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Detection of *Anaplasma marginale*-infected tick vectors by using a cloned DNA probe

(hemoparasite/tick-borne disease/epizootiology)

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ABSTRACT **Anaplasmosis is the most widely distributed of several important tick-borne diseases that constrain cattle production throughout much of the world. Evaluation of the effectiveness of disease control strategies that integrate vaccination with tick control requires the ability to monitor tick and cattle infection rates. To detect *Anaplasma marginale* in ticks and bovine erythrocytes, a 2-kilobase DNA fragment from a cloned *A. marginale* gene coding for a surface protein having a M_r of 105,000 was prepared and evaluated as a probe. The probe was species specific and detected *A. marginale* DNA derived from infected bovine erythrocytes and adult *Dermacentor* ticks infected either as nymphs or adults. Tick infection was confirmed by microscopy and test feeding on a susceptible calf. The sensitivity of the probe is suitable for detecting infected ticks in experimental and field epizootiology studies.**

The tick-borne, hemoparasitic diseases are among the most devastating to cattle worldwide and include the rickettsial diseases, anaplasmosis and cowdriosis, and the protozoal diseases, babesiosis and theileriosis. These diseases, enzootic principally in countries with tropical and subtropical climates, place over one-half billion cattle at risk to one or more of the infectious agents. In various regions of Africa, for example, all four diseases are enzootic. Anaplasmosis, caused by *Anaplasma marginale*, has the greatest worldwide prevalence†† and is the only one occurring in the United States (1).

Anaplasma is transmitted mechanically by biting flies and biologically by ticks‡‡. Tick transmission of *A. marginale* has been documented in several geographic areas of the world, including the United States, where *Dermacentor* species are the principal recognized vectors (2). Ticks can transmit infection to susceptible cattle after acquiring the parasite from acutely infected or chronic carrier cattle (3, 4).

Effective control of this disease, as with other tick-borne diseases, requires a thorough knowledge of the nature and extent of the problem in enzootic areas. There is, however, little detailed and accurate epizootiologic information on anaplasmosis, primarily due to difficulty in identifying infected ticks and chronic carrier cattle. Only recently has *A. marginale* been detected in individual ticks, by labor-intensive methods involving histology and light and electron microscopy (5), and prevalence data in cattle continue to be based on serologic methods that lack sensitivity in identifying chronic carrier infections (6). Consequently, there is need for a specific, sensitive, and practicable method for critically analyzing tick infection rates, bovine carrier status, and the relationship of these parameters to outbreaks of the disease.

In the present study, we prepared a 2-kilobase (kb) DNA fragment from within the gene coding for an *A. marginale* surface peptide (Am 105L; M_r 105,000) for use as a probe. The probe was specific for homologous DNA associated with three geographic isolates of *A. marginale*-infected erythrocytes and two isolates associated with infected midgut tissues of three tick vector species. No hybridization occurred with control erythrocytes, tick tissues, bovine DNA, or other hemoparasite DNA, including an ovine species of *Anaplasma*. The sensitivity appeared to be suitable for determining tick infection levels and rates and for identifying chronically infected cattle.

MATERIAL AND METHODS

Preparation of Infected Erythrocytes. Each of three geographically distinct isolates of *A. marginale*—Florida, Virginia, and Idaho (hereafter designated FAM, VAM, and IDAM, respectively) (7)—was inoculated into a different splenectomized calf. Blood collected at peak parasitemia was depleted of leukocytes, the erythrocytes were washed and counted, and the number of infected erythrocytes was determined from Giemsa-stained thin films. Controls included sheep erythrocytes infected with an Idaho isolate of *Anaplasma ovis* (8), *in vitro* cultures of bovine erythrocytes infected with *Babesia bovis* (9), uninfected bovine erythrocytes, and uninfected bovine erythrocytes to which was added 1×10^7 bovine leukocytes per ml. All samples were adjusted to the same packed cell volume and stored as frozen aliquots without cryoprotectant. For use, each sample was freeze-thawed three times to lyse cells and a volume containing 5×10^7 *Anaplasma*-infected erythrocytes was added to 1 ml of phosphate-buffered saline (0.01 M phosphate/0.14 M NaCl, pH 7.2) (PBS). Samples were centrifuged at 30,000 $\times$ *g* for 30 min to remove excess hemoglobin, and each pellet was resuspended in 100 μl of a Tris buffer (100 mM Tris·HCl, pH 7.5/150 mM NaCl/12 mM EDTA) (TEN buffer) containing 1% $NaDodSO_4$ and 100 μg of proteinase K per ml (PK buffer). The samples were incubated at 37°C overnight. In one experiment, the samples were further

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††Smith, C. A., Proceedings of the Fifth National Anaplasmosis Conference, February 28–29, 1968, Stillwater, OK, pp. 112–115.

‡‡Howell, D. E., Proceedings of the Fifth Anaplasmosis Conference, February 28–29, 1968, Stillwater, OK, pp. 164–166.

processed by phenol/chloroform extraction of nucleic acids (10). Each sample was denatured and diluted in TEN buffer containing normal bovine erythrocyte ghosts. Serial dilutions (1:10) resulted in 2.5×10^5 through 2.5×10^{-2} infected erythrocytes per 5 μl, with each dilution containing 10^6 erythrocyte ghosts per 5 μl.

Infection of Ticks. *Dermacentor variabilis* and *Dermacentor andersoni* nymphs were applied to a splenectomized calf with an ascending VAM parasitemia, whereas *Dermacentor occidentalis* males were applied to a different splenectomized calf during an ascending IDAM parasitemia. All fed ticks were removed after 6 days, when the parasitemias of VAM and IDAM were 27% and 50%, respectively. Control groups of ticks for each species were fed similarly on *A. marginale*-negative calves. All ticks were placed in a humidity chamber (93% relative humidity) at 26°C with a 12-hr photoperiod for 15–30 days during which time they molted to adults. They were then held at 37°C for 3 days to enhance development of *A. marginale* colonies. Ten ticks from each group were individually cleaned with 70% ethanol and cut longitudinally into halves.

Preparation of Tick Tissues for Histology. For *D. variabilis* and *D. andersoni*, the midgut tissue was dissected from one-half of each tick and fixed immediately in 0.2 M sodium cacodylate buffer containing 2% glutaraldehyde. For *D. occidentalis*, the entire half-tick was fixed in PBS containing 2% glutaraldehyde before dissecting the midgut tissues. The *D. variabilis* and *D. andersoni* tissues were post-fixed in 0.2 M sodium cacodylate buffer containing 2% osmium tetroxide. Subsequently, all fixed tissues were washed several times, dehydrated through a graded series of ethanol solutions, and infiltrated with epoxy resin (Dow epoxy resin 736, Polyscience, Warrington, PA) using propylene oxide as the intermediate solvent. For light microscopy, semithin (0.25 μm) sections were stained for 2 min with toluidine blue [1% (wt/vol) toluidine blue crystals dissolved in distilled water containing 1% sodium borate; filtered through a 0.22-μm filter]. For electron microscopy, ultrathin (silver-reflective) sections were prepared as described (11).

Preparation of Tick Tissues for Hybridization. Midgut tissues were excised from the tick halves complementary to those processed for histology and stored in a microcentrifuge tube at −70°C until used. One hundred microliters of PK buffer was added to each sample and solubilized at 37°C overnight. Some samples were denatured and 5 μl was applied directly to nitrocellulose or after making 1:10 serial dilutions in TEN buffer. Nucleic acids were ethanol precipitated from other samples. The precipitates were suspended in 10 μl of TEN buffer, denatured, and neutralized and dilutions were made in TEN buffer. Five microliters of each dilution was applied to nitrocellulose.

Isolation and Nick-Translation of the Probe. The gene coding for Am 105L, one of two complexed *A. marginale* surface polypeptides (Am 105U and Am 105L) (12), has been cloned and expressed in *Escherichia coli* (13). Briefly, *A. marginale* DNA isolated from bovine infected blood was digested to completion with *Bgl* II. The resulting fragments were ligated into the *Bam*HI site of the pBR322 plasmid using T4 DNA ligase. *E. coli* HB101 cells were transformed to ampicillin resistance. The library containing 3000 recombinants was screened with an antibody specific for Am 105, and plasmid DNA from a positive clone (pAm 113) containing a 3.9-kb insert sequence, was digested with *Sst* I. The resulting 2-kb fragment from within the 3.9-kb insert sequence was isolated from agarose gels and nick-translated with [^{32}P]dCTP.

Hybridization Conditions. Five microliters of each sample dilution was applied to nitrocellulose (Bio-Rad) and baked. For hybridizations, nitrocellulose was prehybridized overnight at 42°C by using a modified Denhardt's solution (0.05 M sodium phosphate, pH 6.5/0.75 M NaCl/75 mM trisodium citrate/0.02% polyvinylpyrrolidone 40/0.02% bovine serum albumin/0.02% Ficoll/0.2% $NaDodSO_4$/20% dextran sulfate/50% formamide/100 μg of sheared salmon sperm DNA per ml). Denatured 2-kb probe (specific activity = 3×10^7 to 1×10^8 cpm/μg of DNA) was allowed to react for 24 hr at 42°C. Each nitrocellulose sheet was washed twice at room temperature for 20 min with 0.3 M NaCl/0.03 M trisodium citrate containing 50% formamide and 0.1% $NaDodSO_4$, three times at room temperature for 20 min with 0.3 M NaCl/0.03 M trisodium citrate containing 0.1% $NaDodSO_4$, and four times at 65°C for 15 min with 15 mM NaCl/1.5 mM trisodium citrate containing 0.1% $NaDodSO_4$. The sheets were then exposed to Kodak X-Omat AR film for various times.

RESULTS

Specificity and Sensitivity of the Probe. The probe was first evaluated by hybridizing to infected and control erythrocyte samples. The probe was specific for *A. marginale* DNA, as evidenced by the lack of hybridization to extracted and unextracted DNA from bovine leukocytes, *A. ovis* and *B. bovis* samples. Also, the reaction with unextracted *A. marginale* samples was only slightly less intense than with extracted nucleic acids (Fig. 1 *A* and *B*). The sensitivity of the probe hybridized to homologous, unlabeled 2-kb fragment was 0.5 pg (Fig. 2). Moreover, hybridization sensitivity was nearly equal with three *A. marginale*, geographically distinct isolates (Fig. 2). The 2-kb probe detected 2.5×10^2 infected erythrocytes per 5 μl, an amount equivalent to 0.001% parasitemia in 5 μl of whole blood.

To determine the applicability for detection of infected ticks, the probe was hybridized to solubilized normal tick midgut tissue and normal midgut tissue to which known quantities of infected erythrocytes were added. No hybridization occurred with normal midgut tissue, and tick tissue did not interfere with the detection of *A. marginale* DNA (data not shown).

Detection of Infected Tick Tissue. Three species of *Dermacentor* ticks were used in these experiments. Males and females of *D. andersoni* and *D. variabilis* were exposed as nymphs and tested as adults; for *D. occidentalis*, of which only males were used, the ticks were exposed and tested as adults. This design, in which *D. andersoni* and *D. variabilis*

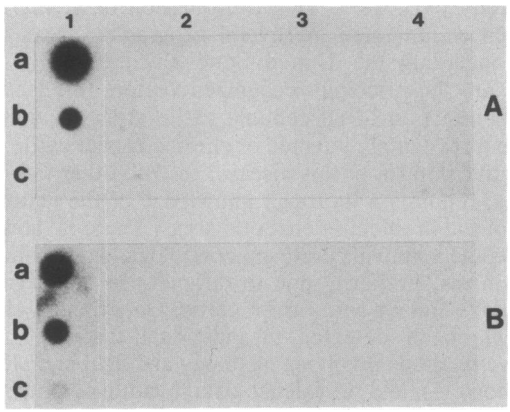

FIG. 1. Specific hybridization of the 2-kb probe with *A. marginale* DNA. (*A*) Dilutions of PK buffer-treated samples: lane 1, 1:10 dilutions of FAM-infected erythrocytes starting with 2.5×10^4 per 5 μl; lane 2, same as lane 1 except normal bovine blood with an additional 1×10^7 leukocytes per ml; lane 3, same as lane 1 except *A. ovis*-infected ovine erythrocytes; lane 4, same as lane 1 except *B. bovis*-infected bovine erythrocytes. (*B*) Same samples as in *A* after phenol/chloroform extraction and ethanol precipitation.

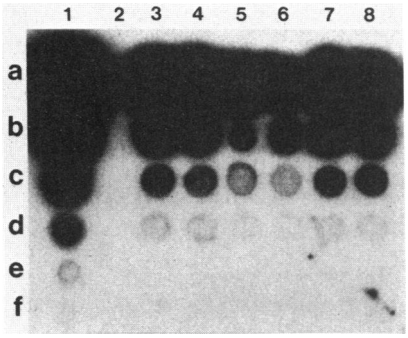

FIG. 2. Sensitivity of the 2-kb probe with homologous 2-kb DNA and erythrocytes infected with three isolates of *A. marginale*. Lane 1, 1:10 dilutions of 2-kb DNA starting with 5 ng; lane 2, 1:10 dilutions of *A. ovis*-infected erythrocytes starting with 2.5×10^5 per 5 μl; the remaining lanes are the same as lane 2 except that in lanes 3 and 4 the erythrocytes are infected with FAM, lanes 5 and 6 the erythrocytes are infected with VAM, and lanes 7 and 8 the erythrocytes are infected with IDAM.

were exposed to a VAM isolate and *D. occidentalis* was exposed to an IDAM isolate, permitted evaluation under conditions of transstadial and intrastadial development of the parasite in the tick vector. Ten ticks from each of the exposed and control groups (Table 1) were dissected and the individual midguts were divided into halves. One-half was evaluated microscopically and the other half was evaluated by hybridization. *A. marginale* colonies were microscopically detected in 43 of the 50 exposed midgut tissues (Fig. 3 *A* and *B*), whereas *A. marginale* DNA was detected in all 50 of the exposed tissues. The 50 control midgut tissues were uniformly negative by histologic and hybridization techniques (Fig. 4 *A–C* and Table 1). Titration of the ethanol-extracted DNA from each exposed tick midgut tissue sample indicated a variable degree of infection within each group. *A. marginale* DNA was detected in all exposed ticks when nucleic acid from 21% of the total midgut tissue was spotted and, in some, when as little as 0.4% was spotted.

A parallel group of exposed *D. occidentalis* (32 males) from the same IDAM-infected calf used to obtain ticks for histology and hybridization was held at 26°C for 18 days and then test fed for 7 days on a susceptible splenectomized calf. The calf developed clinical anaplasmosis on day 28 postinfestation, further confirming the presence and biological transmission of infective *A. marginale*.

DISCUSSION

We have developed and evaluated a DNA probe for use in detecting *A. marginale*-infected vector ticks and infected cattle blood. The probe, a 2-kb fragment, was obtained by digestion of a cloned 3.9-kb insert sequence containing the Am 105L gene (13). When whole *A. marginale* genomic DNA was cut with restriction enzymes that do not cut within the probe sequence and analyzed by Southern blotting, it was revealed that at least four bands occurred using the 2-kb probe (13). The presence of at least partially homologous copies may contribute to the sensitivity of the probe where the equivalent of 0.5 pg of homologous fragment is detected from ≈200 infected erythrocytes. The number of individual organisms (initial bodies) associated with each infected erythrocyte ranges from 1 to 16 depending on the stage of development. Therefore, the amount of homologous DNA per initial body is not known. The genome size of *A. marginale* has been calculated to be ≈340 kb with a molar percentage of guanine plus cytosine of 33 based on melting temperature values. Renaturation reactions appear to follow simple second-order kinetics with a $C_0t/2$ of 0.6, indicating that the genome does not have a great number of repeated sequences (14). However, the sensitivity exceeds that obtainable by examination of stained blood films and thus has promise in identification of chronically infected cattle. These cattle are often blood film negative and serologically negative but their blood can cause infection when inoculated into susceptible splenectomized calves (15).

Although other tick-borne pathogens—e.g., hemoprotozoans and the Rocky Mountain spotted fever rickettsia—are readily detected in tick hemolymph smears either directly or by fluorescent antibody techniques (16, 17), there is only one report of *A. marginale* in tick hemolymph (18). Indeed, fluorescence techniques generally have been unreliable for detecting *Anaplasma* in tick tissues. Consequently, a nucleic acid probe seemed appropriate. The amount of *A. marginale* DNA associated with tick midgut colonies is also unknown. However, we could detect *A. marginale* DNA in each individual infected tick midgut, observing a variable degree of hybridization among the 50 infected tick samples. Titration of each tick tissue sample resulted in a reaction equivalent to that of 0.5 pg of homologous DNA from as little as 0.4% of the total midgut in some ticks.

When one section of each half-tick midgut was examined microscopically for the presence of *A. marginale* colonies, no correlation was found between the colony density (number of colonies per 0.1 mm^2, or percentage of tissue occupied by colonies) and hybridization titrations with the corresponding half midguts. In seven midguts from *A. marginale*-exposed ticks, no colonies were observed yet hybridization revealed the presence of *A. marginale* DNA. These results should be considered in light of evidence that colony distribution within the midgut tissue apparently is not random (D.S., unpublished observations). If the hybridization results using dilutions of tick tissue samples reflect differences in individual infection levels, it should be possible to determine the minimal tick infection level required for trans-

Table 1. Summary of the results of tick exposures to *A. marginale*-infected and control cattle

Tick species	Tick sex	Tick stage fed*	Status of cattle	Histologically positive/total	Hybridization positive/total
D. variabilis	♀	Nymph	Infected (VAM)	7/10	10/10
D. variabilis	♀	Nymph	Control	0/10	0/10
D. variabilis	♂	Nymph	Infected (VAM)	6/10	10/10
D. variabilis	♂	Nymph	Control	0/10	0/10
D. andersoni	♀	Nymph	Infected (VAM)	8/10	10/10
D. andersoni	♀	Nymph	Control	0/10	0/10
D. andersoni	♂	Nymph	Infected (VAM)	10/10	10/10
D. andersoni	♂	Nymph	Control	0/10	0/10
D. occidentalis	♂	Adult	Infected (IDAM)	10/10	10/10
D. occidentalis	♂	Adult	Control	0/10	0/10

*Nymphal *D. variabilis* and *D. andersoni* and adult *D. occidentalis* ticks were fed, removed, and processed as described in the text.

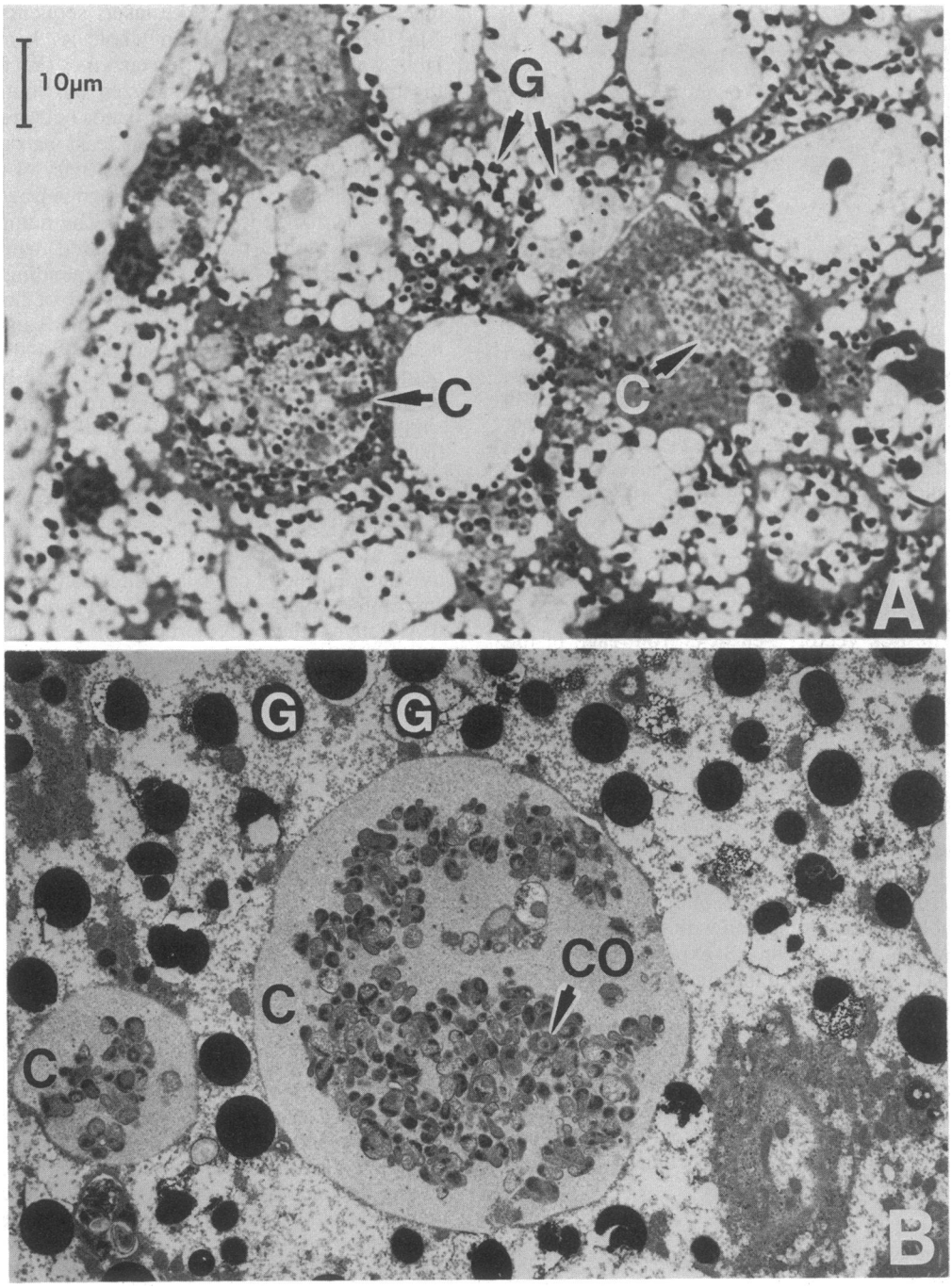

FIG. 3. Colonies of *A. marginale* within tick midgut epithelium. (*A*) *D. occidentalis* midgut epithelium containing colonies of an Idaho isolate of *A. marginale*. (*B*) Electron micrograph of an individual colony. C, *A. marginale* colony; G, electron-dense granules; CO, individual organisms within the colony. (×4500.)

mission as well as the minimal bovine parasitemia level required to infect ticks.

There was no apparent difference in hybridization results between tick tissues derived from adults that had acquired infection as nymphs (transstadial) and those that acquired infection as adults. We felt it was important to test ticks under both acquisition conditions since most of the studies concerned with the development of *A. marginale* in ticks have used nymphs for feeding on parasitemic cattle and have subsequently done morphologic characterization of *A. marginale* colonies from adult midgut tissues (5). However, there is a growing body of evidence indicating that the most likely natural mode of transmission is by interhost transfer of, particularly, male ticks that acquire infection as adults (intrastadial) (2, 19).

The probe was species specific under the conditions used for hybridization, as evidenced by failure to hybridize with *A. ovis* DNA. The use of 50% formamide at 42°C and washing at a final temperature of 65°C is relatively stringent and would not allow stable hybridization with <70–75% homology in base pairing (20). In addition to hybridization stringency, the use of a small cloned probe probably enhanced specificity relative to the use of whole genomic probes (21).

The gene from which the probe was derived codes for Am 105L, a polypeptide that is on the surface of *A. marginale* and is complexed with another surface polypeptide, Am 105U (13, 22). The results of this study demonstrate that the Am 105L gene is conserved among three United States isolates and can be detected in tick-associated colonies.

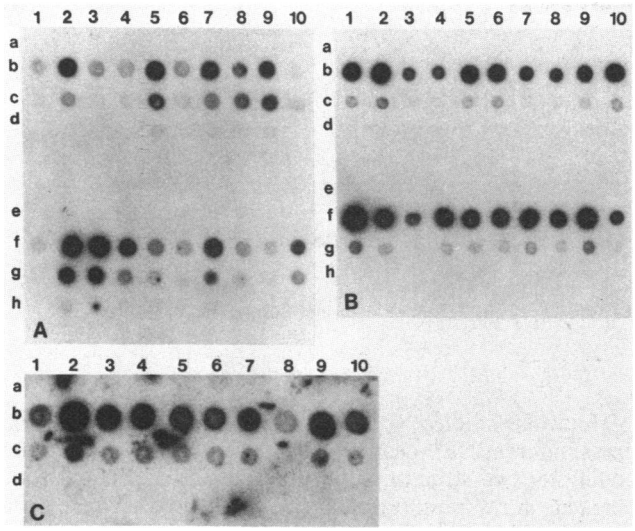

FIG. 4. Hybridization of the 2-kb probe with midgut tissues from three species of ticks exposed to two isolates of *A. marginale*. Nucleic acid was prepared from individual tick midgut samples (see text). (*A*) *D. variabilis* ticks: row a, 10 male ticks fed on an uninfected calf (unexposed controls), 5 μl = 21% of total tick midgut; row b, 10 male ticks fed on a calf infected with a Virginia isolate of *A. marginale* (VAM), 5 μl = 21% of total tick midgut; rows c and d, dilutions of row b, where 5 μl = 4.0% and 0.4% of the total tick midgut, respectively; row e, 10 unexposed control female ticks, same percentage as row a; row f, 10 female ticks fed on the same VAM-infected calf, same percentage as row b; rows g and h, dilutions of row f, same percentages as rows c and d. (*B*) *D. andersoni ticks*: all rows represent the same conditions as in *A*. (*C*) *D. occidentalis* ticks: exposed male ticks were fed on a calf infected with an Idaho isolate of *A. marginale* (IDAM): all rows represent the same conditions as in *A*.

There are other antigenic determinants associated with *A. marginale* that are apparently isolate restricted, as defined by other monoclonal antibodies (7). Probes from sequences coding for these may allow for detection of specific isolates.

The 2-kb probe appears to be suitable for determining the (*i*) susceptibility and infection thresholds of various tick species to different isolates of *A. marginale*, (*ii*) prevalence and incidence of infected ticks within enzootic areas, (*iii*) relationship between tick infection and outbreaks of the disease, and (*iv*) specific tick tissues that support development of the parasite. The use of this probe for *A. marginale* and of probes for other tick-borne etiologic agents will allow rational tick control strategies to be combined with vaccination to reduce disease losses.

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1. Ristic, M. (1977) in *Parasitic Protozoa*, ed. Kreier, J. D. (Academic, New York), Vol. 4, pp. 235–249.
2. Marchette, N. J. & Stiller, D. (1982) in *Ecological Relationships and Evolution of the Rickettsiae*, ed. Marchette, N. J. (CRC, Boca Raton, FL), Vol 2, pp. 97–126.
3. Kocan, K. M., Hair, J. A., Ewing, S. A. & Stratton, L. G. (1981) *Am. J. Vet. Res.* **42,** 15–18.
4. Zaugg, J. L., Stiller, D., Coan, M. E. & Lincoln, S. D. (1986) *Am. J. Vet. Res.* **47,** 2269–2271
5. Kocan, K. M. (1986) in *Morphology, Physiology and Behavioral Ecology of Ticks*, eds. Sauer, J. & Hair, J. A. (Horwood, West Sussex, England), pp. 472–505.
6. Goff, W. L., Johnson, W. C. & Kuttler, K. L. (1985) *Am. J. Vet. Res.* **46,** 1080–1084.
7. McGuire, T. C., Palmer, G. H., Goff, W. L., Johnson, M. I. & Davis, W. C. (1984) *Infect. Immun.* **45,** 697–700.
8. Magonigle, R. A., Eckblad, W. P., Lincoln, S. D. & Frank, F. W. (1981) *Am. J. Vet. Res.* **42,** 199–201.
9. Goff, W. L. & Yunker, C. E. (1986) *Exp. Parasitol.* **62,** 202–210.
10. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 458–462.
11. Kocan, K. M., Hair, J. A. & Ewing, S. A. (1980) *Am. J. Vet. Res.* **41,** 1966–1976.
12. Palmer, G. H. & McGuire, T. C. (1984) *J. Immunol.* **133,** 1010–1015.
13. Barbet, A. F., Palmer, G. H., Myler, P. J. & McGuire, T. C. (1987) *Infect. Immun.* **55,** 2428–2435.
14. Bear, P. D. & Philpott, M. S. (1987) *Curr. Microbiol.* **15,** 171–175.
15. Maas, J., Lincoln, S. D., Coan, M. E., Kuttler, K. L., Zaugg, J. L. & Stiller, D. (1986) *Am. J. Vet. Res.* **47,** 528–533.
16. Riek, R. F. (1964) *Aust. J. Agric. Res.* **15,** 802–821.
17. Burgdorfer, W. & Lackman, D. (1960) *J. Infect. Dis.* **107,** 241–244.
18. Kocan, K. M., Oberst, R. D., Ewing, S. A., Hair, J. A. & Barron, S. J. (1983) *Am. J. Vet. Res.* **44,** 798–801.
19. Stiller, D., Leatch, G., Kuttler, K. L. & Johnson, L. W. (1987) *Am. J. Vet. Res.*, in press.
20. Lawrie, J. M., Jackson, P. R., Stiteler, J. M. & Hockmeyer, W. T. (1985) *Am. J. Trop. Med. Hyg.* **34,** 257–265.
21. Howley, P. M., Israel, M. A., Law, M. F. & Martin, M. A. (1979) *J. Biol. Chem.* **254,** 4876–4883.
22. Palmer, G. H., Barbet, A. F., Davis, W. C. & McGuire, T. C. (1986) *Science* **231,** 1299–1302.