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Inhibition of *Ehrlichia canis* and *Babesia canis* transmission among ticks fed together on dogs vaccinated with Bm86 antigen

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ABSTRACT

GAVAC (Heber Biotec S.A, Havana, Cuba) is a commercially available vaccine developed with the *Rhipicephalus* (*Boophilus*) *microplus* Bm86 recombinant antigen. Bm86 is a "concealed" antigen that is present in the plasmatic membrane of tick gut epithelial cells with unknown function so far. It is well known that after vaccination in the last fifteen years in Cuba, there was a significant decrease of babesiosis (*Babesia bovis* and *Babesia bigemina*) and anaplasmosis (*Anaplasma marginale*) in cattle. A reduced transmission capacity of ticks fed on tick-immune animals and humans has been reported for several tick-borne pathogens. Recent experiments have demonstrated that an anti-tick vaccine may contribute to the control of tick-borne pathogens not only by decreasing the exposure of susceptible hosts to ticks, but also by reducing the vector capacity of ticks. In this study, the potential of Bm86 vaccination to interfere with pathogen transmission among ticks was evaluated by using as experimental model the brown dog tick *Rhipicephalus sanguineus* and the tick-borne *Babesia canis* and *Ehrlichia canis* pathogens. Dogs, vaccinated and not vaccinated, were infested with pathogen-infected ticks and non-infected nymphs of *R. sanguineus*. After feeding, the pathogen transmission to newly molted adults from co-feeding uninfected nymphs was studied by conventional PCR and qPCR. Results suggest that the anti-Bm86 antibodies could be able to block the transmission of *B. canis* and/or *E. canis* from infected to non-infected ticks.

Keywords: *Rhipicephalus sanguineus*; *Ehrlichia canis*; *Babesia canis*; Tick-Borne Diseases; Dogs; Bm86

1. INTRODUCTION

Ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans. Perhaps the most serious impact comes through their capacity to vector the infectious agents that cause diseases in humans and other animals [1,2].

Babesiosis is a disease caused by intraerythrocytic protozoan parasites of the genus *Babesia*, transmitted by ixodid ticks. In Latin America there are two species, *B. bovis* and *B. bigemina*, which infect cattle, are both transmitted by the one-host tick *R.* (*Boophilus*) *microplus* [3]. *R. sanguineus* is the major vector of *Babesia* species, which infect dogs and it is invariably present in areas where canine babesiosis is endemic [4,5]. This tick species is a three-host tick that feeds primarily on dogs and occasionally on other hosts, including humans [6-8].

Ehrlichia species comprise a group of rickettsial agents that are obligate intracellular bacteria and reside within a cytoplasmic vacuole of infected eukaryotic cells [9]. *Anaplasma marginale* and *Anaplasma phagocytophilum* are closely related, but principally ruminants are cited as susceptible hosts [10]. *Ehrlichia canis* is a Gram-negative coccoid to ellipsoidal bacterium, occurring intracy-

toplasmically, either singly or in compact inclusions (morulae) in dog bone marrow derived cells. The first site of development of rickettsiae in ticks occurs in gut cells, but many other tick tissues subsequently become infected, including the salivary glands from where the rickettsiae are transmitted to the host during feeding [11].

The use of chemical pesticides constitutes the primary measure for tick control. Alternative strategies are required to control ticks as populations across the globe continue to evolve resistance to commercially available acaricides [12,13]. An understanding of the biological intricacies underlying vector-host-pathogen interactions is required to reach appropriate levels of innovation in sustainable tick management. GAVAC™ (Heber Biotec S.A, Havana, Cuba) is a commercially available vaccine against ticks, which uses the *R.B. microplus* Bm86 antigen. Bm86 is a "concealed" antigen that is present in the plasma membrane of tick gut epithelial cells with unknown function so far [14,15]. It has been shown to be present in *R.B. microplus* strains from different regions of the world suggesting a certain degree of conservation of the gene [16-18]. Bm86 has demonstrated efficacy in the control of tick infestations in cattle under an integrated management system diminishing dramatically the frequency of acaricide applications [19]. It is a fact that after vaccination over the last fifteen years in Cuba, there is a significant decrease of babesiosis (*B. bovis and B. bigemina*) and anaplasmosis (*A. marginale*) in cattle [17, 19-21]. The efficacy of the Bm86 antigen on the biotic potential of *R. sanguineus* was investigated very recently. In this previous study, it was concluded that the Bm86 antigen used as a vaccine for dogs reduced the viability and biotic potential of this tick species [22].

A reduced transmission capacity of ticks fed on tick-immune animals and humans has been reported for several tick-borne pathogens [23-25]. For example, people who express an immune reaction against the vector tick *Ixodes scapularis* appear to acquire Lyme disease less frequently than those who experience no such immune response [26]. For insect vectors, seroconversion of humans against sand fly vectors correlates with development of protective immunity to leishmaniasis [27]. Recent experiments have demonstrated that an anti-tick vaccine may contribute to the control of tick-borne pathogens not only by decreasing the exposure of susceptible hosts to ticks but also by reducing the vector capacity of ticks [28,29].

Herein, we evaluate the potential of Bm86 dog vaccination to interfere with *B. canis* and *E. canis* transmission among ticks, by investigating if anti-Bm86 antibodies are able to block the dog-supported transmission of these two pathogens from infected *R. sanguineous* ticks to uninfected nymphs feeding together.

2. MATERIALS AND METHODS

2.1. Hosts, Hemoparasites and Ticks

The trial was conducted under controlled conditions at the Department of Veterinary Pathology of the Faculty of Agronomic and Veterinary Sciences-FCAV, Jaboticabal campus, São Paulo State University-UNESP, Brazil. Dogs of different breeds and sexes, an average of 3 months old and weighting approximately 5 kg were used throughout the study. The animals were fed with a commercial pellet diet (Big Boss™) and water ad libitum. Dogs were maintained in separated boxes until the end of the experiment and were handled according to international guidelines for experimentation with animals. After infection with hemoparasites, dogs received medical attention. Once the experiments were over, dogs were included in an adoption program.

R. sanguineus ticks used in this study was obtained from a Brazilian strain maintained at FCAV-UNESP-Jaboticabal, Brazil [30]. Maintenance conditions comprised a biochemical oxygen-demand incubator-BOD with 80% relative humidity at 28˚C, and a 12-h photoperiod. *B. canis* and *E. canis* were obtained from stocks maintained at FCAV-UNESP-Jaboticabal, Brazil [31,32].

2.2. Infection of Ticks with *E. canis* and *B. canis*

Two healthy dogs without antibodies against *E. canis* and *Babesia* sp. were inoculated by intravenous injection with 1 mL of frozen dog blood infected either with *E. canis* or *B. canis* laboratory stocks, respectively. The dog infected with *B. canis* was *splenectomized* 24 hours before the pathogen inoculation. The body temperature and hematocrit of the animals were measured daily throughout the test. Confirmation of a dog's infection with *B. canis* or *E. canis* was performed by thin blood smears stained with Giemsa and evaluated by standard microscopy methods. Whole-blood samples were collected from dogs, with EDTA used as anticoagulant. One hundred naive *R. sanguineus* nymphs were fed during the parasitemia of each infected dog. Engorged dropped-off ticks were kept in the incubator until molt. Genomic DNA from 5 whole individual adult ticks newly molted was used to test infection prevalence of naive ticks with each pathogen. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, USA, # 69506) according to the manufacturer's instructions. The samples were eluted in a final volume of 100 µL. Five µL from each eluted individual DNA were used to perform specific *Babesia* and *Ehrlichia* PCR. The *Babesia* specific PCR amplified a 400-bp fragment of *Babesia ssu-r* DNA [33] using the primers, forward (5' AATACCCAATCCTGAC ACAGGG 3') and reverse (5' TTAAATACGAATGCC CCCAAC

3'). In the case of *Ehrlichia*-specific PCR, a 350 bp fragment of the *dsb* gene [34] was amplified with primers forward (5' ATGAATTGCAAAAAAATTCTTA TA 3') and reverse (5' TTAGAAGTTAAATCTTCCT CC 3'). PCR reactions were prepared in a final volume of 25 µL containing tick DNA prepared as described above plus 2.5 µL of 10× Taq polymerase buffer, 1.5 mM $MgCl_2$, 10 pmol of each oligonucleotide, 200 µM dNTPs and 1.25 units of Taq polymerase.

The reaction mixtures were subjected to an initial denaturation of 5 min at 95˚C, followed by 30 cycles of denaturation-annealing-extension (for *Ehrlichia* PCR: 30 seconds at 95˚C, 1 minute at 52˚C and 2 minutes at 72˚C and for *Babesia* PCR: 1 minute at 95˚C, 1 minute at 55˚C and 1 minute at 72˚C) and a final extension of 5 minutes at 72˚C. Amplification products were visualized on an ethidium bromide-stained 1% agarose gel in TAE 1×.

2.3. Dog Immunizations with Gavac Tm

Six healthy dogs without previous antibodies to hemoparasites and negative PCR for *Babesia* and *Ehrlichia* were allocated at random into two experimental groups as follows:

Group 1: (n = 4) Dogs were immunized with 2 mL of GAVAC™ (100 µg of Bm86 antigen) by subcutaneous injection on days 0, 14 and 28.

Group 2: (n = 2) Negative controls. Dogs were immunized by subcutaneous injection on days 0, 14 and 28 with 2 mL of PBS 1X with a similar composition to GAVAC, but lacking the active pharmaceutical principle, in a 60/40 proportion of water/oil using VG Montanide 888 adjuvant (prepared to 10% in mineral oil).

The general behavior and body temperature of the animals were monitored daily throughout the test. Animal serum samples were taken on days 0, 7, 14, 21, 28, 35, 42, 49 and 56. The antibody response against Bm86 was evaluated by indirect ELISA. Purified recombinant Bm86 (1 µg/well) was used to coat ELISA plates overnight at 4˚C. Sera were serially diluted in base 1:2 in PBS 1X. Each serum was assayed using three replicates. The plates were incubated with the diluted sera for 1 h at 37˚C and then incubated with 1:10,000 anti-dog IgG-HRP conjugate (Sigma) for 1 h at 37˚C. The color reaction was developed with a substrate solution containing O-phenylenediamine 0.4 mg/mL in 0.1 M citric acid and 0.2 M Na_2HPO_4, pH 5.0 and 0.015% hydrogen peroxide. The reaction was stopped with 2.5 M H_2SO_4 and the OD490 nm was determined. The antibody titer was established as the reciprocal of the highest dilution at which the mean OD of the serum in question was three times the mean OD of the serum in negative controls. The mean antibody titer in the vaccinated group was determined from individual values in each dog.

2.4. Infestations of Vaccinated Dogs with Infected and Non Infected Ticks

Two feeding chambers were glued to shaved flanks of each vaccinated and control dog one day before the tick infestation, as described previously [30]. On day 36 of the experiment, (when anti Bm86 titers reached 1:3000), three dogs (two vaccinated and one control) were infested with 10 females and 5 males infected with *B. canis* in one chamber to guarantee infected ticks on each dog according to the infection prevalence determined before. The other three dogs were infested with *E. canis* infected ticks (20 females with 10 males) in one chamber in the same way described for the other pathogen. After 5 days for the *B. canis* infected group, and 10 days for the *E. canis* infected group, when the parasitemia was the highest and guaranteed the ingestion of a greater number of parasitized erythrocytes, which subsequently must result in a higher incidence of infection in the adult ticks [31,35], each dog was infested with 100 naive nymphs in the empty chamber. After ticks were released inside chambers, Elizabethan collars were placed on the dogs to avoid chamber removal.

DNA was extracted from blood samples of each dog with patent parasitemia (on day 5 or 11 after dogs were exposed to *B. canis* or *E. canis* infected ticks, respectively). The same specific PCR tests described above were performed to determine the presence of both pathogens. Sera were collected from experimental dogs after day 15 post-infection to be used in an Indirect Immunofluorescence Assay (IFA) described previously [32]. Briefly, antigen slides (sections of specific pathogen infected ticks) were flooded with serial dilutions in base two of test serum from 1:40 to 1:1280. Appropriate positive and negative control sera were included with each run. The slides were placed in a humidified chamber and incubated at 37˚C for 45 min; they were rinsed three times for 5 min each time in PBS, air-dried and then 10 µL of anti-dog IgG (KPL) labeled with fluorescein isothiocyanate at 1:30 dilution in PBS containing 0.01% Evans blue was applied. Incubation and rinse procedures were repeated as above. Mounting fluid containing buffered glycerin, pH 9.6 was placed on each slide, and covered with a cover slip. The slides were examined on a fluorescence microscope using a 40× objective (Olympus, BX-FLA).

2.5. Pathogen Infection Prevalence in Naive Ticks Feeding Together with Infected Ticks

Engorged dropped-off naive nymphs were collected and kept in a BOD incubator having a 12 h light: 12 h dark photoperiod, 28˚C and 80% relative humidity, until molt. Ten newly molted naive ticks from each group were analyzed by conventional PCR, as described above,

for the presence of pathogens. The infection percentage of naive ticks was calculated for each experimental group.

Remaining newly molted naive ticks from each group were pooled into five groups with the same quantity of both sexes in each group and genomic DNA was analyzed by quantitative real time PCR using a Rotor-Gene 3000 Detection System (Corbett, Life Science). Briefly, 5 µL template DNA prepared from whole ticks in the same way as described above for individual ticks using a DNeasy Blood & Tissue Kit (Qiagen, USA, # 69506) was mixed with 6.5 µL of 2× SYBR Green PCR master mix (Quantitect SYBR Green PCR kit, Qiagen, USA, # 204143) and 0.3 µM of each primer (forward and reverse primers) in a final volume of 12.5 µL. The amplification program was 15 minutes at 95˚C and 45 cycles of 15 seconds at 94˚C, 30 seconds at 56˚C and 30 seconds at 72˚C. All reactions were run in triplicate.

Specific gene amplification efficiency in the real-time PCR was analyzed with a 2-fold dilution series of genomic tick DNA containing the genes of interest. Plots of log copy numbers of the tested gene at different dilutions versus the corresponding cycle threshold (CT) were generated. The slope of the linear plot is defined as −(1/log E), where E is the amplification efficiency. Thus, the quantity of target sequence relative to a reference gene can be calculated using the formula 2(−ΔCT), where ΔCT = (CT target − CT reference) [36]. Tick actin was used as reference gene. Oligonucleotides used are summarized in the **Table 1**.

Quantitative data obtained from ticks fed on vaccinated and non vaccinated groups infected with specific pathogens were compared using t tests (Prism, version 4.0 for Windows; GraphPad Software, USA).

3. RESULTS

3.1. Infection Prevalence of Ticks Fed on Dogs Intravenously Inoculated with Pathogens *E. canis* and *B. canis*

The dog inoculated with *B. canis* showed increased temperature after the fifth day post-infection (p.i.), when the haematocrit started to drop from 35% to 24% on day

Table 1. Specific oligonucleotides used in quantitative real time PCR.

Name	Sequence	Amplicon lenght
Actin R	CGCACGATTTCACGCTCAG	139 pb
Bc18S F	ACCCATCAGCTTGACGGTAGG	
Bc18S R	ACCTCCCTGTGTCAGGATTG	154 pb
Ec16S F	TCGCTATTAGATGAGCCTACGT	
Ec16S R	GAGTCTGGACCGTATCTCAGT	123 pb

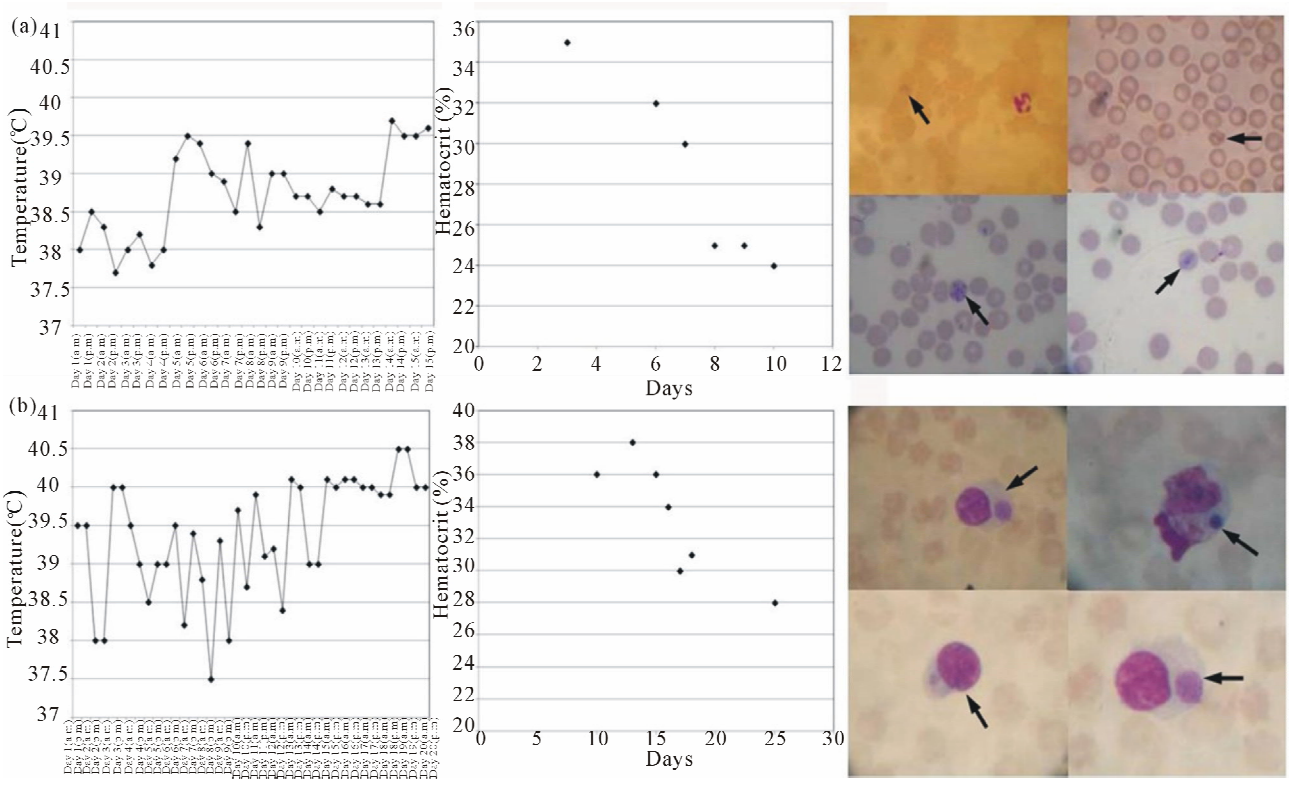

Figure 1. Temperature, hematocrit and blood smears stained with Giemsa in the splenectomized dog inoculated with *B. canis* (panel (a)) and in the dog inoculated with *E. canis* (panel (b)). Arrows indicate typical *Babesia* trophozoites in (a) and characteristic *Herlichia* morulae in (b).

10 p.i. Typical *Babesia* trophozoites including piriform, ameboid and Maltese cross forms were observed in peripheral blood since day 6 p.i. (**Figure 1(a)**), but parasi taemia became very low after three days.

In the case of the dog inoculated with *E. canis*, the highest temperatures were observed after day 11. The haematocrit started to drop from 38% to 28% on day 13 p.i. *Ehrlichial* morulae were observed in peripheral blood of this dog after day 15 p.i. (**Figure 1(b)**).

Two of five (2/5) and 1/5 nymphs become infected when they are fed on *B. canis* and *E. canis* experimentally inoculated dogs, respectively, under the laboratory conditions. Representative images of ethidium bromide-stained gel electrophoresis of the specific amplicons of tick infection are presented in **Figure 2**. The remained adult ticks were used to infest vaccinated dogs.

3.2. Antibodies against Bm86 Seem Interfere with *E. canis* and *B. canis* Transmission among Ticks

There was no change in normal behavior, nor any fever or clinical signs of disease in any of the animals during the immunization experiment (data not shown). Local inflammatory responses down the vaccine application in one side, were evident in two out of four vaccinated dogs, but after two or three days of treatment, disappeared. This issue should be taken in consideration to address changes in the vaccine composition when recommended for pet dogs. Specific titers against Bm86 were obtained only in animals immunized with GAVAC™ (**Figure 3**). On day 35, the anti-Bm86 titer average was

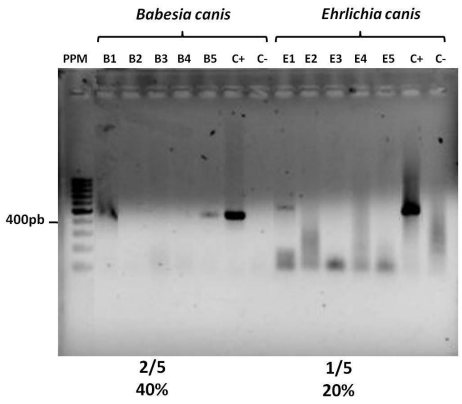

Figure 2. Infection frequency in ticks fed on dogs intravenously inoculated with *E. canis* and *B. canis*. Electrophoresis in a 1% agarose gel in 1X TAE. PPM, Molecular Weigth Marker. B1-5 Amplification products in the *B. canis* specifc PCR from individual tick genomic DNA. E1-5 Amplification products in the *E. canis* specifc PCR from individual tick genomic DNA, C+ and C-Positive and negative controls in each specific PCR.

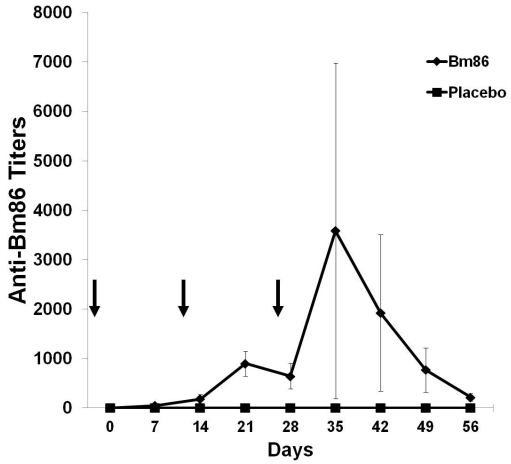

Figure 3. Antibody titers against Bm86 antigen from immunized dogs measured by indirect ELISA. Data are expressed as the reciprocal of the antibody titer average in each experimental group (Vaccinated with GAVAC, n = 4 and negative controls, n = 2). The antibody titer in each vaccinated dog was determined as the last serum dilution with an average OD greater than three times the average OD of the sera in the negative control group. Standard deviations are represented. Arrows indicate immunization days.

Table 2. Dog infection after infected tick exposure as determined by IFA and conventional PCR.

Dogs	Infected tick exposure with	IFA *E. canis*	PCR *E. canis*	IFA *B. canis*	PCR *B. canis*
1 (Control)	*B. canis*	−	−	weak	+
2 (Control)	*E. canis*	ND	+	ND	−
3 (Vaccinated)	*B. canis*	−	−	−	+
4 (Vaccinated)	*B. canis*	−	−	+	+
5 (Vaccinated)	*E. canis*	+	ND	−	−
6 (Vaccinated)	*E. canis*	+	+	−	−

ND: Not determined.

higher than 1:3000.

Table 2 shows the dog infections with pathogens after exposure to infected ticks as determined by IFA and conventional PCR. The low numbers of dogs preclude any statistical analysis of the effect of vaccination with GAVAC™ on infection of the dogs with *E. canis* or *B. canis*, but these results suggest that the anti-Bm86 anti body titers didn't interfere with the pathogens' transmission from ticks to host during tick feeding.

The infection percentage of naive ticks fed together with infected ticks on vaccinated dogs was lower than on control dogs for both pathogens as determined by conventional specific PCRs from genomic DNA of individual ticks (**Figure 4(a)**). For *B. canis*, 67% of the uninfected nymphs feeding on the control dog became in-

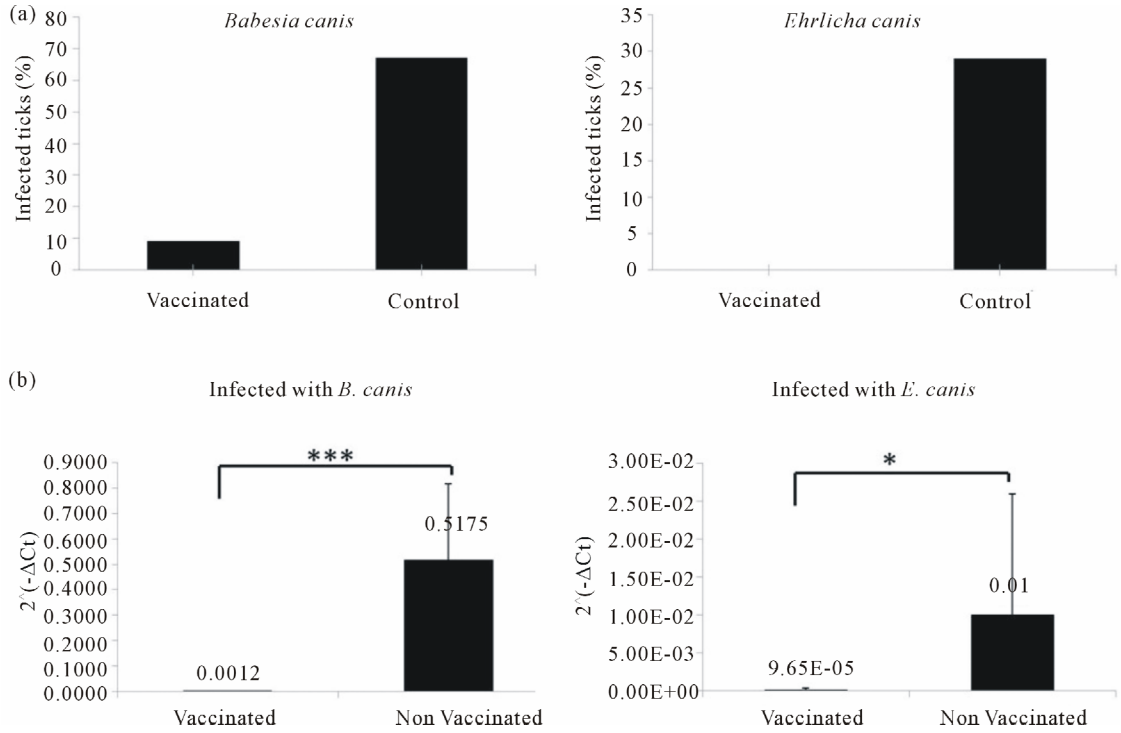

Figure 4. Incidence of infection of naive nymphs fed together with *E. canis* and *B. canis* infected ticks determined by conventional pathogen specific PCRs from individual tick genomic DNA (a) and by pathogen specific Real Time PCRs from five different pools of tick genomic DNA from each experimental group (b). Actin gene was used to normalize. Standard deviations are represented. Statistical significances are represented by asterisks ($^{*}p < 0.05$; $^{***}p < 0.001$).

fected as compared to only 9% on the vaccinated dogs. In the case of *E. canis* also, fewer nymphs became infected on the vaccinated dogs compared with the controls (0% compared to 29%). The qPCR results confirmed the results shown by conventional PCR. Statistically significant differences were found in the average quantity of pathogen DNA between naive tick DNA pools co-feeding with infected ticks on vaccinated dogs compared to the control group for both pathogens (**Figure 4(b)**).

4. DISCUSSION

For tick-borne transmission of *E. canis* and *B. canis*, pathogens are transmitted from an infected tick to the uninfected dog on which it feeds. These pathogens infect host cells and then are transmitted from the infected dog to uninfected ticks cofeeding on the animal. Conventional PCR and qPCR results suggest that immunization with the commercial GAVACTM vaccine provided transmission blocking activity because fewer nymphs became infected compared with the controls as demonstrated by the significantly lowered presence of pathogen DNA in ticks fed on the vaccinated dogs.

The anti-tick vaccine, Gavac, induces an antibody-mediated response that results in rupture of the midgut, tick mortality, and reduced reproductive output [16,19]. This experiment was not designed to assess the effect of Bm86 immunization on *R. sanguineus* tick feeding and tick survival, as demonstrated previously [22], but rather the transmission-blocking effects of GAVACTM. Results indicate that it could affect the likelihood of nymphs acquiring the infection. It is a challenge to speculate on the possible functions of Bm86 in the gut of ticks and its role in the vector capacity of the ticks.

It is known that Bm86 is a membrane-bound glycoprotein expressed mainly on the surface of the digestive tract of *R.B. microplus* ticks [37,38]. Its expression is restricted to a few sites on the digestive cell membrane, in the microvilli exposed to the gut lumen [16,39]. The function of Bm86 has not been elucidated; however, it has been speculated that Bm86 is involved in endocytosis of the tick's blood meal [38,40]. Lysis of midgut digestive cells mediated by anti-Bm86 antibodies during tick feeding occurs in ticks that feed on Bm86 vaccinated cattle, resulting in leakage of blood meal into the tick hemocoel [41]. In addition, it has been shown that this protein contains several epidermal growth factor (EGF)-like domains that may be involved in blood coagulation and cell growth [38,42]. Recent studies silencing of the Bm86 gene via RNA interference (RNAi) are contradictory [43, 44]. Understanding the role of the Bm86 protein in tick biology is critical for understanding the effect of anti-

bodies against Bm86 on ticks and how that could interfere with pathogen transmission.

Both *B. canis* and *E. canis* are pathogens infective to the tick. Certain aspects of the *Babesia* life cycle inside ticks, especially the initial phase in the tick gut lumen are still not well known. However, it is known that after a tick has ingested infected blood, there is a rapid destruction of red blood cells (hemolysis), releasing intracellular forms of *Babesia* in the lumen of the tick gut. The fact that only a small proportion of blood forms survive intestinal digestion to continue its cycle within the tick has led to the acknowledge that there is a sexual stage of *Babesia* [45]. Ingested piroplasms develop into male and female gametes within a cell of the intestinal epithelium in the tick. The microgametes fuse with macrogametes to form motile zygotes [43]. The zygotes then multiply and the ‘‘vermicules’’ that result break through the intestinal epithelium towards the hemolymph and invade numerous organs of the tick, including ovaries. In the salivary glands cells, “vermicules” will develop into infectious forms [45]. When the tick attaches to a new host, maturation of the sporozoites takes place and the host is infected with saliva from the tick. Probably, all of the described process in the tick gut requires an intact intestinal epithelium, which is destroyed by antibodies against Bm86 or these antibodies block, in some way, the entry of *Babesia* piroplasms into cells of the tick intestinal epithelium.

E. canis has a complex and largely unknown life cycle inside the *R. sanguineus* tick. *Ehrlichia*e attach to a cell receptor through an adhesin, to enter the host cell [31]. Probably, the mechanism for entering the tick gut cells, its first site of development inside ticks, is the same. After this initial development, many other tick tissues subsequently become infected, including the salivary glands from where the rickettsiae are transmitted during feeding [11]. Defining the binding specificities of *Ehrlichia*e in a given tick species may lead to the development of a novel type of disease control whose mode of action would be based on competing for the ligands that bind to the pathogen receptors or preventing adhesion to host tissues (transmission-blocking), thereby preventing infection. These data show that antibodies against Bm86 could be blocking, in some way, the *E. canis* transmission to the *R. sanguineus* tick. Whether this blocking effect is due to destruction of midgut cells or is due to a more specific effect remains to be clarified. Further studies are suggested to corroborate this finding.

In conclusion, it appears that high antibody titers against Bm86 could help reducing the infection incidence of naive ticks fed together with *B. canis* and *E. canis* infected ticks, which suggests the potential of Bm86 vaccination for controlling tick-borne diseases by reducing the vector capacity of the ectoparasite.

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