence of *B. bigemina*

Name	Sequence	Position (−strand)	Calculated T_m (°C)[a]
BbgUS1-1R	5′GTGGTTAACGAACTAATAAACGCCGCC3′	209–183	78
BbgUS2-1R	5′GGAACATCGCAAAAGCGATTCGCAAG3′	258–233	78
BbgUS3-1R	5′GGTACTCTGGTGAGGTTGTACATC3′	501–478	74
BbgUS8-1R	5′GGACAGGACAAACTCGATGGATGC3′	1399–1376	76
JD119	5′AAAAAGCCAGCGAAAAGACCCAAC3′	644–621	73
JD113	5′CTCAAAACCAACAAAATAGAACCAAGGTCC3′	747–718	77
JD115	5′CCGACACGATGCACACTAAAGATTACCCAA3′	1437–1408	79

[a] T_m, melting temperature.

0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 10 mM sodium phosphate (pH 6.8), 10× Denhardt's solution, 1 mM EDTA, and 100 μg of denatured, sonicated salmon sperm DNA per ml. Hybridizations were performed in the same solution for 12 to 16 h by the addition of 1.5 pmol of labeled oligonucleotides and 100 μg of denatured, sonicated salmon sperm DNA per ml. Hybridized filters were washed two times at room temperature for 10 min each time and one time at 5°C less than the hybridization temperature in 6× SSC–0.1% SDS for 20 min. A final stringent wash was performed at the hybridization temperature in 2× SSC–0.1% SDS for 1 min. Autoradiograms were prepared from the membrane by exposing X-OMAT AR film for about 20 h at −70°C with an enhancing screen. Comparable images were also obtained in as little as 2 h by storage phosphor autoradiography (Phosphor Imager; Molecular Dynamics, Inc.).

RESULTS

Selecting the oligonucleotides for DNA diagnostic probes. Recently, we have reported the complete nucleotide sequence of the three SSrRNA units of *B. bigemina* (16). The SSrRNA sequence was compared with available SSrRNA sequences of the most commonly encountered bovine hemoparasites, including *B. bovis* (unpublished data), *Theileria annulata* (M64243, M34845), *Cowdria ruminantium* (X61659), *Anaplasma marginale* (M60313), and *Trypanosoma brucei* (M12676) and with that of a related apicomplexan parasite, *Plasmodium berghei* (M14599), by using programs provided on the University of Wisconsin Genetics Computer Group sequence analysis package (4). The regions unique to *B. bigemina* were identified (data not shown). Oligonucleotide probes were made complementary to seven unique regions shown in Table 1. Their positions, sequences, and calculated melting temperatures are presented in the table.

The sensitivity and specificity of these probes were tested by hybridizing them to slot blots of various amounts of *B. bigemina* total RNA and to bovine and *B. bovis* total RNA controls. *B. bovis* was selected as a negative control, since it is the hemoparasite species most closely related to *B. bigemina* and most likely to confound clinical samples. Blots were hybridized at 63°C with all seven oligonucleotides as described in Materials and Methods (data not shown). These probes specifically identified as little as 20 pg of the *B. bigemina* total RNA and did not cross-react with 100 ng of either bovine or *B. bovis* RNA (i.e., 5,000-times-higher concentration). Since they also showed no significant homology with the rRNA sequences of the other major hemoparasites of cattle or other related protozoans, they may be considered specific for *B. bigemina*.

The hybridization of oligonucleotides to rRNA appears to be affected either by secondary structure or by posttranscriptional modification of the rRNA; thus, some sequences may not be suitable as probes (10). To identify the oligonucleotides that form good DNA-RNA hybrids, all seven probes were labeled and independently hybridized to *B. bigemina* RNA at three different temperatures (55, 60, and 65°C) (not shown). Oligonucleotides 3-1R, 8-1R, and JD115 hybridized in all three temperatures (60°C gave maximum hybridization); JD119 hybridized moderately at 55°C; and oligonucleotides 1-1R, 2-1R, and JD113 showed very poor hybridization. Therefore, 3-1R, 8-1R, and JD115 and 60°C were selected for detecting the parasites in bovine blood.

The specificity and sensitivity of the mixture of these three probes were verified at 60°C (Fig. 1). These probes detected *B. bigemina* RNA at levels as low as 20 pg per slot (i.e., 0.012 fmol) without cross-reacting with as much as 20 fmol of *B. bovis* cloned ribosomal DNA or 100 ng (60 fmol) of total RNA from normal bovine blood. This level of sensitivity was the same as that achieved with all seven oligonucleotides previously.

Detection of *B. bigemina* in bovine blood. *B. bigemina* cultivated in vitro in bovine erythrocytes were counted in thin blood smears by light microscopy following staining by

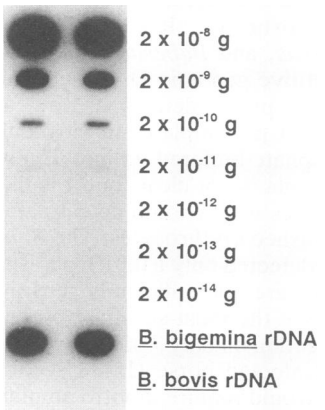

FIG. 1. Slot blot of *B. bigemina* total RNA probed with 3-1R, 8-1R, and JD115. Total RNA from culture-derived parasites was slot blotted as pairs of samples in the amounts shown. ^{32}P-labeled probes were prepared individually from each of the three oligonucleotides with specific activities ranging from 1,800 to 2,400 Ci/mmol. The slot blot was hybridized at 60°C under conditions described in Materials and Methods with an equal mixture of the three probes. The autoradiogram shown was exposed for 20 h. The *B. bigemina* rDNA control was 20 fmol of the cloned SSrRNA gene from unit C (16). The *B. bovis* small ribosomal DNA control was 20 fmol of a clone generated from polymerase chain reaction-amplified DNA (unpublished data).

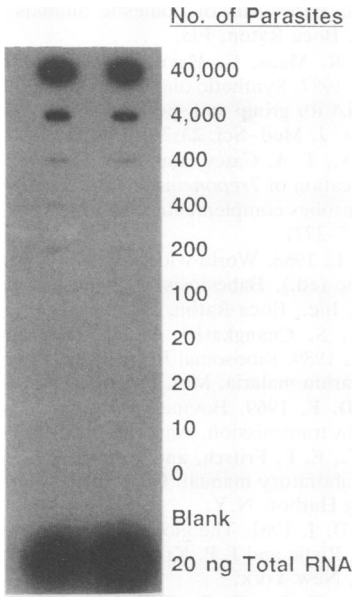

FIG. 2. Detection of blood-stage forms of *B. bigemina*. Nucleic acids from culture-derived parasites were applied to the slot blot. The samples in the pair of slots fourth from the top, containing 400 parasites, were extracted from parasites mixed into 200 μl of whole bovine blood; those in the other pairs of slots were extracted from parasites mixed into 20 μl of bovine blood. Probe hybridizations were performed by using oligonucleotides 3-1R, 8-1R, and JD115 as described in the legend to Fig. 1.

Diff-Quick stain (Fisher Scientific). More than 1,000 erythrocytes per slide were examined to accurately determine the parasitemia of cultures used in these experiments. The hematocrit of each culture was determined by using a hemocytometer, and the number of parasitized erythrocytes per ml of culture was calculated. The required volume of culture was mixed with normal bovine blood to yield parasitemias ranging from 2×10^{-2} to 5×10^{-6}%, and RNA was isolated from all samples. Slot blots were prepared with these RNA samples and hybridized with the mixture of ^{32}P-labeled oligonucleotide probes prepared from 3-1R, 8-1R, and JD115 (Fig. 2). These probes detected as few as 100 infected erythrocytes mixed in 20 μl of bovine blood (10^{10} erythrocytes per ml). The parasitemia under these conditions is equivalent to 5×10^{-5}%. Increased sensitivity of detection was demonstrated by extracting a larger volume of blood. RNA isolated from 400 parasites mixed in 20 or 200 μl of blood (i.e., 2×10^{-4} to 2×10^{-5}% parasitemia) was slot blotted and hybridized. The two preparations yielded similar results, and RNA isolated from 200 μl of normal bovine blood alone did not give any detectable signal.

Detection of *B. bigemina* in experimentally infected animals. Three castrated Holstein bull calves were experimentally infected with 1,000 (calf 2010) or 10,000 (calves 2057 and 2069) *B. bigemina*-infected bovine erythrocytes. These animals were observed for clinical symptoms of babesiosis for 20 days postinoculation. A drop of 10 to 25% in the packed cell volume and a mild transient fever (days 8 to 10) were observed in calves 2010 and 2057, whereas in calf 2069, no clinical symptoms were observed (data not shown). Analysis of thick blood smears by light microscopy showed one parasite in calf 2010 on day 8 and one parasite in calf 2057 on days 9 and 10. This represents one parasite per 50 fields,

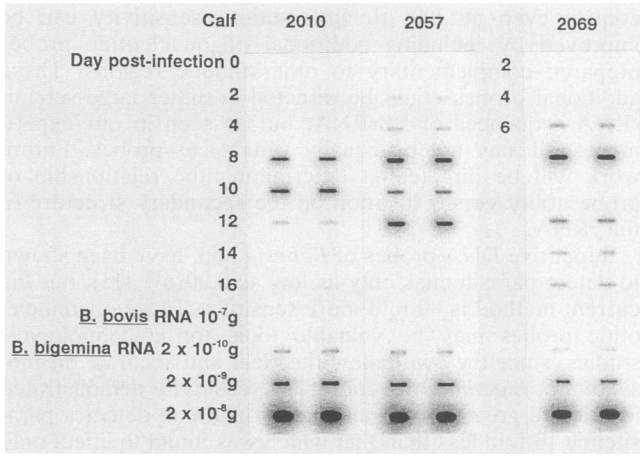

FIG. 3. Detection of *B. bigemina* in experimentally infected calves. Total RNA isolated from the erythrocytes of experimentally infected calves were applied to the slot blot. Each slot contained RNA extracted from the equivalent of 200 μl of blood. Three different amounts of *B. bigemina* total RNA and *B. bovis* RNA were used as controls. Probe hybridizations were performed with oligonucleotides 3-1R, 8-1R, and JD115 as described in the legend to Fig. 1. The images shown were obtained after a 2-h exposure by storage phosphor autoradiography and were similar to those obtained by X-ray film autoradiography for 20 h (not shown). Samples from calves 2010 and 2057 were not available on day 6. Samples from calf 2069 were not available on day 0.

where each field contained about 800 to 1,000 erythrocytes (~0.002% parasitemia). No parasites were detected in thick smears from calf 2069 examined in similar fashion. Blood samples (~1 ml) collected every other day up to day 16 were centrifuged to remove plasma, and the erythrocytes were resuspended in phosphate-buffered saline and stored at −70°C for 16 months. Total RNA was isolated from these samples, and slot blots were prepared in duplicate by using an aliquot of each sample equivalent to 200 μl of blood. Three amounts (20, 2, and 0.2 ng) of total RNA from *B. bigemina* and 100 ng of RNA from *B. bovis* were included in the slot blots as controls. Blots were hybridized with oligonucleotides 3-1R, 8-1R, and JD115, and an image was obtained by using autoradiography and phosphorimaging. Phosphorimaging results are presented in Fig. 3. Positive signals were observed for calves 2010 and 2057 from day 2 to day 14, whereas in calf 2069, signals were detected from day 6 to day 16. Peak parasitemias were observed on day 8, 10, or 12 in all three animals. These results correlated with the observed clinical symptoms in two animals. Although there were no clinical symptoms in calf 2069, the probes detected the infection on 6 of 8 days tested.

DISCUSSION

Diagnostic methods for detecting *B. bigemina* and *B. bovis* in bovine blood at the levels found in subclinical infections would be valuable for cattle rearing in the tropical areas of the world. We have utilized oligonucleotide probes unique and complementary to the SSrRNA sequence of *B. bigemina* for detecting parasites in bovine blood at levels as low as 2×10^{-5}% parasitemia. Although not demonstrated here, it may be possible to increase the sensitivity even further by testing a larger blood sample (>200 μl). This is possible, since RNA used in these experiments is highly purified (2), resulting in a low background for the uninfected blood

control even at 200 μl. In addition, sensitivity can be improved by including additional oligonucleotide probes prepared complementary to other unique regions. These additional probes could be directed at either large-subunit rRNA sequences or SSrRNA, but as seen in our experiments, all may not be equally suitable as probes. Future work will be directed at determining the relationship of probe utility versus location on the secondary structure of the rRNA.

Repetitive DNA probes of *Babesia* spp. have been shown to detect parasitemias only as low as 0.0016% (15), but the current method is 80-fold more sensitive. These oligonucleotide probes may be valuable tools for epidemiological studies, since they will allow the clear and accurate diagnosis of a *B. bigemina* infection. The sensitivity demonstrated is 100-fold greater than microscopy and can detect a parasitemia 10-fold less than that which was found to infect only 50% of engorging ticks (6). In addition, our results indicate that rRNA is a stable target and can be recovered from samples properly stored for at least 16 months. Studies on three experimentally infected Holstein bull calves demonstrated that this test will detect subclinical infections with the parasite, including the very early stages of infection (day 2 postinfection). At present, the dynamics of cycling of parasitemia in the host and the minimum parasitemia occurring in carrier animals are largely unknown because of insensitive methods of accurately detecting parasitemia. The method described here is sensitive enough to greatly extend the information available on such parameters and may be used to determine the strategies for applying DNA probes to the diagnosis of babesiosis. It is also possible to amplify the rRNA target sequences in vitro and further increase sensitivity, if required.

ACKNOWLEDGMENTS

We thank David Allred for the generous gift of blood samples from experimentally infected calves.

This work was supported by USAID grant DAN-4178-A-00-7056-00.

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