

Detection of *Theileria annulata* in Blood Samples of Carrier Cattle by PCR

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We report the detection of *Theileria annulata*, the causative agent of tropical theileriosis, by PCR in blood samples obtained from carrier cattle. The assay employs primers specific for the gene encoding the 30-kDa major merozoite surface antigen of *T. annulata*. A 721-bp fragment was amplified from blood samples taken monthly from calves experimentally infected with one of four different stocks of *T. annulata* originating in either Mauritania, Portugal, Spain, or Turkey. At the end of the experiment, five animals carried the infection for 12 months and two animals remained infected for 15 months. DNAs from six other *Theileria* species, *T. parva*, *T. mutans*, *T. sergenti*, *T. buffeli*, *T. velifera*, and *T. taurotragi*, were not amplified. Moreover, DNAs from four other hemoparasites (*Anaplasma centrale*, *Anaplasma marginale*, *Babesia bovis*, and *Babesia bigemina*) were also not amplified. As a control, primers derived from the small subunit rRNA gene of *Theileria* spp. amplified a 1.1-kb DNA fragment from all *Theileria* species examined but not from the other four hemoparasites. As few as two to three parasites per μl of infected blood in a 50- μl sample volume were detected by Southern or microplate hybridization with a *T. annulata*-specific cDNA probe. In addition, 92 field samples obtained from cattle in Spain were tested; 22% were positive in blood smears, 40% were positive by immunofluorescent antibody test, and 75% were positive for *T. annulata* by PCR. The method provides a useful diagnostic tool for detecting *T. annulata* carrier cattle.

Theileria annulata, a protozoan parasite of cattle and domestic buffalo (*Bubalus bubalis*), is transmitted by ticks of the genus *Hyalomma*. It causes tropical theileriosis, a disease which is present in northern Africa and southern Europe, extending through the Middle East, India, and southern Russia into China (25). The disease threatens an estimated 250 million cattle and acts as a major constraint on livestock production and improvement in many developing countries. Mortality varies from 90% in introduced exotic breeds to 5% or less in indigenous breeds (15). *Theileria* parasites enter the bovine host during tick feeding as sporozoites, which rapidly invade mononuclear leukocytes. Here, they mature into macroschizonts and induce proliferation of the host cell. Macroschizonts develop further into microschantons and ultimately into merozoites, which are released from the leukocyte. The merozoites invade erythrocytes and develop into piroplasms. Tropical theileriosis is a lymphoproliferative disease in its early phases and is accompanied by enlargement of lymph nodes. On development of pyrexia, a lymphodestructive phase which is associated with a pronounced leukopenia is initiated. The disease is further characterized by a marked anemia (for a review, see reference 23).

Diagnosis of clinical *T. annulata* infection in cattle is usually based on the detection of macroschizonts in Giemsa-stained lymph node biopsy smears. After recovery, a long-lasting car-

rier state occurs, in which low numbers of erythrocytes remain infected with *Theileria* piroplasms (15). Such carriers are important contributors to the infection within *Hyalomma* ticks. Hence, detection of piroplasms in carrier animals is an important epidemiological parameter. However, *Theileria* piroplasms may be difficult to find in stained blood smears. More important, it is generally not possible to discriminate *T. annulata* from nonpathogenic *Theileria* species that may occur simultaneously within the same bovine host.

In addition, serological tests such as the indirect immunofluorescent antibody test (IFA) can be used to detect circulating antibodies by using either piroplasms or cultured macroschizonts as the antigen (16). However, cross-reactivity with antibodies directed against other *Theileria* species limits the specificity of the IFA (5, 14). Moreover, antibodies tend to disappear in long-term carriers, whereas *Theileria* piroplasms persist. Therefore, animals with a negative serological test can still infect ticks. The advent of PCR (18) has allowed the development of sensitive diagnostic assays for *Theileria parva* (4) and *Theileria sergenti* (24).

Here, we report the use of PCR for sensitive and specific amplification of *T. annulata* DNA from blood samples obtained from carrier cattle. Primers were derived from the gene encoding the 30-kDa major *T. annulata* merozoite surface antigen (21, 22). The specificity of the PCR was determined with DNA from six *Theileria* species other than *T. annulata* and four other hemoparasites. Detection of PCR-amplified DNA by agarose gel electrophoresis was compared with that by Southern hybridization or microplate hybridization with a *T. annulata*-specific cDNA probe. Finally, PCR was compared with conventional detection methods by using a panel of blood

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TABLE 1. Oligonucleotide primers used to amplify 30-kDa and SSU rRNA gene sequences of *T. annulata*

Primer	Sequence ^a	Position ^b	Characteristic
30-kDa gene			
N516	GTAACCTTTAAAAACGT	234–250	<i>T. annulata</i> specific
N517	GTTACGAACATGGGTTT	954–938	<i>T. annulata</i> specific
SSU rRNA gene			
989	AGTTTCTGACCTATCAG	278–294	<i>Theileria</i> specific ^c
990	TTGCTTAAACTTCCTTG	1376–1359	<i>Theileria</i> specific ^c
1347	TGCACAGACCCAGAGG	650–634	<i>T. annulata</i> specific ^c

^a The 5'-to-3' primer sequence is given.

^b The position within the gene sequence that corresponds to the 5' and 3' ends of each primer is given.

^c Primers were previously described by Allsopp et al. (1).

samples from cattle exposed to infected *Hyalomma* ticks in southwest Spain.

MATERIALS AND METHODS

Parasite stocks. The stocks (11) of *T. annulata* used in this study were isolated in Turkey (20), Spain (10), Mauritania (12), and Portugal (13). The stocks of other *Theileria* species included *T. parva* (Pugu 1, Tanzania), *T. mutans* (Katsina, Nigeria), *T. sergenti* (Japan), *T. buffeli* (Brisbane, Australia), *T. velifera* (Lugurni, Tanzania), and *T. taurotragi* (Chilington, Zimbabwe). Other hemoparasites examined were *Anaplasma centrale* (Korea), *Anaplasma marginale* (Zaria, Nigeria), *Babesia bovis* (Australia), and *Babesia bigemina* (Runka, Nigeria). In addition, *T. annulata* (Turkey) was cultivated in lymphoblastoid cell cultures. Cultures were maintained at 37°C in RPMI 1640 (Gibco, Gaithersburg, Md.) supplemented with 20% heat-inactivated fetal calf serum, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml).

Test samples. Experimental calves (*Bos taurus*) approximately 1 year of age were maintained in experimental tick-proof pens. The animals were experimentally infected with one of the four different stocks of *T. annulata* originating in Mauritania (calves 178 and 338), Portugal (calves 222 and 328), Spain (calves 292 and 293), or Turkey (calf 184). Calves were infected either by application of infected *Hyalomma* ticks or by intravenous injection of infected blood. Monitoring of experimental animals was carried out by the taking of daily rectal temperatures, blood smears, and biopsy smears from swollen lymph nodes. Blood samples in citrate solution were collected weekly for 2 months postinfection and were collected thereafter at intervals of 1 month for up to 12 months (calves 178, 222, 292, 328, and 338) or 15 months (calves 184 and 293) postinfection, after which the experiments were terminated. Two-milliliter aliquots were stored at -20°C until used for PCR. Blood samples in citrate solution were obtained from 92 animals maintained in the Extremadura region (Spain) and exposed to a natural tick challenge. Giemsa-stained blood smears were examined for the presence of parasites, and blood samples were analyzed by IFA (16), PCR, and Southern blot hybridization.

Processing samples for PCR. Blood samples (9 ml each) were collected in tubes containing 1 ml of 0.1 M (3.2%) buffered citrate solution (Becton Dickinson Vacutainer Systems, Rutherford, N.J.). Samples were processed as previously described (2). Briefly, 500 µl of saponin lysis mixture (0.22% NaCl, 0.015% saponin, 1 mM EDTA) was added to 50 µl of blood, and the mixture was then centrifuged at 10,000 × g for 1 min. Pellets were washed three times with 0.5 ml of saponin lysis mixture, resuspended in 100 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8], 0.5% Tween 20, 100 µg of proteinase K per ml), and incubated at 56°C for 1 h. After 10 min of incubation at 95°C, 5 µl was taken for the PCR. Cell culture samples infected with *T. annulata* were processed as described above, except that the pellets were washed in distilled water. Ground-up tick material infected with *T. taurotragi* was processed as described above. *T. annulata* piroplasm DNA was purified from bovine blood with 25% parasitemia as previously described (6). Samples of 10 ng each were used as a positive control in the PCR. To determine the threshold of detection, blood with 25% parasitemia was serially diluted with noninfected blood up to 0.000006% parasitemia, corresponding to 15 parasites per 50-µl sample volume. These samples were processed for PCR as described above.

PCR. Two sets of primers were used: N516/N517 (primer set A) and 989/990 (primer set B) (Table 1). PCR was performed in a final reaction volume of 100

µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ (primer set A) or 4 mM MgCl₂ (primer set B), 0.1% Triton X-100, 200 µM (each) deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase (Promega, Madison, Wis.), 40 pmol of primers B or 160 pmol of primers A, and 5 µl of processed sample. The reactions were performed in an automatic DNA thermal cycler (Perkin-Elmer, Foster City, Calif.) for 30 cycles. Each cycle consisted of a denaturing step of 1 min at 94°C, an annealing step of 1 min at 55°C (primer set A) or 1 min at 60°C (primer set B), and an extension step of 1 min at 72°C.

Detection and characterization of PCR products. Amplified products were separated by electrophoresis on a 1% agarose gel and were subsequently transferred (19) to Nytran membranes (Schleicher & Schuell, Dassel, Germany). To detect the PCR fragments generated by primer set N516/N517, the *Pst*I-*Xho*I fragment of pTa30ms1 containing the complete cDNA encoding the 30-kDa major merozoite antigen (22) was used as a probe. The fragment was labeled with digoxigenin-11-dUTP by using the DIG DNA labelling kit (Boehringer, Mannheim, Germany). Hybridization was performed overnight at 58°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1% blocking reagent (Boehringer)-0.1% *N*-lauroylsarcosine-0.02% sodium dodecyl sulfate (SDS). Filters were washed twice with 2× SSC-0.1% SDS for 5 min at room temperature and twice with 0.1× SSC-0.1% SDS for 5 min at 58°C. The presence of hybrids was visualized by using the DIG luminescent detection system (Boehringer).

Microplate hybridization. Hybridization on microtiter plates was performed as previously described (24). After heat denaturation at 95°C for 5 min, 10 µl of PCR-amplified product was ethanol precipitated and subsequently diluted with 90 µl of a mixture of 10 mM sodium phosphate, 10 mM EDTA, and 1.5 M NaCl (pH 7.0) in Maxisorp microplate wells (Nunc, Roskilde, Denmark). Plates were sealed and immersed in a water bath at 37°C for 2 h. Thereafter, they were washed five times with PBS (pH 7.2) containing 0.05% Tween 20 (Tween-PBS). Hybridization solution (100 µl) containing 5 ng of heat-denatured biotinylated cDNA probe (prepared as described above but labelled with biotin-16-dUTP instead of digoxigenin-11-dUTP), 5× SSC, 0.05% Tween-20, and 5 mM EDTA was added to the wells. Plates were again sealed and incubated in a water bath at 58°C overnight. After plates were washed five times with Tween-PBS, a mixture of 100 µl of streptavidin-horseradish peroxidase conjugate (Boehringer) diluted 1:1,000 in Tween-PBS, 1% bovine serum albumin, and 0.1% Triton X-100 was added, and this mixture was then incubated at 25°C for 1 h. Plates were washed five times with Tween-PBS, and 100 µl of substrate (0.01% H₂O₂ and 0.04% ortho-phenylenediamine dihydrochloride [Sigma, St. Louis, Mo.] in 0.05 M sodium phosphate-0.02 M citric acid buffer [pH 5.0]) was then added to each well. After incubation at 25°C for 30 min, 50 µl of 4 N H₂SO₄ was added to stop the reaction. The A₄₉₀ was determined with a microplate colorimetric reader (Bio-Rad, Richmond, Calif.). Samples with an optical density greater than 0.1 were considered positive (24).

RESULTS

Specificity of the PCR. The specificity of the *T. annulata* primer set N516/N517 (Table 1) was examined with DNA from seven *Theileria* species, namely, *T. annulata*, *T. parva*, *T. mutans*, *T. sergenti*, *T. buffeli*, *T. velifera*, and *T. taurotragi* (Fig. 1), and four other hemoparasites, namely, *A. centrale*, *A. marginale*, *B. bovis*, and *B. bigemina* (Fig. 2). The expected 721-bp fragment (21, 22) was generated from *T. annulata* DNA but was not generated from any of the other *Theileria* species (Fig. 1A). The specificity was confirmed by Southern hybridization with the 30-kDa-specific cDNA probe of *T. annulata*, henceforth called the cDNA probe (Fig. 1B). *Theileria*-specific small subunit (SSU) rRNA primers 989 and 990 (Table 1) were used as a positive control and amplified the expected 1,098-bp fragment from all *Theileria* species examined (Fig. 1C). Only a very weak band was obtained from *T. taurotragi* DNA (Fig. 1C, lane 10). The specificity of the test was further confirmed, since neither *Anaplasma* nor *Babesia* DNA sequences could be amplified with the *T. annulata* N516/N517 primer set or the *T. annulata* SSU rRNA 989/990 primer set (Fig. 2A and C, respectively). A slightly larger DNA fragment was amplified from *B. bigemina* DNA, but this fragment did not hybridize with the cDNA probe (Fig. 2A and B, lane 7). The weak hybridization signal seen in Fig. 2B, lane 6, is probably an artifact, because it was not detected on other autoradiograms.

Sensitivity of the PCR. To determine the detection limit of this PCR, agarose gel electrophoresis and Southern hybridization (Fig. 3) results were compared with those from microplate hybridization with the cDNA probe. The expected 721-bp frag-

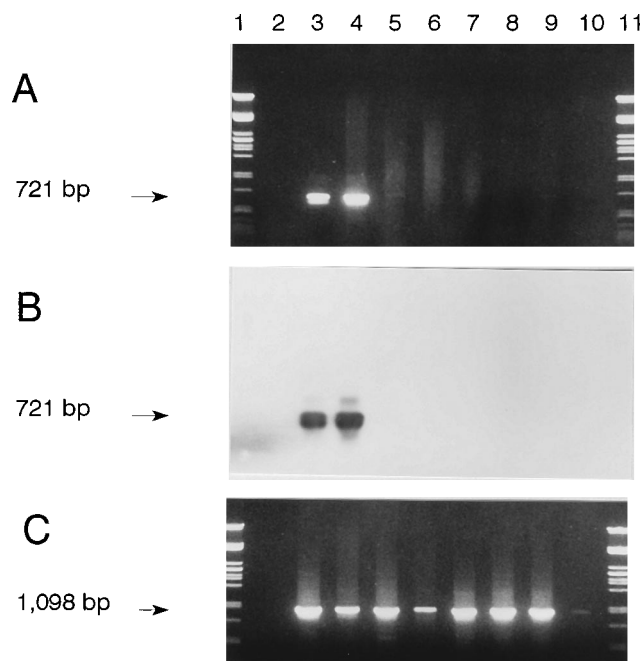


FIG. 1. Agarose gel electrophoresis (A) and Southern blot hybridization (B) of amplified DNA from different *Theileria* species by using primer set N516/N517 and the cDNA probe. (C) Agarose gel electrophoresis of amplified DNA from different *Theileria* species by using primer set 989/990. Lanes: 1 and 11, molecular size markers (lambda DNA digested with *Pst*I); 2, distilled water; 3, *T. annulata*; 4, *T. annulata* (cell culture); 5, *T. parva*; 6, *T. mutans*; 7, *T. sergenti*; 8, *T. buffeli*; 9, *T. velifera*; 10, *T. taurotragi*.

ment was detected by electrophoresis from a blood sample with 0.00038% parasitemia, corresponding to 19 parasites per μ l (Fig. 3A, lane 3). The lowest detection limit of Southern or microplate hybridization was 0.000048% parasitemia, corresponding to two to three parasites per μ l (Fig. 3B, lane 6). The molecular size marker was omitted from the autoradiogram because of cross-hybridization with the cDNA probe (Fig. 3B).

Detection of *T. annulata* in carrier cattle by PCR. Primer set N516/N517 was used in the PCR performed on blood samples taken monthly for 15 months from calf 184 experimentally infected with *T. annulata* (Ankara, Turkey). In addition, PCR was performed on blood samples taken from animals infected with three other stocks of *T. annulata* (Mauritania, Portugal, or Spain). The 721-bp fragment was generated in all samples tested, except in samples taken before infection. The specificity of the amplified DNA fragment was confirmed by Southern hybridization with the cDNA probe (data not shown). Finally, a comparison of results from PCR, IFA, and Giemsa-stained blood smears for detecting *T. annulata* in field-collected samples was made. Twenty of 92 (22%) cattle were positive by blood smears, and 36 of 91 (40%) cattle were positive by IFA, whereas 68 of 91 (75%) cattle were positive by PCR and Southern hybridization. All animals that were positive by blood smears were also positive by IFA as well as PCR.

DISCUSSION

Seven species of *Theileria* are known to infect cattle; of these, *T. parva* and *T. annulata* are of major importance (25). The other five species are less pathogenic, and some of them may confuse the epidemiology of theileriosis. Various criteria and methods have been used to identify these parasites (11, 25). It is difficult to differentiate *Theileria* species solely on the

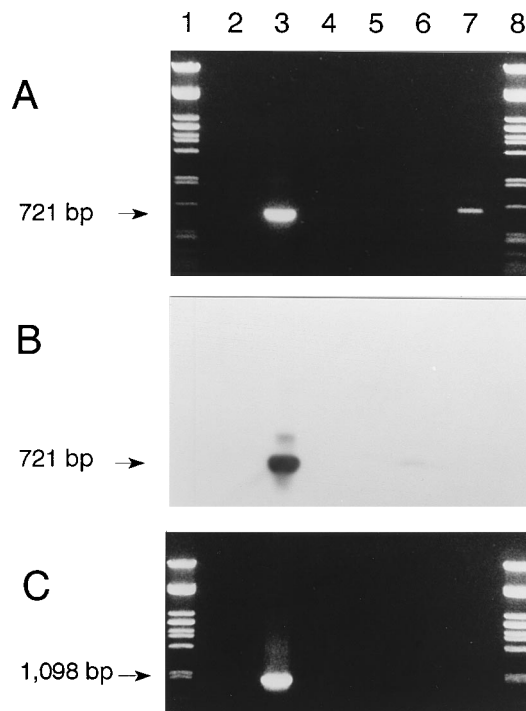


FIG. 2. Agarose gel electrophoresis (A) and Southern blot hybridization (B) of amplified DNA from different hemoparasites by using primer set N516/N517 and the cDNA probe. (C) Agarose gel electrophoresis of amplified DNA from different hemoparasites by using primer set 989/990. Lanes: 1 and 8, molecular size markers (lambda DNA digested with *Pst*I); 2, distilled water; 3, *T. annulata*; 4, *A. centrale*; 5, *A. marginale*; 6, *B. bovis*; 7, *B. bigemina*.

basis of the morphology of the piroplasm and schizont stages, and confusion may arise if mixed infections occur. For instance, *T. mutans* is a widespread and usually avirulent parasite of cattle and buffalo (*Syncerus caffer*) in sub-Saharan Africa. The piroplasms of *T. mutans* are predominantly oval and round but resemble those of *T. annulata*. With IFA, cross-reactions have been observed among *T. annulata*, *T. parva*, *T. mutans*, and *T. taurotragi* (5, 9, 14). Under field conditions, it is impor-

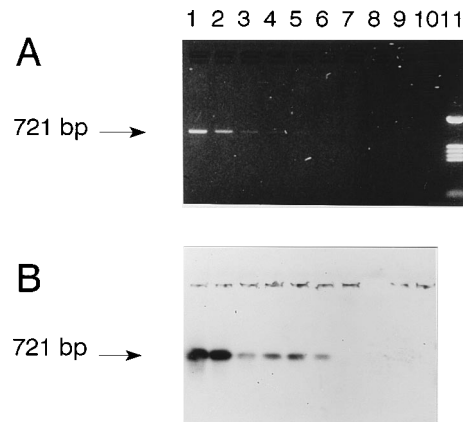


FIG. 3. Threshold of detection of *T. annulata* in blood samples by PCR. Results from agarose gel electrophoresis (A) and Southern blot hybridization (B) of amplified DNA from serial dilutions of infected blood samples by using primer set N516/N517 and the cDNA probe are shown. Lanes: 1 to 10, blood samples with 0.0061, 0.0015, 0.00038, 0.00019, 0.000095, 0.000048, 0.000024, 0.000012, 0.000006, and 0.0% parasitemia, respectively; 11, molecular size marker (pBlue-script [Stratagene, La Jolla, Calif.] digested with *Hinf*I).

tant to be able to distinguish infections caused by *T. annulata* from mild infections caused by *T. mutans* and those caused by *T. buffeli*, which occur worldwide. Geographic distribution and vector specificity may help in the identification of a *Theileria* species, and in most cases, it is necessary to use a combination of methods (for a review, see reference 11).

With the availability of sequenced parasite genes and PCR, it is possible to detect parasites within samples of blood (4, 8, 17, 24). In this study, we developed a PCR assay for improved detection of *T. annulata* in blood samples from carrier cattle. Previously, we amplified a 372-bp fragment by using primer set 989/1347 (Table 1) from all blood samples taken monthly from calf 184, who had carried the infection for over 15 months (7). However, because the SSU rRNA sequences of *T. annulata* highly resembled those of other species examined (1), it was not possible to deduce a *T. annulata*-specific probe from within the amplified SSU rRNA gene fragment. On the other hand, this method has recently been improved with primers derived from the large subunit rRNA gene sequences (3). Primers derived from the gene encoding the 30-kDa major merozoite surface antigen were used in the present study. The cDNA sequence has been reported elsewhere (21, 22). With primer set N516/N517, a 721-bp product was amplified from blood samples from calf 184, and the specificity of this fragment was confirmed by Southern blot hybridization with the 30-kDa-specific cDNA probe.

Successful amplification of all four stocks of *T. annulata* from distant geographic regions indicates that the targeted gene sequence is conserved within *T. annulata*. Nevertheless, additional stocks remain to be tested. Moreover, the specificity of PCR was also demonstrated by the failure to amplify target sequences from *Theileria* species other than *T. annulata*. A *Theileria*-specific primer set based on the SSU rRNA gene generated the expected DNA fragment in all *Theileria* species examined, confirming the presence of *Theileria* DNA. The weak band seen with *T. taurotragi* DNA could be due to the different way in which that sample was processed. Whole blood was used in all samples tested, except to generate *T. taurotragi* DNA, for which ground-up tick material was used.

The lowest detection limit of the PCR was two to three parasites per μ l of infected blood, which corresponds with a parasitemia of 0.000048%. There was no difference in the sensitivity of Southern hybridization compared with that of microplate hybridization, whereas agarose gel electrophoresis was approximately three fourfold dilution steps less sensitive (Fig. 3A). The advantage of microplate hybridization is that the PCR-amplified product can be identified in an enzyme-linked immunosorbent assay format (24). However, our PCR samples required precipitation before hybridization to obtain comparable results. Further investigations are necessary to overcome this drawback.

Amplification of parasite DNA is far more sensitive than parasite detection by light microscopy or IFA, as demonstrated with field samples from Spain. Piroplasms were detected in only 20 of 92 animals by microscopic examination, and 36 animals were positive by IFA, whereas 68 animals were positive by PCR.

Our results demonstrate that this PCR assay detects *T. annulata* parasites at low parasitemias in carrier cattle. Discrimination of *T. annulata* from nonpathogenic *Theileria* species and other hemoparasites that may occur simultaneously in the same carrier animal is feasible. In addition, PCR can be used to determine whether animals that are translocated from regions where theileriosis is endemic are carriers of *T. annulata*. The sensitivity of the PCR will also facilitate monitoring of animals after vaccination with attenuated macroschizont-in-

fect cell cultures. Blood samples taken from vaccinated animals can be tested for the appearance of the piroplasm stage either directly after vaccination or after natural tick challenge. It will be important to determine whether vaccinated animals become carriers of *T. annulata* and thus a source of infection for *Hyalomma* ticks.

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