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A Cloned DNA Probe Identifies *Cowdria ruminantium* in *Amblyomma variegatum* Ticks

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Heartwater, caused by *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma*, is a constraint to ruminant animal production in sub-Saharan Africa. This rickettsial disease could spread from endemically infected areas of sub-Saharan Africa and certain Caribbean islands to other countries, including the United States, in which *Amblyomma* ticks exist. To detect *C. ruminantium* in tick vectors and animals, we made DNA probes from *C. ruminantium* DNA isolated from endothelial cell cultures. Two clones were evaluated; pCS20 from Crystal Springs (Zimbabwe) strain DNA had a 1,306-bp insert, and pCR9 from Kiswani (Kenya) strain DNA had a 754-bp insert. Both DNA probes detected 1 ng of Crystal Springs DNA; however, the pCS20 probe had a 10-fold-greater ability to discriminate between *C. ruminantium* DNA and DNA from other organisms. Also, the pCS20 probe did not hybridize to 400 ng (highest amount tested) of DNA from bovine cells, 3 protozoa, 3 rickettsiae, and 12 bacteria. In all experiments, *C. ruminantium* DNA was detected in midguts from 99 of 160 *Amblyomma variegatum* nymphs infected as larvae and in midguts from 38 of 80 adult ticks infected as nymphs but not in midguts from control nymphs and adults. The presence of *C. ruminantium* in nymphs and adults was confirmed by transmission of heartwater to goats. The DNA sequences of both probes were determined; synthetic oligonucleotides from pCS20 are recommended as DNA probes for *C. ruminantium*.

Heartwater, caused by *Cowdria ruminantium*, is an important rickettsial disease of cattle, sheep, goats, and some wild ruminants (8). In sub-Saharan Africa, it is a very significant impediment to ruminant livestock production, similar in importance to theileriosis, babesiosis, anaplasmosis, and trypanosomiasis (29). Animals infected with highly virulent strains exhibit fever, diarrhea, and nervous system involvement resulting in circling and disorientation (25). Mortality from heartwater can be high when susceptible ruminants are introduced into endemic areas (25, 29).

C. ruminantium is transmitted biologically by ticks of the genus *Amblyomma* (9, 10, 22, 26). *Amblyomma variegatum* is the most important of 12 *Amblyomma* species shown to transmit *C. ruminantium* because it is the most widely distributed species (41) and is an efficient heartwater vector. Even though ticks (*A. maculatum* and *A. cajennense*) capable of transmitting *C. ruminantium* are present in both North America and South America (39, 40) and heartwater occurs in the Caribbean (7, 40), the disease has not been detected on the American continents.

Demonstrating *C. ruminantium* in ticks and in live animals is difficult and often requires subinoculation into susceptible animals. *C. ruminantium* differs from several other rickettsiae and protozoa because it is not detectable by light microscopic examination of stained blood smears, even during times when the blood is infectious for ruminants (8, 15). After infection of ruminants, organisms multiply in the cytoplasm of endothelial cells (8, 11, 15, 28) and possibly leukocytes (20, 32). At present, definitive diagnosis of heartwater in animals is made by finding colonies of *C. ruminantium* in endothelial cells of stained smears of brain tissue (31) obtained by biopsy or postmortem. In infected ticks, colonies of the organism are found in thick or thin sections of the midgut by light and electron microscopy (4, 16, 43). Since current procedures are unsuitable for the rapid detection of *C. ruminantium* in either live animals or ticks, a method for screening large numbers of samples for *C. ruminantium* is needed.

One method for detecting rickettsiae and other infectious organisms in host samples, including arthropod vectors, is nucleic acid hybridization. Usually an RNA or a DNA probe derived from cloned DNA fragments of an organism is used in laboratory and field studies (2, 13, 24, 36, 38). In this study, two clones of *C. ruminantium* DNA were made and sequenced: a 1,306-bp insert in pCS20 from the Crystal Springs (Zimbabwe) strain and a 754-bp insert in pCR9 from the Kiswani (Kenya) strain. The specificity and sensitivity of DNA probes made from pCS20 and pCR9 inserts were evaluated with DNA from animals, ticks, and various infectious agents. On the basis of the results, we propose the use of nucleic acid probes and primers based on the *C. ruminantium* DNA insert in pCS20 in further studies.

MATERIALS AND METHODS

Infection of goats with *C. ruminantium*. Goats were infected to feed ticks and to provide blood for cell culture infections. Blood stabilates in 10% dimethyl sulfoxide of two strains of *C. ruminantium*, Kiswani (18) and Crystal Springs (42), were stored in liquid nitrogen. Twenty-seven Galla-Toggenburg cross goats were infected intravenously with 8 ml of Kiswani stabilate, and 3 goats were infected with 8 ml of Crystal Springs stabilate. Goats were examined for clini-

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cal signs, and rectal temperatures were recorded daily. The diagnosis was confirmed in dead goats by postmortem lesions and the demonstration of colonies of *C. ruminantium* in stained brain smears (31).

***C. ruminantium* DNA isolation.** In vitro cultures of irradiated bovine aortic endothelial cells (5, 42) were inoculated with 2 ml of blood from goats acutely infected with the Kiswani and Crystal Springs strains. Organisms released into 25 ml of culture medium (30) were partially purified by differential centrifugation. Following centrifugation at 1,200 × *g* for 10 min at 4°C to remove cellular debris, organisms were pelleted at 10,000 × *g* for 15 min at 4°C and washed three times with cold 0.01 M phosphate–0.15 M sodium chloride (pH 7.4) (PBS). DNA was extracted and resuspended in 10 ml of 0.15 M sodium chloride–0.1 M EDTA (pH 8.0) as previously described (1, 23). To determine whether the partially purified organisms were infectious for goats, we resuspended organisms from 25 ml of medium in 1 ml of cold PBS, diluted the suspension 1:10, and inoculated 2 ml of each strain intravenously into each of two goats.

Identification of *C. ruminantium* DNA clones. For cloning, DNA from each strain was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and partially digested with *Sau*3A to a median size of 2 kbp. Three hundred nanograms of digested DNA was ligated, with T4 DNA ligase, to 100 ng of *Bam*HI-cleaved and dephosphorylated pUC19 (23). *Escherichia coli* JM109 cells were transformed with ligated plasmids by the calcium chloride method (34), and transformants were selected on medium (0.8% tryptone, 0.8% yeast extract, 0.5% sodium chloride, 1.5% agar) containing 100 μg of ampicillin per ml, 0.004% 5-bromo-4-chloro-3-indol-β-D-galactopyranoside, and 0.002% isopropyl-β-D-thiogalactopyranoside. Plasmid DNAs from 74 transformants (34 from Kiswani DNA and 40 from Crystal Springs DNA) were prepared by alkaline lysis (6), digested with *Eco*RI, and electrophoresed on 0.6% agarose gels. Plasmids with inserts were digested with *Kpn*I and *Xba*I, Southern blotted (37), and probed with infected and uninfected endothelial cell DNAs labeled with biotin-7-dATP (19). Probe binding was detected with a streptavidin-alkaline phosphatase conjugate and a color substrate (BlueGene; GIBCO BRL, Gaithersburg, Md.). Two recombinant plasmids, pCS20 from Crystal Springs DNA and pCR9 from Kiswani DNA, hybridized only to *C. ruminantium*-infected-cell DNA.

Characterization of pCR9 and pCS20 DNA inserts. Insert DNAs were removed from pCS20 and pCR9 with *Xba*I and *Kpn*I, electrophoresed on 0.6% agarose gels, recovered by electroelution, and labeled with [^{32}P]dCTP with a random primer labeling system or by nick translation (GIBCO BRL). The probes were hybridized to restriction endonuclease-digested Crystal Springs strain DNA after Southern blotting. Probe specificity was evaluated by hybridization with DNAs isolated from *C. ruminantium*-infected endothelial cells, uninfected endothelial cells, goat leukocytes, calf thymus, *Anaplasma marginale*, *Trypanosoma brucei*, *Babesia bigemina*, *B. bovis*, *Ehrlichia risticii*, a new bovine *Chlamydia*-like organism, *E. coli*, *C. pneumoniae*, *Campylobacter jejuni*, *C. fetus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Clostridium perfringens*, *Actinobacillus pyogenes*, and *Listeria monocytogenes*. The sources of these DNAs have been described elsewhere (9a). For hybridization (36), the DNAs were applied to a nitrocellulose membrane with a vacuum manifold apparatus (Hybriblot; GIBCO BRL).

Infection of ticks with *C. ruminantium*. The tick colony was derived from an engorged adult female *A. variegatum* tick collected from a cow in the Narok district of Kenya. To obtain infected nymphs, we applied larvae to skin patches 5, 6, and 7 days after inoculating goats with the Kiswani strain (3, 14). Control nymphs were prepared by applying larvae to uninfected goats. Engorged larvae dropping off goats 7 to 11 days after attachment were collected and allowed to moult into nymphs for 16 to 21 days at 28°C and 80% relative humidity. Nymphs were allowed to harden for 7 days under the same conditions, engorged on an uninfected goat for 7 to 12 days, and allowed to harden again for 13 days. Nymphs were washed with distilled water and 70% ethanol, and the midguts were removed and stored individually at −70°C.

Infected adult ticks were obtained by allowing uninfected nymphs to feed on infected goats. Controls fed on uninfected goats. After moulting from nymphs, adult ticks were allowed to harden for 20 days, dried for 1 day at 37°C, and washed with water and ethanol, and the midguts were removed and stored individually at −70°C. Five midguts were homogenized in 20 ml of minimal essential medium (MEM) and diluted 1:70 with minimal essential medium containing 3.5% bovine serum albumin, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Eight milliliters of this suspension was inoculated intravenously into each of eight goats. The same amount of midgut suspension from five control adult ticks was inoculated into each of three goats.

For hybridization, individual tick midguts were solubilized (13) for 18 h at 37°C in 200 μl of 0.1 M Tris-HCl (pH 7.5) containing 0.15 M sodium chloride, 0.012 M EDTA, 1% sodium dodecyl sulfate, and 100 μg of proteinase K per ml and centrifuged for 2 min in a Microfuge. DNA was extracted from the supernatant (34), precipitated with ethanol, resuspended in 200 μl of TE buffer, denatured with 0.3 M NaOH, and neutralized with 1 M ammonium acetate. Fifty microliters, representing 25% of the midgut DNA, was applied to nitrocellulose membranes with a vacuum manifold, baked for 2 h at 80°C, hybridized with the pCR9 probe (19), and processed for autoradiography (13). In one experiment, the midgut DNA from adult ticks was resuspended in 100 μl of TE buffer, and 10 μl was applied to nylon membranes and hybridized with the pSC20 probe.

DNA sequencing. Double-stranded DNA of plasmid inserts was sequenced by dideoxy nucleotide chain termination (35) with Sequenase (United States Biochemical). Oligonucleotide primers specific for pUC19 were used in the first sequencing reactions. Additional primers were then synthesized on the basis of the sequences obtained.

Indirect fluorescent-antibody test. Sera from three goats, two infected with the Kiswani strain and one infected with the Crystal Springs strain, were used in an indirect fluorescent-antibody test to identify *C. ruminantium* in cell cultures. *C. ruminantium* organisms partially purified from 5 ml of cell culture medium as described above were resuspended in 1 ml of PBS, and 50 μl was added to a well of a Teflon-coated microscope slide with 10 wells, immediately withdrawn, and transferred serially to all 10 wells. Slides were dried, fixed in acetone for 7 min, wrapped in paper and aluminum foil, and stored at −70°C. Serial dilutions of sera (50 μl) in PBS were applied to wells and incubated at 37°C in a humid chamber for 30 min. Slides were washed, stained with fluorescein isothiocyanate-conjugated rabbit anti-goat immunoglobulin G, and examined.

Nucleotide sequence accession numbers. The GenBank accession number for the pCS20 nucleotide sequence derived from *C. ruminatium* Crystal Springs DNA (see Fig. 7) is X58242, while the GenBank accession number for the pCR9

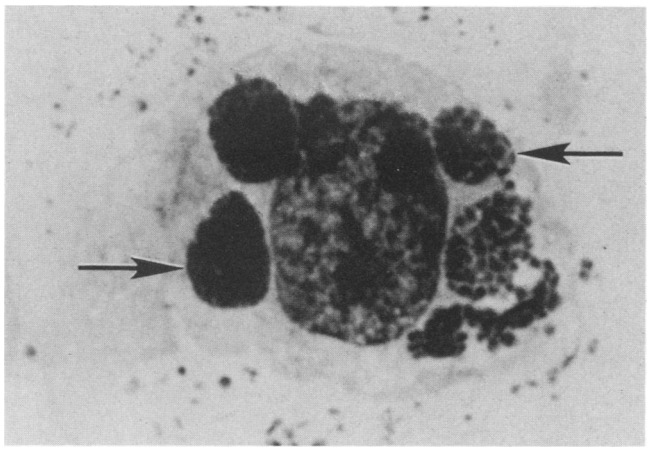

FIG. 1. Colonies (arrows) of *C. ruminantium* in the endothelial cell cytoplasm of an in vitro culture. Note individual *C. ruminantium* organisms outside the endothelial cell membrane.

nucleotide sequence derived from *C. ruminantium* Kiswani DNA (data not shown) is X58243.

RESULTS

In vitro propagation of *C. ruminantium*. *C. ruminantium* was propagated in endothelial cell cultures (42) infected with blood from goats rickettsemic with the Kiswani and Crystal Springs strains. Replication was monitored by the presence of cytoplasmic colonies (Fig. 1), with uninfected endothelial cell cultures as controls. To confirm the identity of organisms in cell cultures, we stained infected cells by indirect fluorescent-antibody staining with sera from two goats infected with the Kiswani strain and one infected with the Crystal Springs strain. Homologous antibody titers ranged between 20,000 and 80,000, with heterologous antibody titers being two- to fourfold lower. Titers of preinfection sera were less than 200, and infected goat sera did not react with uninfected endothelial cells. Moreover, following inoculation of two goats each with each strain from cultures, all four goats died and all had *C. ruminantium* colonies in vascular endothelial cells in stained brain smears.

***C. ruminantium* DNA clones.** Southern blots of the plasmid inserts were probed with biotin-7-dATP-labeled DNAs isolated from Kiswani strain-infected and uninfected endothelial cells. Inserts in two recombinant plasmids, pCS20 (Crystal Springs) and pCR9 (Kiswani), hybridized to Kiswani strain-infected-cell DNA but not to uninfected-cell DNA. Gel electrophoresis of restriction endonuclease-digested DNA fragments demonstrated that pCS20 and pCR9 contained inserts of approximately 1,500 and 750 bp, respectively.

To confirm that the pCS20 and pCR9 inserts were *C. ruminantium* DNA and to determine whether copies of the same or related genes were present in the genome, we probed Southern blots of Crystal Springs strain DNA. [^{32}P]dCTP-labeled pCR9 and pCS20 DNA probes bound to several fragments of *Pst*I-digested Crystal Springs DNA (Fig. 2A and B).

DNA probe specificity and sensitivity. The specificity of the pCS20 probe was higher than that of the pCR9 probe. The pCR9 probe yielded detectable hybridization signals with 40 ng of *E. coli*, *C. fetus*, and *C. perfringens* DNAs and with 400 ng of *A. marginale* and *S. aureus* DNAs. In contrast, the

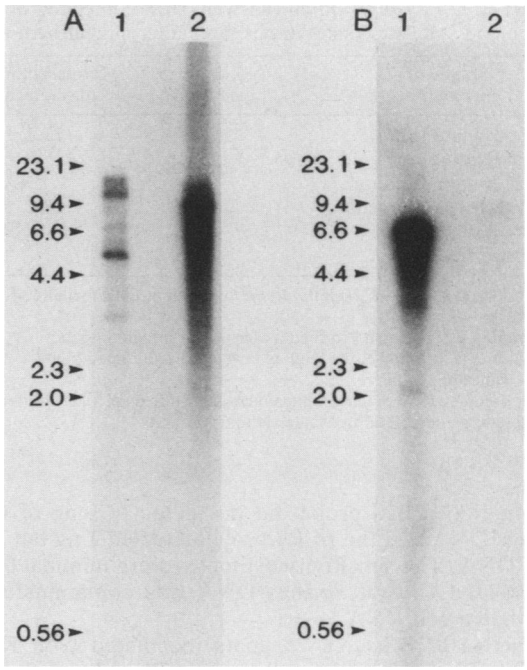

FIG. 2. Southern blot of *C. ruminantium* Crystal Springs DNA (lanes 1) and *E. coli* DNA (lanes 2) after *Pst*I digestion. (A) Hybridization with [^{32}P]dCTP-labeled pCR9 insert DNA (10^8 cpm/μg). (B) Hybridization with [^{32}P]dCTP-labeled pCS20 insert DNA (10^8 cpm/μg). DNA markers of known sizes (kilobase pairs) are indicated on each panel.

pCS20 probe yielded no detectable signals with the highest level of DNA tested (400 ng) from any organism examined. These included *T. brucei*, *A. marginale*, *B. bigemina*, *B. bovis*, and *E. coli* (Fig. 3). Additionally, the pCS20 probe yielded no detectable signals with 400 ng of DNA from calf thymus, *E. risticii*, a new bovine *Chlamydia*-like organism, *C. pneumoniae*, *C. jejuni*, *C. fetus*, *S. aureus*, *P. aeruginosa*, *S. faecalis*, *C. perfringens*, *A. pyogenes*, and *L. monocytogenes*. Hybridization of the pCR9 probe, but not the pCS20 probe, with *E. coli* DNA was confirmed in Southern blots (Fig. 2).

The sensitivity of the pCS20 DNA probe was determined in dot blot hybridizations; the pCS20 probe detected 10 and 1 ng of Crystal Springs strain DNA (Fig. 3). In blots not

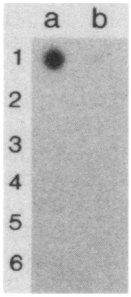

FIG. 3. Dot blots of various DNAs hybridized with [^{32}P]dCTP-labeled pCS20 insert DNA (10^8 cpm/μg). In row 1, lanes a and b contain 10 and 1 ng of *C. ruminantium* Crystal Springs DNA, respectively. Rows 2 to 6 contain 400 ng (lane a) and 40 ng (lane b) of *A. marginale*, *T. brucei*, *B. bigemina*, *B. bovis*, and *E. coli* DNAs.

TABLE 1. *C. ruminantium* (Kiswani strain) infection of goats with blood stabilate, tick nymphs, and adult midgut homogenate

Source of infection	No. dead/no. inoculated[a]	Incubation period (days, mean ± SD)
Blood stabilate (8 ml)	24/25	11.8 ± 3.3
Nymph feeding[b]	9/10	15.0 ± 4.3
Control nymph feeding[c]	0/5	NA[d]
Adult midgut homogenate[e]	8/8	12.2 ± 2.4
Control adult midgut homogenate[e]	0/3	NA

[a] All dead goats had *C. ruminantium* colonies in endothelial cells of brain smears.
[b] Nymphs were allowed to feed as larvae on infected goats.
[c] Nymphs were allowed to feed as larvae on uninfected goats.
[d] NA, not applicable.
[e] Each goat was inoculated intravenously with 8 ml of a diluted midgut homogenate as described in Materials and Methods.

shown, the pCS20 probe did not detect 0.1 ng of Crystal Springs DNA and the pCR9 probe detected 1 ng but not 0.1 ng of DNA. The sensitivity estimates were minimal because the isolated Crystal Springs DNA was contaminated with endothelial cell DNA.

Infection of ticks. Of 25 goats inoculated with Kiswani stabilate for tick feeding, all became ill and one recovered (Table 1). Confirmation of the infected status of tick nymphs allowed to feed as larvae on infected goats was obtained by successful transmission of heartwater to 10 goats during nymphal engorgement. Five goats used to engorge nymphs allowed to feed as larvae on uninfected goats remained asymptomatic (Table 1). Analogously, the presence of *C. ruminantium* in midguts of adult ticks allowed to feed as nymphs on infected goats was confirmed by parenteral inoculation of a midgut homogenate into eight goats. All three goats inoculated with a midgut homogenate from adult ticks allowed to feed as nymphs on uninfected goats remained asymptomatic (Table 1).

Detection of *C. ruminantium* DNA in ticks. Both probes proved to be effective at detecting *C. ruminantium* in ticks. The pCS20 probe detected *C. ruminantium* DNA in at least 28 of 70 midguts of adult ticks allowed to feed as nymphs on infected goats (Fig. 4). With the pCR9 probe, *C. ruminantium* DNA was detected by dot blot hybridization in 29 of 60 midguts of nymphs allowed to feed as larvae on infected goats but not in 10 midguts of nymphs allowed to feed as larvae on uninfected goats (Fig. 5). In another experiment (data not shown), the pCR9 probe detected *C. ruminantium* DNA in 70 of 100 midguts of nymphs allowed to feed as larvae on infected goats but not in 10 midguts of nymphs allowed to feed as larvae on uninfected goats. The pCR9 probe hybridized to midgut DNAs from 10 adult ticks allowed to feed as nymphs on infected goats but not from 10 adult ticks allowed to feed as nymphs on uninfected goats (Fig. 6).

Sequences of pCR9 and pCS20 DNA inserts. The nucleotide sequences of both strands of the pCS20 and pCR9 DNA inserts were determined. The 1,306-bp insert in pCS20 contained two long open reading frames of 513 (nucleotides 205 to 717) and 459 (nucleotides 717 to 1175) nucleotides (Fig. 7). There were no obvious ribosome binding sites associated with the open reading frames, and it is not known whether they were transcribed. The 737-bp insert in pCR9 (data not shown) contained one long open reading frame of 243 nucleotides. Even though the open reading frame started with an ATG codon, there was no obvious ribosome binding site upstream of the ATG. Both insert sequences contained approximately 70% A+T, which caused numerous potential prokaryotic promoter consensus sequences to be found.

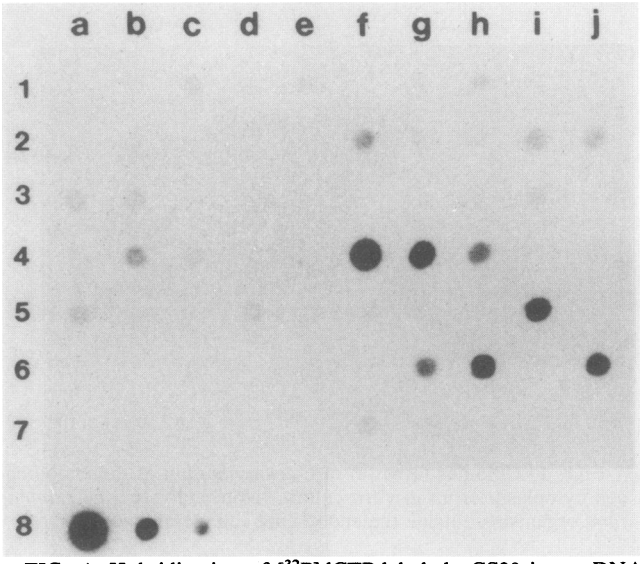

FIG. 4. Hybridization of [^{32}P]dCTP-labeled pCS20 insert DNA (10^8 cpm/μg) with midgut DNA from 70 adult ticks allowed to feed as nymphs on a goat infected with *C. ruminantium* Kiswani (rows 1 to 7, lanes a to j). Row 8 contains 100, 10, 1, and 0.1 pg of unlabeled insert DNA, in lanes a to d, respectively.

DISCUSSION

Currently available control procedures for heartwater are inadequate. As a base for significant progress on heartwater control, more needs to be known about the epidemiology of the causative organism. A sensitive and specific detection

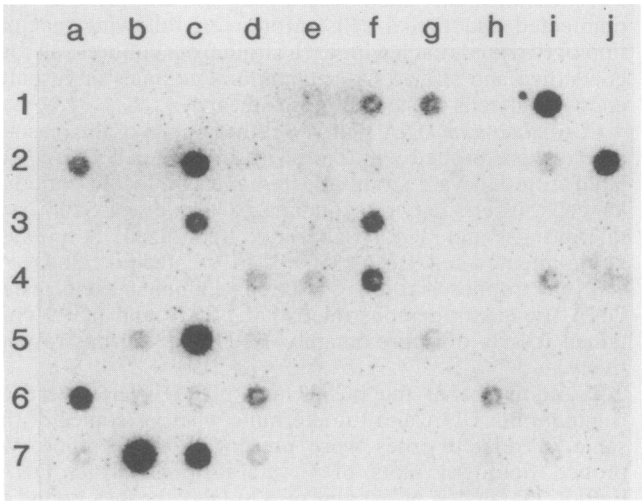

FIG. 5. Hybridization of [^{32}P]dCTP-labeled pCR9 insert DNA (10^7 cpm/μg) with midgut DNA from tick nymphs allowed to feed as larvae on either *C. ruminantium*-infected goats (60 nymphs) or uninfected goats (10 nymphs). Midgut DNAs from the 10 nymphs allowed to feed as larvae on uninfected goats are in positions 1h, 2g, 3b, 4h, 5f, 5i, 5j, 6g, 7f, and 7j. All other positions contain midgut DNAs from the 60 nymphs allowed to feed as larvae on infected goats.

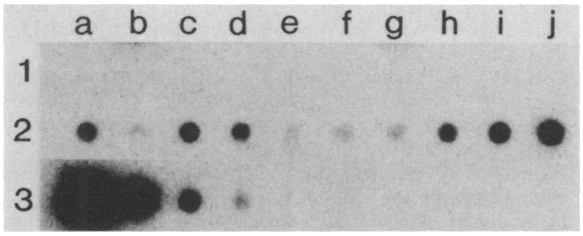

FIG. 6. Hybridization of [^{32}P]dCTP-labeled pCR9 insert DNA (10^7 cpm/μg) with midgut DNAs from 10 adult ticks allowed to feed as nymphs on an infected goat (row 2, lanes a to j) and from 10 allowed to feed on an uninfected goat (row 1, lanes a to j). Row 3 contained 100, 10, 1, 0.1, and 0.01 ng of unlabeled pCR9 insert DNA in lanes a to e, respectively.

method for *C. ruminantium* is a prerequisite for such studies. It was for this purpose that the current experiments were done to develop a DNA probe to detect *C. ruminantium* organisms in cell cultures, ticks, and mammalian hosts.

To isolate *C. ruminantium* genomic DNA for cloning, we used a bovine aortic endothelial cell culture system for growing *C. ruminantium* (5, 42). This system supported the growth of strains from Kenya (Kiswani) and Zimbabwe (Crystal Springs) in quantities adequate to yield DNA for the present cloning experiments. The identity of the organism in cultures was confirmed by animal inoculation, histologic appearance, and serologic detection with antiserum to *C. ruminantium*. Even though the strains used originated from different countries, biological and immunological characterizations are incomplete.

Both the pCS20 and the pCR9 probes hybridized to *C. ruminantium* DNA at a minimum sensitivity threshold of 1.0 ng but failed to yield a detectable signal with 400 ng of normal bovine DNA. The specificity of the pCS20 probe, however, was greater than that of the pCR9 probe, which yielded signals with three axenic bacteria at levels as low as 40 ng. In contrast, the pCS20 probe did not yield signals with 400 ng of DNA from 3 protozoal, 3 rickettsial, and 12 bacterial organisms. Therefore, the minimum specificity ratios were 400 for the pCS20 probe and 40 for the pCR9 probe. The actual specificity ratio for the pCS20 probe may be improved if this probe is evaluated with more highly purified *C. ruminantium* DNA.

Because currently available methods for the detection of *C. ruminantium* in ticks (12) are unsatisfactory for several reasons (21, 43), DNA probes were evaluated with infected tick tissues. Ticks for hybridization were exposed as either larvae or nymphs by allowing them to engorge on goats inoculated with Kiswani blood stabilate. Tick infection was confirmed by transmission to goats. Midgut DNA was probed because this tissue was previously shown to be a site of *C. ruminantium* replication (9, 17, 18). The pCS20 probe detected *C. ruminantium* DNA in 28 of 70 adult ticks allowed to feed as larvae on infected goats. *C. ruminantium* DNA

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1     CTAGAGAACGAAAAGGCACTCCAATATTCTTGAAAGAAGATCACAAATGCTATATCAAAAATTATCAAATATGATTTTAAAAATACTCAATTGCTTAATG

101   AAGCACTAACTCACCCAAGTGTTCTTTCTAAAGACAATAATAATTTCAATTACGAAAGACTTGAATTTTTAGGAGATGCTGTGCTAAATTTAGTAATTTC

201   CGAAATGCTATTCAATATTTTTCCCTATGATACAGAAGGTAACCTCGCAAAAAAGAAAACAGCTTTGGTTTGTGGAACAAAACTAGTAGAAATTGCACAA

301   TCTATAAATTTAGGAATATTTATTATCATGTCAGATGGAGAAAGGAGTTGTGGTGGAGCTAAAAATTCTAACAACTTAGAAAATGCATTAGAAGCACTAA

401   TAGGTGCTATATATCTTGATGGAGGATTAAAAGCAGCAAAAGACTTTATTTTTCTATTCTGGAAAAATTCTGCAACACATATGAAAGTACCACCACAAGA

501   TGCAAAAACTATCTTACAAGAATGGGCACAGAGTAAAGGATTTCCCGCACCAAGTTATCATATAATAAACAAATCTGGTCCAGATCACAATCCTTGCTTT

601   ACTGTAGAAGTAAGAATCGATTCACATGAAACATTACATGCAACTGGTCATAACAAAAAACTAGCTGAACAGAAAGCTGCTAGTTTAATGTTAGAAAAAA

701   TTAATTACAAAATAAAATGACACAAAAAGCAAAAAATACTATCTATTTATTAATATTAATCATACTATCTATGTTATGTTTAGTATATGCTTCAGTACCA

801   TTATACAGCATATTCTGTAAAGTTACAGGATACGGAGGAACTGTCAGAACAACTACAGTAACACAATCTAAACTCGGTAAGACTACCATTAAAATAAGAT

901   TTAATGCAGACATAAATAAAGAACTTCCATGGAAATTTTATCCTGAAATACCTTATACAACTGTCAAACCAGGAGAACAAAAATTAATTTTTTATAGAGC

1001  AGAAAATTTAACTAATGAATACGTGTCAGGTATGGCTGTATATAATGTTACCCCTTACAAAGTAGGTAAATATTTCAATAAGGTAGCCTGCTTCTGTTTT

1101  ACCAAACAAACGTTATCTCCTTATCAAAAAACTATTATGCCTGTATCATTTTTTATAGATCAAGTCTATATAAATTAGACAAGAATTATTGTACAATATT

1201  ATATAAATAAACGGCCTACAAATAAAGAGACACACAATGCAGTGTTACATATTGTATATTACCTTAATAAAGTTTCCACAAATATCAAATAACAGATTAA

1301  ATTAAT
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FIG. 7. Nucleotide sequence of the *C. ruminantium* DNA insert in pCS20. Start codons of open reading frames are in boxes, and stop codons are underlined.

was detected by the pCR9 probe in 99 of 160 nymphs allowed to feed as larvae on infected goats and in all of 10 adult ticks allowed to feed as nymphs on infected goats. All control ticks examined were negative. These results demonstrated that DNA probes detected *C. ruminantium*-infected ticks irrespective of the stage at which infection occurred. Differences in signal intensity between ticks were interpreted as reflecting differences in the numbers of organisms they contained (2, 24).

The DNA sequences of both probes were determined, and the sequence for pCS20 is presented (Fig. 7). We propose that the sequence of the pCS20 probe, which had a higher specificity ratio, be used to make synthetic oligonucleotides for DNA probes and to make synthetic primers for polymerase chain reactions (27, 33).

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