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A Cloned DNA Probe for *Cowdria ruminantium* Hybridizes with Eight Heartwater Strains and Detects Infected Sheep

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The DNA probe pCS20, which was cloned from the DNA of the Crystal Springs heartwater strain from Zimbabwe, cross-reacted with DNAs of heartwater strains from all endemic areas, including four heartwater strains from Zimbabwe, two strains from South Africa, one strain from Nigeria, and the Gardel strain from the Caribbean island of Guadeloupe. By nucleic acid hybridization, the pCS20 DNA probe detected *Cowdria ruminantium* DNA in all DNA preparations made from plasma samples from infected sheep before and during the febrile reaction. Synthetic oligonucleotides were prepared for amplification of specific *C. ruminantium* DNA sequences by the polymerase chain reaction (PCR). Amplification of two DNA products (181 and 279 bp) from pCS20 DNA and *C. ruminantium* genomic DNA of heartwater strains was demonstrated. In contrast, amplification of these products or any other products was not possible from genomic DNAs of *Anaplasma marginale*, *Babesia bigemina*, *Trypanosoma brucei brucei*, *Escherichia coli*, and bovine endothelial cells. The cross-reactivities of the ^{32}P-labeled PCR products with genomic DNAs from several heartwater strains were similar to those with the pCS20 DNA probe. A nucleic acid-based test that uses hybridization assays and PCR provides a sensitive method for the detection of heartwater in both animals and ticks and has applications in epidemiological studies for the disease, which may allow for improved disease control.

Heartwater is an economically important disease of ruminant livestock caused by a rickettsial agent, *Cowdria ruminantium*, and transmitted by ticks of the genus *Amblyomma* (24). In susceptible animals, heartwater can cause mortalities ranging from 20 to 90% (24). To control such high mortalities and to fully understand the epidemiology of the disease, sensitive and practical diagnostic methods are needed to detect the organism in vivo. Definitive diagnosis of heartwater in animals is made by demonstrating the presence of the organisms within endothelial cells of Giemsa-stained brain smears made from biopsy specimens or at the time of death (16). This method is impractical for routine use; in addition, it does not have the sensitivity required to detect the organisms in animals that have recovered but that may be carriers of the disease (1, 24). In carriers, heartwater is detected by transfer of blood to susceptible hosts or by transmission via ticks (1, 24). While carrier animals may be detected by serological assays, these tests do not detect the organisms in vivo.

To detect heartwater in infected animals, we used the Crystal Springs strain-derived pCS20 DNA probe, which has been described previously for the detection of *Cowdria ruminantium* (Kiswani strain) in *Amblyomma variegatum* ticks (25). DNA probes have been developed to detect protozoa, bacteria, viruses, and rickettsiae in arthropod and animal hosts by nucleic acid hybridization and have been found to have high sensitivities and specificities (3, 7, 9, 21, 22). To further improve the sensitivity of nucleic acid-based diagnostic tests, the polymerase chain reaction (PCR) has been used to amplify DNA sequences (14, 17) which are specific to an organism. These include the DNA sequences of *Plasmodium falciparum* (23), *Trypanosoma brucei* and *Trypanosoma congolense* (13), *Rickettsia rickettsii* and *Rickettsia typhi* (2), *Toxoplasma gondii* (19), cytomegalovirus (6), and hepatitis B virus (11).

In this study we showed that the pCS20 DNA probe can detect heartwater-infected animals by nucleic acid hybridization and that the sensitivity of this test may be improved by amplification of *C. ruminantium*-specific sequences by PCR.

MATERIALS AND METHODS

Animals. Ten female Dorper sheep were used in this study. All sheep were serologically negative for heartwater on the basis of results of the indirect fluorescent-antibody test (20). Five sheep (4536, 4556, 4564, 4572, and 4573) were each infected with 3 ml of the Crystal Springs tissue culture-derived *C. ruminantium* organisms (5), and five sheep (4532, 4557, 4565, 4566 and 4570) were uninfected controls. All sheep were bled before infection and from day 3 after infection until the time of death or recovery. Giemsa-stained brain biopsy specimens and brain smears from the infected sheep were prepared during the febrile reaction and at the time of death, respectively, for confirmation of heartwater by the identification of *C. ruminantium* colonies within capillary endothelial cells (16, 24). In addition, the sheep were necropsied to further confirm heartwater by the presence of hydropericardium, hydrothorax, and widespread hemorrhages on serosal and mucosal surfaces (15). In a

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second experiment, five Merino sheep (010, 011, 012, 013, 014) were infected with 10^5 in vitro-cultured Crystal Springs organisms, which were quantitated by the fluorescein diacetate viability staining method (12). The organisms were purified on discontinuous Percoll gradients ranging from 0 to 40% in phosphate-buffered saline (PBS). The purified organisms were harvested from the 0% layer and, prior to quantitation, were washed once in PBS by centrifugation at 30,000 × *g* for 30 min. Plasma was prepared from sheep blood that was collected before infection, on day 0, and on days 5 and 14 (during the febrile reaction) after infection. At the time of death, Giemsa-stained brain smears were prepared to confirm heartwater (16).

Preparation of DNA. Fresh plasma was prepared from uncoagulated EDTA sheep blood and was centrifuged at 30,000 × *g* for 30 min at 4°C to pellet the *C. ruminantium* organisms. The supernatant was discarded, and DNA was extracted from the pellet by solubilizing the pellet with 200 μl of buffer containing 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.012 M EDTA, 1% sodium dodecyl sulfate, and 5 mg of lysozyme per ml at 37°C for 30 min. Proteinase K (100 μg/ml) was added, and the samples were incubated overnight. The samples were extracted once with phenol-chloroform-isoamyl alcohol; and the DNA was precipitated with 2 volumes of absolute ethanol, dried, and resuspended in 250 μl of Tris-EDTA buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing 1 μg of herring sperm DNA (as carrier DNA). The DNA samples were denatured with 0.4 N NaOH at 37°C for 30 min and applied to nylon membranes (Genescreen; DuPont) with a hybridot vacuum manifold (GIBCO BRL Life Technologies Inc.).

DNA from *C. ruminantium* strains was prepared from in vitro cultures of infected bovine endothelial cells (5). DNA from the following strains was prepared: Crystal Springs, Zwimba, Nyatsanga, Lemco T3, and Palm River (Zimbabwe strains) (5); Ball 3 (10) and Welgevonden (8) (South African strains); Nigeria; and the Gardel strain from Guadeloupe. The Gardel strain was kindly provided by E. Camus in infected *A. variegatum* ticks, which were fed on susceptible sheep. During the febrile reaction, the sheep blood was collected and plasma was inoculated into endothelial cell cultures. Supernatants of infected cultures (when all cells were infected and lysed by infection) for all strains were centrifuged at 2,000 × *g* for 10 min to reduce endothelial cell contamination. The organisms were pelleted, washed three times in PBS by centrifugation at 30,000 × *g* for 30 min at 4°C before the DNA was extracted by standard methods (18), and applied to nylon membranes as described above. DNA concentrations were estimated by ethidium bromide staining after agar gel electrophoresis (18).

pCS20 DNA probe. The pCS20 DNA probe was prepared from a Crystal Springs strain genomic DNA library prepared in plasmid pUC19 (25). Initial characterization of the pCS20 DNA probe revealed two open reading frames (ORFs) in the 1,306-bp insert. To isolate the probe from plasmid DNA, the plasmid was digested with *Xba*I and *Kpn*I restriction endonucleases, and the insert was isolated from low-melting-point agarose gels (18). For hybridization assays, 100 ng of this gel-purified probe or PCR products was labeled with [α-^{32}P]dCTP by the random primer extension method (18).

Hybridization of pCS20 and PCR-amplified DNA probes. The nylon membranes blotted with the DNA samples were prehybridized overnight at 42°C in a hybridization buffer containing 10% dextran sulfate in formamide and were hybridized to an [α-^{32}P]dCTP-labeled pCS20 probe or PCR-amplified products for 16 h (18). After hybridization, the blots were washed under highly stringent conditions and were exposed to X-ray films record the hybridization signals (18). If necessary, the probes were stripped off the nylon membranes by washing the blot at 42°C for 30 min in 0.4 N NaOH; this was followed by a wash with 0.2 M Tris-HCl (pH 7.5)–0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 42°C for another 30 min. The treated blots were exposed to X-ray film, to determine complete stripping of the probes, before the blots were again hybridized to fresh probes.

PCR amplification. Oligonucleotides AB 126 (GTAACAC AATCTAAACTCGGTAAG) and AB 127 (CAGCCATACC TGACACGTATTCAT; ORF-1), and AB 128 (ACTAGTAG AAATTGCACAATCTAT) and AB 129 (TGATAACTTGGT GCGGGAAATCCTT; ORF-2) were synthesized from the two ORFs of the pCS20 DNA probe (25). Amplification of 181- and 279-bp fragments (from ORF-1 and ORF-2, respectively) was attempted by PCR of 5 ng of template DNA from *C. ruminantium* Crystal Springs, Welgevonden, Welgevonden-infected cell DNA, Nyatsanga, and Gardel; 5 ng of DNA from *Anaplasma marginale*, *Babesia bigemina*, *Trypanosoma brucei brucei*, and *Escherichia coli* organisms; and 5 ng of DNA from uninfected bovine endothelial cells. The amplification of the two PCR products was conducted in separate reactions. Each PCR mixture contained a volume of 50 μl and included 10 mM Tris-HCl (pH 8.3); 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1.5 mM $MgCl_2$; 50 mM KCl; 0.001% gelatin (GeneAmp DNA amplification kit; Perkin-Elmer Cetus Corporation, Norwalk, Conn.); oligonucleotides at 0.1 μM; and 5 ng of template DNA. The reaction mixture containing the DNA was denatured at 95°C for 10 min and was cooled on ice. *Taq* polymerase (1.25 U) was added to the reaction mixture, which was then overlaid with 50 μl of paraffin oil and placed in a Coy Tempcycler (Coy Laboratory Products) that was set to cycle 45 times. Each cycle was set at 94°C for 1 min for DNA denaturation, 55°C for 1 min to allow the primers to anneal, and then at 72°C for 2 min for extension of the reaction. After amplification, the Tempcycler was programmed to 72°C for 10 min to complete the extension of newly formed DNA and then to cool the DNA to 4°C. The paraffin was removed from each reaction mixture, and 10 μl of the amplified DNA sample was analyzed on a 1.5% agarose gel (18). The amplified products were visualized by UV light illumination and photography after ethidium bromide staining (18).

RESULTS

Cross-reactivity of the pCS20 DNA probe. In addition to cross-reaction with the Kiswani strain from Kenya (25), the pCS20 DNA probe cross-reacted with the DNAs of four heartwater strains from Zimbabwe (Lemco T3, Nyatsanga, Zwimba, Palm River), the DNAs of two strains from South Africa (Welgevonden, Ball 3), the DNA of a strain from Nigeria, and the DNA of strain Gardel from the Caribbean island of Guadeloupe. With the exception of the Nyatsanga strain, which showed a diminished but positive reaction with the probe, the probe's reactions with DNAs from all other strains were similar to the reaction with the homologous Crystal Springs strain DNA (Fig. 1). This result shows that the pCS20 DNA probe could the detect DNAs of all tested strains from areas of the world where heartwater occurs.

Detection of *C. ruminantium* DNA in sheep plasma. In one experiment, five sheep (4536, 4556, 4564, 4572, 4573) were inoculated with Crystal Springs strain organisms and five sheep (4532, 4557, 4565, 4566, 4570) were included as

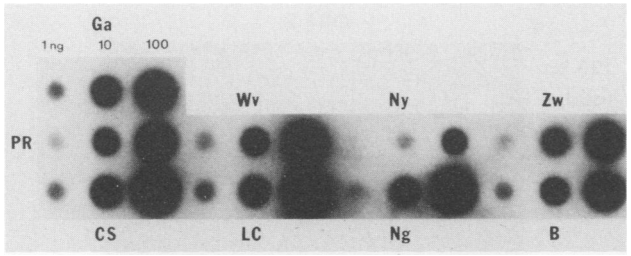

FIG. 1. Hybridization of ^{32}P-labeled pCS20 DNA probe to genomic DNA samples containing 1, 10, and 100 ng of DNA from heartwater strains Gardel (Caribbean) (Ga); Crystal Springs (CS), Lemco T3 (LC), Nyatsanga (Ny), Palm River (PR), and Zwimba (Zw) (all from Zimbabwe); Ball 3 (B) and Welgevonden (both from South Africa) (Wv); and Nigeria (Ng).

uninfected controls. The clinical parameters for these sheep are presented in Table 1. All five infected sheep were found to have *C. ruminantium* colonies in endothelial cells in Giemsa-stained brain smears or biopsy specimens collected at the time of death or during the febrile reaction, respectively. Of the five sheep, three (4556, 4564, and 4572) died, and at necropsy, they were further confirmed to have been infected with heartwater. In the control group, one sheep (4570) died of peritonitis caused by *Corynebacterium pyogenes*.

In this experiment, 5 or 10 ml of plasma from blood samples of all sheep was prepared for DNA probe analysis. *C. ruminantium* organisms in plasma were pelleted and the DNA was extracted. After hybridization with the pCS20 probe, weakly positive signals were generally detectable by day 5 after infection in DNA samples derived from plasma samples of both 5 and 10 ml from all infected sheep (Fig. 2A). The intensity of the signal between animals varied from weak to strong and was probably related to the number of organisms in plasma. Nevertheless, the signal intensity in each infected sheep was above the background signal obtained with DNA samples derived from plasma samples on

TABLE 1. Clinical parameters for sheep infected with Crystal Springs strain

Expt and sheep no.	Infected	Days to febrile reaction	Brain biopsy or smear	Days to death
Expt 1				
4536	+	6	+	
4556	+	8	+	14
4564	+	6	+[a]	10
4572	+	6	+[a]	9
4573	+	6	+	
4532[b]	−		ND[c]	
4557[b]	−		ND	
4565[b]	−		ND	
4566[b]	−		ND	
4570[b]	−		—[d]	18
Expt 2				
010	+	6	+[a]	10
011	+	12	+[a]	19
012	+	11	ND	
013	+	13	+[a]	18
014	+	11	+[a]	17

[a] Positive by brain smear at death.
[b] Control sheep.
[c] ND, not done.
[d] —, The sheep died of peritonitis.

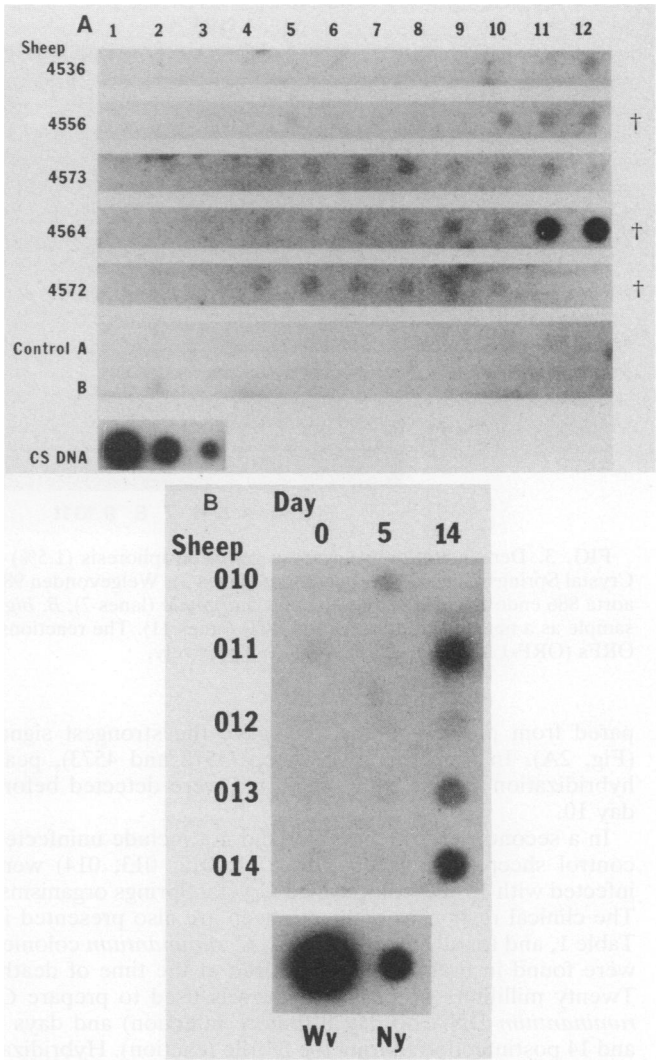

FIG. 2. (A) Hybridization of ^{32}P-labeled pCS20 DNA probe to DNA samples prepared on each day from 5 and 10 ml of plasma from heartwater-infected sheep 4536, 4556, 4573, 4564, and 4572. Wells 1 and 2, samples from day 0; wells 3 and 4, samples from day 3 after infection; wells 5 and 6, samples from day 5; wells 7 and 8, samples from day 6; wells 9 and 10, samples from day 8; wells 11 and 12, samples from day 10. Plasma samples from control sheep are given in rows A and B. In row A, wells 1 to 4 are samples from sheep 4532, wells 5 to 8 are samples from sheep 4557, and wells 9 to 12 are samples from sheep 4565. In row B, wells 1 to 4 are samples from sheep 4566 and wells 5 to 8 are samples from sheep 4570. In all cases, the control sheep samples were from four different days of the experiment (days 0, 5, 8, 14) and from 10 ml of plasma. Row B, wells 9 to 12, has no samples. Positive control DNA samples from the Crystal Springs (CS DNA) heartwater strain, in amounts of 100, 10, and 1 ng of DNA, are given in wells 1 to 3, respectively. †, sheep that died. (B) Hybridization of ^{32}P-labeled pCS20 DNA probe to DNA samples prepared from 20 ml of plasma from sheep 010, 011, 012, 013, and 014 on days 0, 5, and 14 after infection. DNA samples from strains Welgevonden (Wv) and Nyatsanga (Ny) were included as positive controls.

day 0 and from control sheep (Fig. 2A). In three of five sheep, the peak hybridization signal was detected in DNA samples on day 10, which was during the febrile period. Sheep 4564 died on day 10 after infection, and DNA pre-

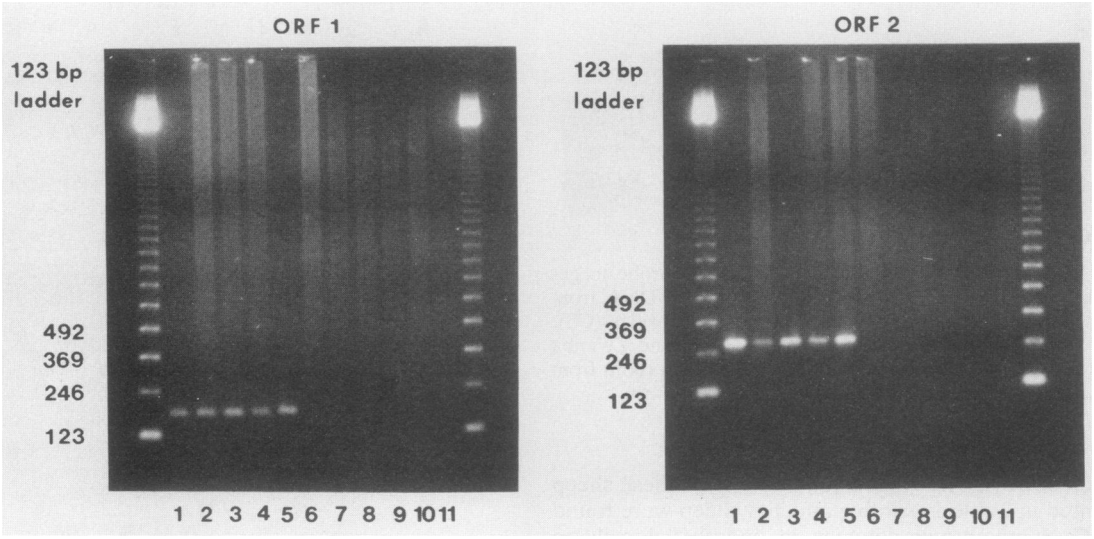

FIG. 3. Demonstration by agarose gel electrophoresis (1.5%) of PCR products amplified from the genomic DNAs of pCS20 (lanes 1), Crystal Springs (lanes 2), Welgevonden (lanes 3), Welgevonden 987-infected cells (lanes 4), Welgevonden 886-infected cells (lanes 5), bovine aorta 886 endothelial cells (lanes 6), *A. marginale* (lanes 7), *B. bigemina* (lanes 8), *T. brucei brucei* (lanes 9), *E. coli* (lanes 10), and no DNA sample as a negative control for the PCR (lanes 11). The reactions show the amplification of DNA products of 181 and 279 bp from the two ORFs (ORF-1 and ORF-2) of pCS20, respectively.

pared from plasma on this day gave the strongest signal (Fig. 2A). In the other two sheep (4572 and 4573), peak hybridization signals were weak and were detected before day 10.

In a second experiment which did not include uninfected control sheep, five sheep (010, 011, 012, 013, 014) were infected with 10^5 Percoll-purified Crystal Springs organisms. The clinical responses of these sheep are also presented in Table 1, and for all sheep that died, *C. ruminantium* colonies were found in brain smears prepared at the time of death. Twenty milliliters of fresh plasma was used to prepare *C. ruminantium* DNA on day 0 (before infection) and days 5 and 14 postinfection (during the febrile reaction). Hybridization of the pCS20 DNA probe with these samples is shown in Fig. 2B. The pCS20 DNA probe hybridized with the DNA sample prepared from sheep 010 on day 5, a signal which was stronger than the reaction signal of the DNA sample from this sheep prepared on day 0. The reaction signals of the DNA probe with the day 5 DNA samples of the other four sheep (011, 012, 013, 014) were not greater than the signals obtained with the day 0 DNA samples for these sheep, and hence, they were considered to be negative reactions. Compared with the day 0 and day 5 samples, stronger hybridization signals were detected with DNA samples from these four sheep prepared on day 14; this was during the febrile reaction. Of these four sheep, three (011, 013, and 014) died, and the one that had the weakest signal on day 14 (012) recovered and survived. Sheep 010 died before day 14 (Table 1).

PCR amplification of *C. ruminantium*-specific DNA. By PCR, oligonucleotides AB 126 and AB 127 (ORF-1), and AB 128 and AB 129 (ORF-2) specifically amplified DNA fragments of 181 and 279 bp, respectively, from pCS20 DNA and from genomic Crystal Springs and Welgevonden *C. ruminantium* DNAs (Fig. 3). These fragments were also amplified from Welgevonden 987-infected cell DNA (Fig. 3), which is estimated to be composed of 1% *C. ruminantium* DNA and 99% bovine cell DNA (data not shown). The two PCR products were not amplified from the DNAs of *A. marginale*, *B. bigemina*, *T. brucei brucei*, or *E. coli* or bovine DNA and from the control reactions that contained no template DNA.

Hybridization of PCR products with DNAs of other heartwater strains. Since the two PCR products were specifically amplified from pCS20 DNA and DNAs of the *C. ruminantium* Crystal Springs and Welgevonden strains, their hybridization reactions with DNAs of other heartwater strains were tested. The aim of this experiment was to establish whether the two PCR-amplified products hybridized to the other *C. ruminantium* DNA samples not used in PCR amplifications, as well as to compare the sensitivities of the PCR-amplified probes with the sensitivity of the whole pCS20 DNA probe. The results show that the two PCR-amplified products generally hybridized to the DNAs of all strains tested, including those from Zimbabwe, South Africa, and Nigeria (Fig. 4). However, the sensitivities of the labeled PCR products were found to be lower than that of the whole pCS20 probe (compare with Fig. 1). The 279-bp fragment had a level of sensitivity between those of the whole pCS20 probe and the 181-bp fragment. In addition to the reduced sensitivity, the reactions of the 181- and 279-bp PCR-derived probes with the Nyatsanga strain DNA were found to be diminished in comparison with their reactions with the other DNA samples. The reaction of the 181-bp PCR-derived probe with the Welgevonden DNA was also diminished in comparison with the reaction with the other DNA samples, even though it was possible to amplify by PCR both the 181- and 279-bp fragments from the Welgevonden strain DNA.

Because the reaction of the PCR probes was much weaker with the Nyatsanga strain DNA, we attempted to amplify the 181- and 279-bp fragments from the Nyatsanga DNA by PCR. Figure 5 shows that it was possible to amplify by PCR the two fragments from Nyatsanga strain DNA, and it was also possible to amplify similar fragments from the Gardel strain DNA. The amplification of the two PCR products from the Gardel strain DNA was carried out as an alternative to the hybridization reactions of the two PCR probes with the Gardel strain DNA.

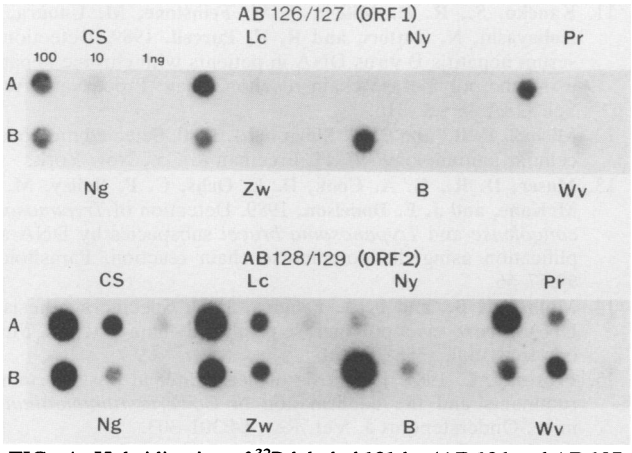

FIG. 4. Hybridization of ^{32}P-labeled 181-bp (AB 126 and AB 127; ORF-1), and 279-bp (AB 128 and AB 129; ORF-2), PCR-amplified *C. ruminantium*-specific DNA fragments to genomic DNA (100, 10, and 1 ng) samples of Crystal Springs (CS), Lemco T3 (Lc), Nyatsanga (Ny), Palm River (Pr), and Zwimba (Zw) (all from Zimbabwe); Nigeria (Ng); and Ball 3 (B) and Welgevonden (Wv) (both from South Africa) heartwater strains.

DISCUSSION

In a previous study (25), we described the pCS20 DNA probe, evaluated its ability to detect *C. ruminantium* DNA, and used it to detect these organisms (Kiswani strain) in ticks. In this report, we showed that the pCS20 DNA probe hybridizes with eight additional *C. ruminantium* strains from the major regions of the world where heartwater occurs, including countries of Africa and the Caribbean island of Guadeloupe. These hybridization reactions showed that the pCS20 probe detects the DNAs of these strains with a similar level of sensitivity for all strains except strain Nyatsanga. DNA from the Nyatsanga strain registered lower intensity signals at all DNA concentrations. This difference probably resulted from a greater contamination with endothelial cell DNA, since the hybridization reactions with the pCS20 DNA probe were conducted under highly stringent conditions to prevent nonspecific hybridization of the probe to the target DNA samples.

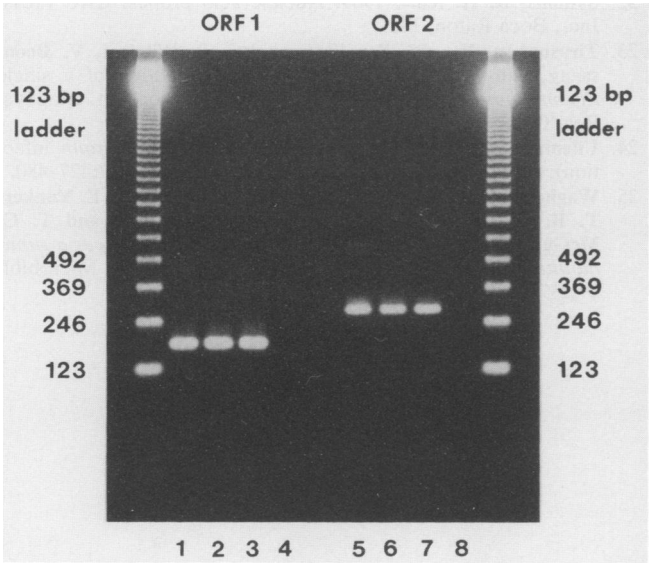

FIG. 5. Agarose gel electrophoresis (1.5%) of the 181-bp PCR product amplified from DNA of pCS20 (lane 1), Nyatsanga (lane 2), Gardel (lane 3), and no DNA (lane 4) and the 279-bp PCR product amplified from DNA of pCS20 (lane 5), Nyatsanga (lane 6), Gardel (lane 7) and no DNA (lane 8). The lane with no DNA sample was included as a negative control for the PCR. The reactions show the amplification of DNA products of 181 and 279 bp from the two ORFs (ORF-1 and ORF-2) of pCS20, respectively.

Detection of *C. ruminantium* DNA in infected sheep plasma samples by hybridization with the pCS20 DNA probe was demonstrated. However, the hybridization signal varied among sheep, probably depending on the level of rickettsemia in individual sheep. Enhanced signals could be obtained by increasing the amount of plasma used to prepare the target DNA. It was possible to detect the organisms in infected sheep before the febrile reaction, which is the first clinical sign of disease (24). No other method can diagnose heartwater at this early phase. The strongest hybridization signals were usually detected during the febrile reaction, when transmission studies indicate that the highest level of rickettsemia occurs (24). The positive hybridization signals in all cases were correlated with the diagnosis of heartwater by use of brain biopsy specimens, brain smears, or necropsy lesions.

PCR has been used to detect both acutely infected animals and chronic carriers of several diseases (2, 6, 11, 13, 19, 23). We evaluated PCR as a method for the diagnosis of heartwater by amplifying DNA products from homologous (Crystal Springs strain) and heterologous (Welgevonden, Nyatsanga, and Gardel strains) genomic *C. ruminantium* DNAs. It was also possible to amplify these products from *C. ruminantium*-infected endothelial cell DNA, which contains approximately 1% *C. ruminantium* DNA (data not shown). These PCR products were specific for *C. ruminantium*, because they were not amplified from bovine DNA or from the DNAs of other organisms. The specificities of the PCR-amplified products for the DNAs of the various heartwater strains by hybridization were similar to that of the pCS20 probe, although the sensitivity level compared with that of the pCS20 probe was lower. As in the reaction of the pCS20 DNA probe, the reaction of both PCR probes with the Nyatsanga strain DNA was diminished compared with the reaction with the other DNA samples. However, since we showed that it is possible to amplify by PCR both the 181- and 279-bp fragments from the genomic DNA of this strain, the weaker signal was probably due to a greater contamination of Nyatsanga strain DNA with endothelial cell DNA. The lower sensitivity of the PCR probes probably resulted from the fact that these probes are shorter compared with the length of the pCS20 probe and, hence, have less incorporated radioactivity. However, these data suggest that the synthetic oligonucleotides AB 126-AB 127 and AB 128-AB 129 could be used for the amplification by PCR of the two fragments, either together or separately, from any of the heartwater DNA samples used in these studies. PCR could play a crucial role in the detection of animals that are heartwater carriers (1), since the rickettsemia in such animals could be either intermittent or too low to be detected by hybridization with the pCS20 DNA probe. This is especially so because the detection of *C. ruminantium* DNA in the plasma of acutely infected sheep was positive but variable in signal intensity, even during the febrile reaction, when peak rickettsemia is expected to exist.

The hybridization and PCRs with the pCS20 DNA probe could be used together or separately to diagnose heartwater

in animals and ticks and provide aid in understanding the epidemiology of heartwater, which should allow for improved control strategies.

ACKNOWLEDGMENTS

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