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Detection and Quantitation of *Anaplasma marginale* in Carrier Cattle by Using a Nucleic Acid Probe

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Cattle which have recovered from acute infection with *Anaplasma marginale*, a rickettsial hemoparasite of cattle, frequently remain persistently infected with a low-level parasitemia and serve as reservoirs for disease transmission. To fully understand the role of these carriers in disease prevalence and transmission, it is essential that low levels of parasitemia can be accurately detected and quantitated. We have developed a nucleic acid probe, derived from a portion of a gene encoding a 105,000-molecular-weight surface protein, that can detect *A. marginale*-infected erythrocytes. The probe is specific for *A. marginale* and can detect 0.01 ng of genomic DNA and 500 to 1,000 infected erythrocytes in 0.5 ml of blood, which is equivalent to a parasitemia of 0.000025%. This makes the probe at least 4,000 times more sensitive than light microscopy. Hybridization of the probe with treated blood from animals proven to be carriers of anaplasmosis showed that parasitemia levels were highly variable among carriers, ranging from >0.0025 to <0.000025%. Parasitemia levels of individual animals on different dates were also variable. These results imply that, at any given time, individuals within a group of cattle may differ significantly in their abilities to transmit disease.

Anaplasma marginale is a rickettsial hemoparasite of cattle and other ruminants. The organism is transmitted via biological and mechanical vectors, such as ticks and biting flies, as well as by contaminated fomites, such as needles (23). Anaplasmosis is of economic importance worldwide and represents a major obstacle to meat and milk production in tropical and subtropical regions (24). A conservative estimate of the annual loss due to anaplasmosis in the U.S. alone amounts to $100 million and includes 50,000 to 100,000 cattle deaths (18). The acute phase of the disease can cause severe anemia, abortions, weight loss, and death (1). Animals that recover from the acute phase may remain persistently infected with low, microscopically undetectable levels of the organism. These animals, known as carriers, serve as reservoirs for disease transmission and allow continual outbreaks to occur (28).

Little information is available about the carrier state of anaplasmosis or the role carrier animals play in disease prevalence and transmission. This lack of knowledge stems from the inability to detect and quantitate the low levels of parasitemia found in carrier animals. The current methods of detection involving serological tests are limited in the diagnosis of carriers by their inability to distinguish between active carriers and animals with antibodies due to prior infections (17). The tests also give no indication of levels of parasitemia present in carrier animals. Currently, the most reliable method of detecting carriers is by subinoculation of infected blood into splenectomized, susceptible calves (17). However, this procedure is too costly and time-consuming for routine use and provides little information concerning the level of parasitemia.

To detect and quantitate *A. marginale* in carriers, an assay is needed that will accurately detect very low levels of parasitemia. Nucleic acid probes, which hybridize only to their complementary sequences, can provide a sensitive and specific tool for diagnosis of infectious diseases (10). In addition, the intensity of the hybridization signals obtained correlates with the number of parasites, thus providing information on the level of parasitemia (5, 6, 13, 27). Previously, we developed a DNA probe based upon a portion of the gene encoding a 105,000-molecular-weight surface protein (designated 105L) of *A. marginale* (4, 12). It was demonstrated that this probe could detect *A. marginale* in infected tick tissue and within erythrocytes (12). Detection of infected erythrocytes indicated the feasibility of using a nucleic acid probe to identify *A. marginale* in carrier cattle.

In this report, we describe the development of an RNA probe based on this gene fragment and show that the RNA probe can be used to identify and quantitate the low, microscopically undetectable levels of parasitemia found in cattle proven to be carriers of anaplasmosis for at least 3 years.

MATERIALS AND METHODS

Source of blood samples. Six steers were experimentally infected with *A. marginale* (Florida isolate) in July 1984. All developed an acute infection and recovered. Parasites could not be detected in Wright-Giemsa-stained peripheral blood smears taken from these cattle on a weekly basis for 1 year following recovery. Blood was obtained for hybridization assays in May, July, and November 1987. Parasites were not detected by microscopic examination on any of these dates. Carrier status was confirmed in November 1987 by drawing 100 ml of blood from each of the six steers and subinoculating individually into splenectomized calves. Wright-Giemsa-stained peripheral blood smears from the recipient calves were examined daily until parasitemia was microscopically evident at a level of 1.0%.

Negative-control blood was obtained from calves under 4

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months of age and demonstrated by splenectomy to be free of *A. marginale* or from an adult cow proven to be negative for anaplasmosis by subinoculation of a blood sample into a splenectomized calf.

Positive-control blood with known parasitemia was obtained from splenectomized calves that had been experimentally infected with isolates of *A. marginale* from Florida; Missouri; South Idaho; Texas; Okanogan, Washington (Washington-O); and St. Croix, U.S. Virgin Islands. The origins and characterizations of the Florida, South Idaho, Texas, and Washington-O isolates have been previously described (19). The St. Croix and Missouri isolates were provided by Gerald Buening (Department of Veterinary Microbiology, University of Missouri-Columbia).

All of the above blood samples were washed three times with phosphate-buffered saline (PBS) (0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4) to remove plasma and buffy coat. They were then either diluted with PBS to provide known concentrations of erythrocytes or diluted with sterile water and further depleted of leukocytes by use of Whatman CF11 cellulose (Whatman, Inc., Clifton, N.J.) columns (2). All samples were stored at −70°C without cryoprotectant.

In vitro cultures of bovine erythrocytes infected with *Babesia bovis* and *Babesia bigemina* were provided by Terry McElwain and Steve Hines (Department of Infectious Diseases, University of Florida, Gainesville). Blood from rats infected with *Trypanosoma brucei* was provided by Sondra Kamper (Department of Infectious Diseases, University of Florida, Gainesville).

Probe specificity. Individual volumes of blood containing 5×10^8 *B. bovis*- and *B. bigemina*-infected erythrocytes and 5×10^8 *T. brucei* organisms were each added to 1 ml of PBS. A volume of blood containing 5×10^8 *A. marginale*-infected erythrocytes added to 1 ml of PBS was used as a positive control. These samples were treated with sodium dodecyl sulfate and proteinase K by previously described methods (12). The DNA was denatured by using NaOH (final concentration, 0.4 N). Tenfold serial dilutions of each sample were made, and 10 μl of each dilution, containing from 10^6 to 10^2 *T. brucei* organisms and from 10^6 to 10^2 *B. bovis*-, *B. bigemina*-, or *A. marginale*-infected erythrocytes, were blotted directly onto charged nylon filters (Zetaprobe; Bio-Rad Laboratories, Richmond, Calif.). Filters were baked for 2 h at 80°C in a vacuum oven.

Probe reactivity with different *A. marginale* isolates. PBS-washed blood from calves infected with Florida, Missouri, South Idaho, Texas, and Washington-O isolates of *A. marginale* was frozen and thawed two times to lyse the erythrocytes. Blood containing 5×10^7 infected erythrocytes was added to 1 ml of PBS. Samples were treated with SDS and proteinase K by previously described methods (12). A 5-μl sample of each dilution, containing from 10^5 to 10^2 infected erythrocytes, was spotted directly onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.), and the filters were baked for 2 h at 80°C in a vacuum oven.

Probe sensitivity. To determine the lowest percentage of infected erythrocytes that could be detected, serial dilutions were made of known numbers of erythrocytes infected with the Florida isolate of *A. marginale*. The infected erythrocytes were added to 0.5-ml samples of uninfected control blood that had been buffy coat depleted and restored to a final concentration of 8×10^9 erythrocytes per ml. This resulted in levels of parasitemia ranging from 0.0025 to 0.00000125%. DNA was extracted as follows. Samples were washed three times in PBS by centrifugation (30,000 × *g*) to remove hemoglobin and suspended in 500 μl of Tris-sodium chloride buffer containing 1% SDS and 0.5 mg of lysozyme per ml. Samples were incubated at 37°C for 1 h. Proteinase K was added to a concentration of 200 μg/ml, and samples were incubated overnight at 37°C. To ensure complete digestion of proteins in this larger volume of blood, proteinase K was again added to make a final concentration of 400 μg/ml, and samples were further incubated at 60°C for 1 h. Samples were transferred to siliconized Microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) and deproteinized by using phenol and phenol-chloroform. NaOH was added to a final concentration of 0.4 N, and the samples were incubated at 37°C for 1 h to denature the DNA. The denatured samples were applied to a charged nylon membrane (Zetaprobe) by using a dot blot manifold (Hybri-Dot Manifold; Bethesda Research Laboratories, Gaithersburg, Md.).

To determine the lowest amount of purified genomic *A. marginale* DNA that could be detected, DNA was isolated by previously described methods (4) from blood infected with *A. marginale* (Florida isolate) at approximately 50% parasitemia. Purified DNA was denatured by using 0.1 volume of 1 N NaOH, and 10-fold serial dilutions were made. A 10-μl sample of each dilution, containing from 10^2 to 10^{-6} ng of DNA, was spotted directly onto nitrocellulose, and the filters were baked for 2 h at 80°C in a vacuum oven.

Detection of ascending parasitemia. The probe was examined for its ability to detect ascending levels of parasitemia following initial challenge. A susceptible, splenectomized calf was inoculated intramuscularly with a volume of blood containing 10^{10} *A. marginale*-infected erythrocytes (Washington-O isolate). Samples of blood were taken from this calf before infection, at the time of inoculation, and daily thereafter until parasitemia was microscopically detectable at a level of 0.1% in Wright-Giemsa-stained peripheral blood smears. Blood samples were buffy coat depleted, suspended in PBS to known erythrocyte concentrations, and frozen at −20°C. A volume of blood containing 4×10^9 erythrocytes was taken from each sample, and the DNA was extracted by the methods described above. DNA was denatured by using NaOH (final concentration, 0.4 N), and the samples were applied to charged nylon membranes (Zetaprobe) by using a dot blot manifold (Hybri-Dot).

Detection of carrier cattle. Blood samples were taken on three different dates from each of the six carrier animals and uninfected controls. Blood was washed in PBS, suspended to a final concentration of approximately 8×10^9 erythrocytes per ml, and frozen and thawed two times to lyse erythrocytes. Samples (0.5 ml) of washed blood were added to PBS, and DNA was extracted and applied to nylon filters as described above.

RNA probe preparation and hybridization. Plasmid DNA containing a previously described *A. marginale* gene insert (pAM97) (4) was digested with *Sst*I, and a 2.0-kilobase (kb) fragment was isolated on a low-melting-point agarose gel (SeaPlaque Agarose; FMC Bioproducts, Rockland, Me.). The fragment was ligated in agarose into the *Sac*I site of dephosphorylated plasmid pGEM-3 (Promega Biotec, Madison, Wis.). By using this construct, *Escherichia coli* HB101 cells were transformed to ampicillin resistance (14). Purified pGEM-3 DNA containing the 2.0-kb *Sst*I fragment insert of 105L *A. marginale* DNA was linearized by using the restriction enzyme *Eco*RV, which cuts close to one end of the insert DNA. The SP6 promoter was used to generate an [α-^{32}P]CTP (DuPont-NEN Research Products, Boston, Mass.)-labeled RNA transcript with a specific activity of 1 ×

10^8 to 2×10^8 dpm/μg of DNA (Riboprobe Gemini System II; Promega Biotec).

Nitrocellulose filters were used for initial experiments. We later substituted nylon because of its ease of handling, potentially increased DNA-binding capacity, and simpler hybridization and washing conditions.

Nitrocellulose filters were hybridized and washed as previously described (12). Nylon filters were prehybridized for at least 6 h at 42°C in a mixture containing 50% formamide, 7% SDS, 1 mM EDTA, 0.25 M NaCl, 0.25 M sodium phosphate (pH 7.2), and 100 μg of sheared, denatured herring sperm DNA per ml. Hybridization was carried out overnight at 42°C. Filters were washed at room temperature for 15 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0)–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS. A high-stringency wash was carried out for 30 min at 60°C by using 0.1× SSC–1% SDS. After being washed, the filters were rinsed briefly in 0.1× SSC at room temperature, and autoradiography was carried out with Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

RESULTS

Proof of carrier status. Six steers were proven to be carriers of *A. marginale* 3 years after initial infection and recovery by subinoculation of their blood individually into six splenectomized, susceptible calves. *A. marginale*-infected erythrocytes were microscopically detectable at a 1.0% level from 15 to 32 days following subinoculation into these susceptible calves.

Specificity of the RNA probe. The probe was specific for *A. marginale*, as evidenced by lack of hybridization to blood infected with *B. bovis*, *B. bigemina*, and *T. brucei*, as well as to bovine leukocyte DNA from uninfected control blood (data not shown). Prior studies, using a DNA probe made from the same cloned gene fragment, demonstrated a lack of hybridization to *Anaplasma ovis* and *B. bovis* (12). The reactivity of the probe with different *A. marginale* isolates was tested. The probe reacted with approximately equal intensity to all five U.S. isolates (Florida, Missouri, South Idaho, Texas, and Washington-O) (Fig. 1). In addition, it was capable of detecting an isolate from St. Croix (data not shown). The specificity of the RNA probe was identical to that obtained from the DNA probe.

Sensitivity of the RNA probe. The sensitivity of the probe was initially determined using 10-fold serial dilutions of purified *A. marginale* DNA. The probe was capable of detecting 0.01 ng of genomic DNA (Fig. 2). The sensitivity of the RNA probe was similar to that of the DNA probe made from the same clone gene fragment. When serial dilutions of *A. marginale*-infected erythrocytes were made, the RNA probe was able to detect 500 to 1,000 infected erythrocytes in 0.5 ml of uninfected blood (Fig. 3). This is equivalent to a parasitemia level of approximately 0.000025%. If the approximate genome size of a single rickettsial organism is assumed to be 1,500 kb (15), 0.01 ng is the equivalent of approximately 6,000 organisms. Thus, each parasitized erythrocyte contained an average of 6 to 12 *A. marginale* organisms. This is consistent with previously published data, wherein 1 to 16 organisms were detected per infected erythrocyte (12, 25).

Detection of ascending parasitemia. The RNA probe would be useful for clinical and epidemiologic studies if it is capable of detecting infections when they are not microscopically evident. A splenectomized calf was subinoculated intramuscularly with 10^{10} *A. marginale*-infected erythrocytes. On day 2 postinfection, a positive signal was obtained with the RNA probe by using 0.5-ml blood samples (Fig. 4). On the basis of hybridization intensity, this parasitemia level remained fairly constant until day 9 postinfection, when it began to increase. A parasitemia level of 0.1% was detected microscopically on day 14 postinfection.

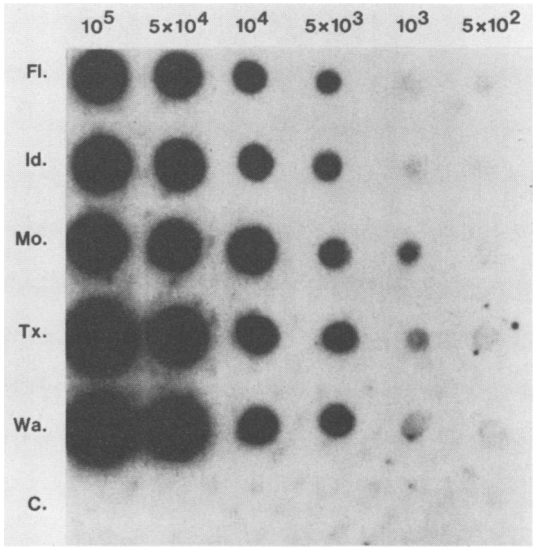

FIG. 1. Reactivity of the 2.0-kb probe with five different U.S. isolates of *A. marginale*. Erythrocytes infected with Florida (Fl.), Missouri (Mo.), South Idaho (Id.), Texas (Tx.), and Washington-O (Wa.) isolates of *A. marginale* were treated to lyse initial bodies, serially diluted (10^5, 5×10^4, 10^4, 5×10^3, 10^3, and 5×10^2 infected erythrocytes), and applied to nitrocellulose. A negative control (C.), consisting of serially diluted uninfected erythrocytes, was treated in the same manner.

Detection of carrier infections. Samples (0.5 ml) of blood were taken from the six carrier animals on three separate dates, applied to nylon filters, and hybridized with the probe. Positive signals were obtained for each animal on at least one occasion; animals 2 and 5 were positive on two different dates, and animals 1 and 4 were positive on all three dates (Fig. 3). Comparison with the infected erythrocyte standards showed that the number of infected erythrocytes present was highly variable among the carrier animals, ranging from $>10^5$ to <500 to 1,000 infected erythrocytes per 0.5 ml of blood. This corresponds to parasitemia levels ranging from >0.0025 to <0.000025%. The parasitemia levels of an individual animal on different dates were also highly variable. For example, animal 2 had a parasitemia level of approximately 0.00025% in May 1987; was below the detection limit of 0.000025% in July 1987; and in November 1987 had a parasitemia level exceeding 0.0025%.

DISCUSSION

In this report we have described the development and use of an RNA probe to detect and quantitate the low, microscopically undetectable levels of parasitemia found in chronic carriers of anaplasmosis. This probe is an RNA transcript made from a 2.0-kb *Sst*I fragment of a gene encoding a portion of an *A. marginale* surface protein. The sensitivity of the probe compares very well with that of probes developed for detection of other procaryotic organisms (11, 21, 22, 26). Light microscopy is capable of detect-

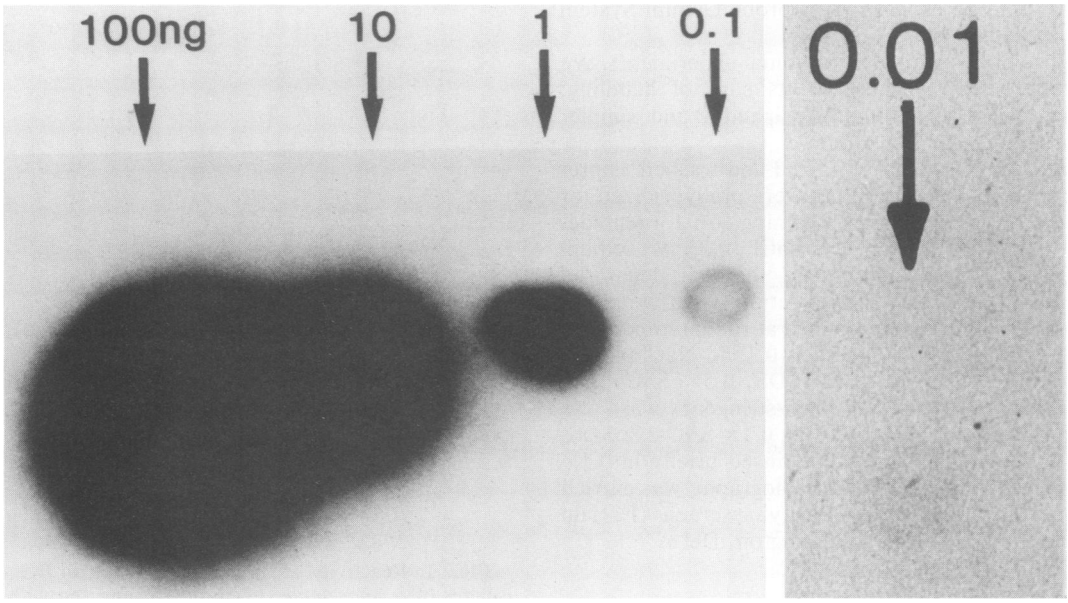

FIG. 2. Sensitivity of the 2.0-kb probe with *A. marginale* genomic DNA. DNA was isolated from *A. marginale*-infected erythrocytes (Florida isolate), and 10-fold serial dilutions were made and applied to nitrocellulose. At right is a hybridization with 0.01 ng of DNA.

ing *A. marginale* parasitemia levels of 0.1 to 0.2%, which is at least 4,000 times less sensitive than the RNA probe. The probe was shown to detect parasitized erythrocytes 12 days prior to microscopic detection. Clearly, the number of days postinoculation required to detect an infected animal would vary with the initial dose and route of inoculation. However, early detection of infections could reduce losses significantly in herd outbreaks of anaplasmosis.

The carrier state of anaplasmosis is presently a poorly understood phenomenon. Until now, it has been impossible to determine the levels of parasitemia present in carrier animals and whether these levels remain stable or rise and fall over time. The variation seen in hybridization intensity implies that, at any given time, individuals within a group of cattle may differ significantly in their abilities to transmit disease. These results have important epidemiologic implications. It is possible that below a certain level of parasitemia individual cattle are no longer capable of transmitting anaplasmosis via biological or mechanical vectors. Both external and internal factors may be capable of altering the level of parasitemia in an individual. For example, immunosuppression of infected cattle (8), environmental or nutritional stress (7), and concurrent infection with other diseases can provoke a recrudescence of clinical anaplasmosis. These factors may contribute to the fluctuations in parasitemia levels observed in the six carriers.

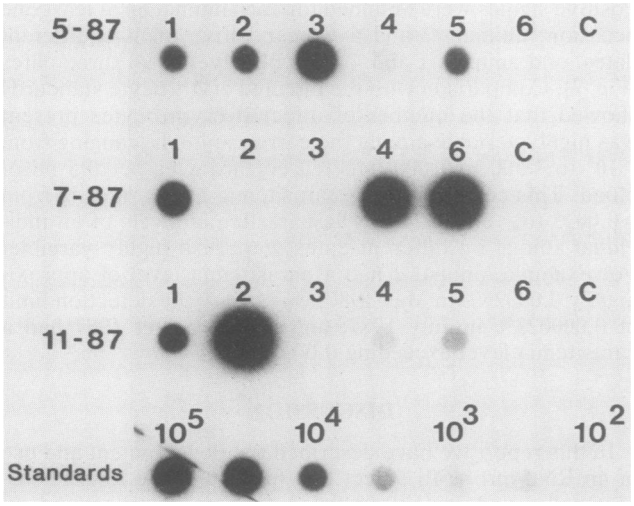

FIG. 3. Detection of carrier cattle. Blood was obtained from six carriers and negative controls (C) on three different dates. DNA was extracted from 0.5-ml samples of washed blood and applied to nylon filters by using a dot-blot apparatus. Standards consisted of DNA extracted from known numbers of infected erythrocytes, which had been serially diluted (10^5, 5×10^4, 10^4, 5×10^3, 10^3, and 5×10^2) and added to 0.5 ml of uninfected, washed blood.

A key epidemiologic question is the importance and prevalence of carriers in enzootically stable regions. Using a test that will distinguish infected animals from those that are immune will allow us to better define the relationship between herd immunity and disease transmission. This information may allow for the design of a model to predict the impact of control measures, including vaccination, on enzootic stability (3).

To fully evaluate the role carriers play in maintaining enzootic stability, it is necessary that the probe be able to detect all carrier animals regardless of parasitemia level. We have not yet obtained this degree of sensitivity. To further increase the sensitivity of this assay, several alternatives are available. A more highly reiterated sequence could be sought, such as those used to detect *Plasmodium falciparum* (20). However, restriction analysis of *A. marginale* genomic DNA has failed to reveal highly reiterated sequences (unpublished data). Another possibility is the development of a probe to detect rRNA (9). However, ribosomal genes are often highly conserved among similar organisms, and ensuring adequate specificity may be difficult. A recent innovation is a polymerase chain reaction which uses a thermostable

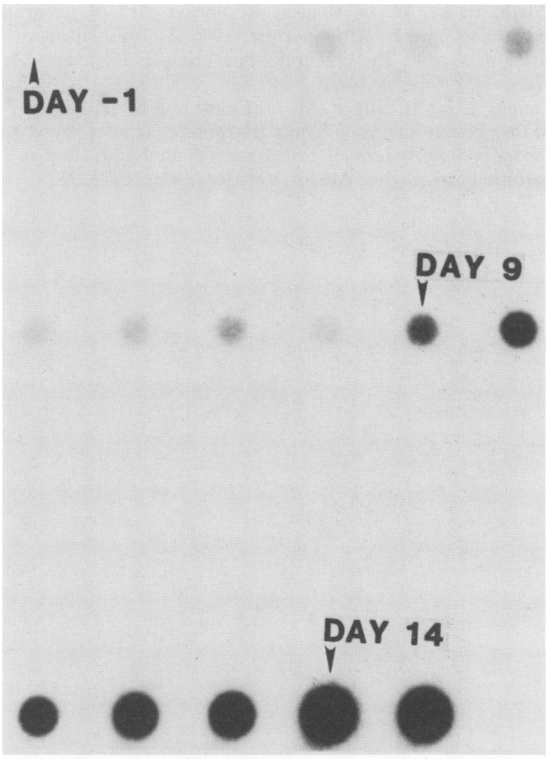

FIG. 4. Detection of ascending parasitemia with the 2.0-kb probe. Blood was taken daily from a splenectomized calf before (day −1) and after subinoculation with *A. marginale* (Washington-O isolate). DNA was extracted from 0.5-ml portions of these samples and applied to nylon filters by using a dot blot apparatus. Samples are arranged chronologically from left to right. On day 14, parasitemia was observed microscopically at a 0.1% level.

DNA polymerase to specifically amplify the target sequence of a probe. This technique, which can amplify a target sequence up to 200,000-fold, has recently been modified for use in lysed cells, without a requirement for isolation of the DNA (16). By using this technique to amplify the target sequence of the RNA probe, we could substantially reduce the time necessary for sample preparation while gaining the potential to detect even the lowest levels of parasitemia found in carrier cattle.

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