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Failure of the *Amblyomma cajennense* nymph to become infected by *Theileria equi* after feeding on acute or chronically infected horses

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ABSTRACT

Tick-borne diseases in horses are caused by the intraerythrocytic protozoan parasites *Theileria equi* and *Babesia caballi*. Although *T. equi* is highly endemic in Latin America, the New World vector of this important parasite is controversial. The aim of this study was to test the ability of nymph *Amblyomma cajennense* ticks acquire infection by *T. equi* following feeding on infected horses. Three experiments were performed: tick acquisition of *T. equi* from an experimentally infected horse, tick acquisition of *T. equi* from naturally infected foals and tick acquisition of *T. equi* from a chronically infected horse. *A. cajennense* adults were dissected and salivary glands were collected in aliquots. Methyl green pyronin staining of the salivary glands did not show the presence of hypertrophy of acini or cell nuclei normally suggestive of *Theileria* spp. infection. The pools of salivary glands were negative for *Theileria* DNA in nested PCR assays. Histopathological analysis failed to detect sporoblast and sporozoites of *T. equi* in salivary gland acini. This study was not able to observe infection of the *A. cajennense* by *T. equi*.

1. Introduction

Equine piroplasmosis is a tick-borne disease of horses caused by the intraerythrocytic protozoan parasites *Theileria equi* and *Babesia caballi*. Equine piroplasmosis is distributed throughout emerging areas of the world, including tropical and subtropical countries. Most of the worldwide equine population is distributed in *Babesia*-endemic areas (Friedhoff et al., 1990).

T. equi is considered to be the most pathogenic tick-borne piroplasma of horses. The disease caused by this agent is characterized by fever, anemia, icterus, lymphadenophathy, hepatomegaly, splenomegaly, hemoglobinuria and bilirubinuria (Schein et al., 1981; Guimarães et al., 1997). The hemolytic anemia is attributed to the destruction of parasitized erythrocytes due to repeated cycles of erythrocyte invasion and parasitic reproduction. This life cycle can lead to anoxia and general inflammatory lesions in many organs, especially the liver and kidneys (Hildebrandt, 1981).

Most of animals from endemic areas can recover from the acute phase of disease and develop the chronic form of the disease, and clinical cases frequently occur in carrier animals after suffering intense physical or stressful situations (Rudolph et al., 1975; De Waal et al., 1988; Oladosu and Olufemi, 1992). In order to comply with international export regulations, exportation of horses or their use in equestrian sports is only permitted if animals have been declared seronegative for *T. equi*. Horses that survive the primary infection become life-long carriers of the parasite and are known to act as sources for subsequent infections (Schein, 1988).

Although *T. equi* is highly endemic to Latin America, the New World vector of this important parasite is controversial. In this region, horses are regularly infested by the time they are foals with tick species *Dermacentor nitens*, *Rhipicephalus microplus* and *Amblyomma cajennense* (Borges and Leite, 1998; Labruna et al., 2001; Costa Pereira et al., 2005). *R. microplus* has been incriminated as a competent vector of *T. equi* (Guimarães et al., 1998a,b; Battsetseg et al., 2002; Ueti et al., 2005). *R. microplus* is a monoxenic tick and transovarial transmission of its protozoan does not occur; therefore, its epidemiological importance has been questioned. Some farms are not infested with *R. microplus* but have horses infected with *T. equi*. This raises the possibility of another vector being involved in transmission of the parasite (Kerber et al., 2009).

Kerber et al. (2009) observed a highly significant statistical association between the abundance of *A. cajennense* on horses and the presence of horses positive for *T. equi*. Although Dennig (1988) and Pfeifer Barbosa (1993) did not observe transmission of *T. equi* by *A. cajennense*, this tick has been incriminated as the probable vector of *T. equi* under natural conditions. The aim of this study was to test the ability of nymph *A. cajennense* ticks acquire infection by *T. equi* following feeding on infected horses.

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2. Materials and methods

2.1. Feeding and molting of A. cajennense

Engorged adult female *A. cajennense* ticks were obtained from naturally infested cattle. The ticks were incubated in a chamber at 28 °C with 85% relative humidity (RH) in order to facilitate egg laying and hatching. Larvae were allowed to feed on calves that had cotton fabric chambers fixed on back of hosts using special glue (BRASCOPLAST® – Brascola LTDA, Brazil). After feeding and detaching from the host, the engorged larvae were incubated in a chamber at 28 °C and 85% RH. Hungry, uninfected nymphs were stored at 18 °C and 85% RH.

To test the ability of A. cajennense nymphs acquire and transmit T. equi in adult stage, three experiments were developed:

- *Experiment 1*. Tick acquisition of *T. equi* from an experimentally infected horse

One horse free of infection by hemoparasites, detected by indirect fluorescent antibody (IFA), was infected with *T. equi* BE/SL strain (Guimarães et al., 1997) by intravenous inoculation. Approximately 200 nymphs were put in a chamber that was attached to the horse when host parasitaemia was 1.5%. After detaching, engorged nymphs were collected when the level of parasitaemia reached 3.2%. Then, nymphs were incubated at 28 °C and 85% RH in order to molt to the adult stage.

- *Experiment 2.* Tick acquisition of *T. equi* from naturally infected foals

Approximately 200 nymphs were put in a chamber attached to a four-month-old foal that was naturally infected with *T. equi.* The foal's level of parasitaemia was 0.8%. After detaching, engorged nymphs were collected and incubated at 28 °C and 85% RH in order to promote molting to the adult stage.

- *Experiment 3.* Tick acquisition of *T. equi* from a chronically infected horse

As in the first experiment, approximately 200 nymphs were put in a chamber attached to a horse with a chronic infection of *T. equi.* The host infection was detected by IFA and nested PCR. After 4 days, engorged nymphs detached from the host, then were collected and were incubated at 28 °C and 85% RH in order to molting to the adult stage.

All *A. cajennense* adults in experiments 1, 2 and 3 remained in the chamber for 50 days after molting. Then, 40 adult ticks of each experimental were fed for 3 days on calves that had cotton fabric chambers fixed in place using special glue (BRASCOPLAST® – Brascola LTDA, Brazil).

2.2. Processing of the ticks

A. cajennense adults (males and females) were dissected and the salivary glands were collected and separated into three aliquots:

(a) The aliquot was spread out on a microscope slide and stained with methyl green pyronin, as described by Walker et al. (1979).
(b) The salivary glands were collected and immersed in a cell lysis solution for subsequent DNA extraction. Genomic DNA was extracted from different tick pools, each containing salivary glands from 10 ticks, and tested by nested PCR.

DNA was extracted from tick salivary glands using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the tissue culture protocol. As a positive control, DNA was extracted from 300 μL of the whole blood of experimental horse infected with *T. equi* BE/SL strain, collected during the acute phase of infection. Doubly distilled water was used as the negative control.

Nested PCR of genomic DNA involved two separate amplification reactions. The first reaction was carried out using primers RIB-19 (5′8CGGGATCCAACCTGGTTGATCCTGC3′) and RIB-20 (5′CCG AATTCCTTGTTACGACTTCTC3′) that are specific for a 1700-bp segment of the 18S rRNA gene from *Babesia* and *Theileria* spp. (Zahler et al., 2000). The reaction mixture comprised 1.2 μL dNTPs (10 mM), 0.15 μL Taq polymerase (0.05 U), 1.5 μL reaction buffer (10X), 0.6 μL of a solution containing the mixed primers (10 μM) and sufficient sterile ultra-pure water to give a final volume of 15 μL. A 1.5-μL aliquot of the DNA template was added to the reaction mixture, and amplification was performed using an Eppendorf Mastercycler® (Eppendorf, Brazil) thermocycler programmed as follows: 94 °C for 5 min (initial denaturation step), 30 cycles each comprising 92 °C for 1 min (denaturation), 54 °C for 1 min (annealing) and 72 °C for 2 min (extension), and a final extension step at 72 °C for 8 min. Following amplification, reaction mixtures were maintained at 12 °C.

The second reaction was carried out using primers BabRumF (5′ACCTCACCAGGTCCAGACAG3′) and BabRumR (5′GTACAAAGGGC AGGGACGTA3′) that amplify a common 420-bp *Babesia* 18S rRNA fragment. The primers were made by aligning sequences from *Babesia* spp. using GenBank (http://www.ncbi.nlm.nih.gov), with accession numbers, (X59607), (U16369), (U07885) and (L31922) (Silveira et al., 2010) and BlastN test was used to confirm if they could amplify *T. equi* samples. These primers were localized of *T. equi* 18s rRNA sequence (accession number: gi|270309056|) with 100% homology to the 3′ end of the primer.

The reaction mixture contained 2.0 μL dNTPs (0.2 mM), 0.25 μL Taq polymerase (0.05 U), 2.5 μL buffer (10X), 1.0 μL of a solution containing the mixed primers (10 μM) and sufficient sterile ultrapure water to give a final volume of 25 μL. An aliquot (2.5 μL) of amplicon obtained in the first reaction was added to each the reaction mixture and amplification was carried out under the conditions described above. PCR amplicons were separated by electrophoresis on 1% agarose gel (40 min, 100 V), stained with gel red (Biotium) and visualized under ultraviolet light. The expected product size was 420 bp.

(c) An aliquot of salivary glands was processed immediately for histopathology as described by Guimarães et al. (1998b). Glands were fixed with 2% glutaraldehyde in a sodium cacodylate buffer solution (pH 7.2) for 1 h at 4 °C and postfixed with 2% osmium tetroxide in cacodylate buffer. The salivary glands were dehydrated in a graded series of ethanol and embedded in epon-araldite resin. After polymerization, 0.5 μm slides were sectioned, stained with a solution of 1% toluidine blue in 0.5% sodium borate (Trump et al., 1961) and examined using a light microscope.

The experimental was conducted in accordance with the protocol of ethical principles in animal research adopted by the Ethics Committee in Animal Experimentation.

3. Results

Patent parasitaemia was detected in blood smears from the horse inoculated with *T. equi* strain BE/SL and the maximum parasitaemia (5.6%) was reported on the 16th day. The animal presented with pyrexia, followed by tachycardia, anemia and pale

mucous membranes.Ticks used in experiments 1, 2 and 3 were analyzed, and the results are as follows:

(a) Methyl green pyronin staining of the salivary glands:

The salivary glands presented acinar cells of uniform size and shape with a centrally placed nucleus that stained a faint blue green with a clear cytoplasm. The presence of hypertrophy of acini or cell nuclei, that is suggestive of *Theileria* spp. infection, was not observed.

(b) nPCR – Molecular analysis:

Nested PCR assays showed that the control was positive. However, all of the pools of tick salivary glands from experiments 1, 2 and 3 were negative for *Theileria* DNA (Fig. 1).

(c) Histopathology studies:

In the ticks used in this study, sporoblasts and sporozoites of *T. equi* were not observed in acinar cells from semi-thin sections of salivary glands of the *A. cajennense* adult (male and female).

4. Discussion

The calving season of the equines is seasonal in Brazil and occurs from August to December. Foals acquire *T. equi* primary infection from 1 to 6 months of age in endemic areas (Ribeiro et al., 1995, 1999). The *A. cajennense* population in the southeast region of Brazil demonstrates a 1-year generation pattern, with distinct peaks of activity for each of the three parasitic stages (Oliveira et al., 2000; Labruna et al., 2002). Larval peaks are observed from April to July, and *A. cajennense* larvae cannot be found on horses from October to February. Nymphal peaks occur from June to October and engorged nymphs cannot be found on horses from January to April. Peaks of *A. cajennense* adult ticks occur from October to March, but adult stage ticks can be found on horses throughout the year. Therefore, at the time of *T. equi* transmission, the tick population on foals is mainly *A. cajennense* nymphs and adults.

In the biological cycle of *Theileria* spp., the zygotes do not multiply in the intestinal cells of the ticks, but they migrate towards the salivary glands in both males and females. The zygotes neither invade other organs nor pass through the ovaries and eggs, resulting in no transovarial transmission of the *Theileria* spp. parasites (Uilenberg, 2006). In the next stage of life, the tick vector attaches to a new host, sporogony and maturation of the sporozoites in the salivary glands occurs, and transmission takes place by the injection of infected saliva. When the nymph is infected, the subsequent adult tick is infective.

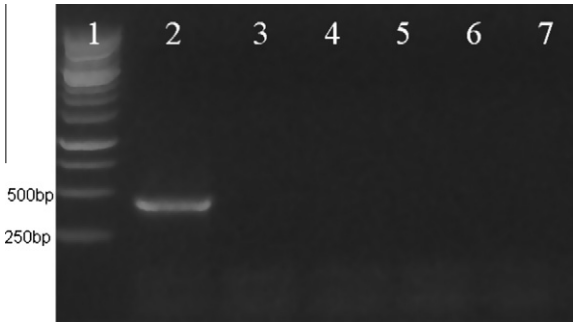

Fig. 1. Agarose gel electrophoresis of the products of nested PCR analysis conducted using *Theileria equi* DNA templates obtained from *Amblyomma cajennense*. Lane 1 shows the GeneRuler™ 1 kb DNA Ladder; lane 2 is the positive control (whole blood from experimental horse infected with *T. equi* BE/SL strain) (420 bp); lanes 3, 4 and 5 show samples with negative results; lane 6 and 7 represent no DNA control (no template, water only) from 1st and 2nd reaction, respectively.

In this study, we analyzed the occurrence of *T. equi* infected nymphs in three different ways.

The first experiment used a horse experimentally inoculated with *T. equi* BH/SL to test the occurrence of nymph infections. The isolate of *T. equi* was infective, as the inoculated equine developed typical theileriosis with detection of the parasite in a blood smear after a pre-patent period of 12 days. Most studies of infections of ticks have used known protozoan samples. A typical practice in laboratories working with vector-borne protozoa is maintaining strains through subinoculation without the arthropod vector of blood in splenectomized animals. These strains, however, may change or lose infectivity for ticks due to the elimination of the sexual cycle. This has been observed in *Babesia bovis* (Dalgliesh and Stewart, 1977) and *T. equi* (Klinckmann, 1981). Guimarães et al. (1998a,b) observed *R. microplus* infected with the *T. equi* strain BE/SL and this strain is currently on the fifth passage in splenectomized animals. In present study, nymphs were unable to be infected with this strain after feeding on an infected horse. Considering the *T. equi* BE/SE strain could be lost the ability to infect ticks after fifth passage in splenectomized animals, the second experiment was developed in order to reject this hypothesis testing others natural *T. equi* strains.

The second experiment was designed to assess the above issue. Nymphs were allowed to feed on a foal naturally infected with *T. equi.* Previous studies have found that transmission of *B. bigemina* to ticks occurs only during the initial phase of parasitaemia, from 4 to 7 weeks postinfection, and the erythrocytic stages (Friedhoff and Smith, 1981). In this experiment, *T. equi* infection in the nymphs was not detected after feeding on a foal.

A. cajennense infection obtained from animals with a chronic *T. equi* infection was attempted in the third experiment. *T. equi* – carrier equines were shown be infective for *R. microplus* (Ueti et al., 2008). Nymphs were allowed to feed on animals with patent parasitaemia, but they did not become infected. It is known that the level of parasitaemia in a horse influences the infection rate of feeding tick vectors (Ueti et al., 2008). However, once established in the tick, *T. equi* parasites replicate to similar levels by the time of transmission, independent of the parasitaemia level during acquisition. Carrier animals with lower parasitaemia can go on to infect a large number of ticks. This can explain the epidemiological importance of these animals for the maintenance of the disease in the environment. However, under our conditions, infection of *A. cajennense* by *T. equi* was not observed.

The lack of parasites in the salivary glands of adult *A. cajennense* may be because the level of *T. equi* infection in the vector ticks is very low to be detected by staining and histopathology methods used or molecular technique. Guimarães et al. (1998b) described the development of *T. equi* in salivary glands of *R. microplus* using the histopathology method and found that the infection rate for the ticks was 80%. This result shows that the histopathology method is sufficiently sensitive to detect of *T. equi* infection in tick salivary gland. However, Howell et al. (2007) showed that transmission could be accomplished even from ticks that were nested PCR and light microscopy negative for *B. bovis* kinetes in hemolymph, suggesting that the most sensitive test to prove the infection is competence transmission feeding. Experiments are in progress in our laboratory to address this hypothesis.

For the methods used in this study, the data suggest that *A. cajennense* nymphs cannot become infected with *T. equi*, and this tick is probably not the biological vector of this parasite in Brazil.

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