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Anti-tick monoclonal antibody applied by artificial capillary feeding in *Rhipicephalus* (*Boophilus*) *microplus* females

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ABSTRACT

The tick *Rhipicephalus microplus* is an ectoparasite harmful to livestock, a vector of disease agents that affects meat and milk production. However, resistance to acaricides reflects the need for alternative tick control methods, among which vaccines have gained increasing relevance. In this scenario, monoclonal antibodies can be used to identify and characterize antigens that can be used as vaccine immunogens. Capillary tube artificial feeding of partially engorged *R. microplus* females with monoclonal antibodies against proteins from the gut of tick were used to test the effects of immunoglobulins in the physiology of the parasite. The results of artificial feeding showed that female ticks over 25 mg and under 60 mg in weight performed better in the artificial feeding process, with a 94–168% weight increase after 24 h of feeding. Results showed that artificial feeding of ticks proved to be a viable technique to study the effects of antibodies or drugs in the physiology of the parasite. One monoclonal antibody (BrBm2) induced decreased oviposition. Moreover, the antigen recognized by BrBm2 was identified as a 27-kDa protein and immunolabeled on digestive vesicles membranes of digestive cells of partially and fully engorged females.

1. Introduction

The cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, is accountable for significant financial loss associated with direct injury and with the transmission of hemoparasites to its host (Rachinsky et al., 2008). This tick has high host specificity (Evans et al., 2000), and therefore cattle is a requirement for the maintenance of laboratory isolates and the appropriate conduction of experiments. This *R. microplus* characteristic makes laboratory studies with this tick more complex and expensive than the research with other ticks species. A low cost alternative for the testing of a new acaricide or vaccine research is the observation of the biological effects caused by inoculation of drugs or antibodies against a target protein into ticks. Previously, laboratory studies were shown to be an alternative to in-field experiments, and today are considered a useful approach in tick control strategies. The adult and larval immersion tests are largely used to test drugs and acaricides (FAO, 2003; Klafke et al., 2006; Ribeiro et al., 2007), as well as to evaluate the pathogenicity of entomopathogenic fungi (Frazzon et al., 2000).

Similarly, it has been shown that the direct inoculation of antibodies in the hemocele affects the reproductive efficiency of engorged *R. microplus* females (Toro-Ortiz et al., 1997; da Silva Vaz et al., 1998). However, the artificial feeding of ticks could be a more interesting technique for the introduction of anti-tick protein antibodies, since it affords to introduce antibodies into the tick in a condition that simulates the natural feeding process more closely.

Although the artificial feeding using capillary tubes does not afford to totally abolish the use of cattle in the tick feeding cycle, it could facilitate the obtainment of engorged females able to oviposit. The technique mitigates the effects of numerous variables that cannot be left aside during the tick feeding process and allows conducting experimental manipulations (Baldridge et al., 2007). It is possible to use this technique in vaccine studies, since polyclonal

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or monoclonal antibodies can be offered either alone or together using one same capillary (Almazán et al., 2005).

Despite all difficulties to feed ixodid ticks artificially, many researchers have been using the technique to infect ticks with pathogens or to investigate the role of artificial feeding in the tick life cycle (Waladde et al., 1996; Burkot et al., 2001). De la Vega et al. (2000) and Abel et al. (2008) used capillary feeding in starving females of *R. microplus* and *Amblyomma cajennense*, respectively. However, as observed by Abel (2004) and Rangel et al. (2008) with *A. cajennense* and *Dermacentor nitens* females, respectively, it seems that partially engorged females are more suitable for the technique, mainly when it is important to assure that a large amount of blood is ingested. So, it is possible to use the technique to introduce antibodies or drugs experimentally into ticks of different species in order to investigate the biological effects produced. The ticks fed using this system are useful for research purposes, e.g. to test the effect of drugs and antibodies, and to study the transmission of blood borne disease organisms and parasites to tick. This method is safe and relatively inexpensive. Additionally, it reduces the need for animal hosts, lowering research costs affording to conduct various blood meal manipulations.

Here we used the capillary tube artificial feeding technique to feed partially engorged *R. microplus* females with bovine blood plus monoclonal antibodies against midgut extract of *R. microplus* fully engorged females. The biological parameters of females obtained after capillary tube artificial feeding were analyzed to determine the efficiency of the method. In addition, the expression sites of the proteins recognized by the monoclonal antibodies were determined.

2. Material and methods

2.1. Tick maintenance

A *Babesia* and *Anaplasma*-free colony of *R. microplus* (Porto Alegre strain) was used in the experiments. Non-parasitic phases of the ticks were reared at BOD incubator with 85% humidity at 27 °C. Artificial infestation of 10-day-old larvae was performed on *Babesia* and *Anaplasma*-free Hereford calves confined in insulated stables. On the 21st day of infestation, fully engorged females were recovered, cleaned and kept in Petri dishes for oviposition.

2.2. Antigen preparation

Fully and partially engorged female ticks were washed in PBS. The dorsal surface was dissected with a scalpel blade. Guts were separated using a fine-tipped forceps and washed in PBS pH 7.2. These materials were kept frozen at −70 °C upon use. Protein extracts were prepared according to Da Silva Vaz et al. (1994). Protein concentrations of the extracts were measured according to the Bradford method with bovine serum albumin as standard.

2.3. Production of monoclonal antibodies (mAbs)

BALB/c mice were intraperitoneally inoculated three times at 10-day intervals with 100 μg of protein extract plus Freund's adjuvant. Spleen cells were fused to SP2-0 myeloma cells (Köhler and Milstein, 1975). Hybridoma culture supernatants were screened for antibodies to gut antigens by ELISA. Cloned hybridoma cells secreting mAbs were inoculated into BALB/c mice previously injected with Pristane to induce ascites formation (Harlow and Lane, 1988). The ascites were dialyzed with 20 mM sodium phosphate buffer, pH 8.4 and applied to a protein G-Sepharose column equilibrated with the same buffer and eluted with 100 mM glycine–HCl buffer, pH 2.7. The fractions containing IgG were dialyzed against PBS. A non-related monoclonal antibody (OC3) against Foot and Mouth Disease Virus (Crowther et al., 1993) was used as control.

2.4. SDS–PAGE and Western blot

For SDS–PAGE and Western Blot analysis, gut extracts in sample buffer containing 2% SDS, 250 mM Tris pH 6.8, 0.025% bromophenol blue, 5% glycerol, 10% β-mercaptoethanol and 5 M urea were applied to SDS–PAGE and transferred to a nitrocellulose membrane at 70 V for 1 h at 4 °C in 12 mM carbonate buffer pH 9.9 (Dunn, 1986). The nitrocellulose sheet was blocked with 5% cow nonfat dry milk–PBS (BLOTTO) for 2 h at room temperature. Monoclonal antibodies were incubated in BLOTTO overnight at 4 °C. After three washes in BLOTTO, goat anti-mouse IgG-phosphatase alkaline conjugate was incubated for 1 h at room temperature. After three washes in PBS and one wash in detection buffer (5 mM $MgCl_2$, 100 mM NaCl in 100 mM Tris pH 9.5), the reaction was performed with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) in the detection buffer.

2.5. Immunohistochemical analysis

Midguts were dissected out of fully and partially engorged female ticks on the drop-off day or on the 20th day of infestation, respectively. Midguts were then fixed in 10% buffered formalin for 24 h. Fixation, embedding and mounting of tissue sections were carried out according to the methods described by Fernandes (1943). Sections (5 μm thick) were cut and serially mounted on slides coated with gelatine–chrom alum (0.5% gelatine, 0.05% chrom alum dissolved in distilled water). The tick tissue was then dewaxed in xylene and hydrated in decreasing ethanol concentrations. Inhibition of endogenous peroxidase was conducted using 3% H_2O_2. Nonspecific reactive sites on tissue sections were blocked with 5% non-fat milk in PBS for 15 min. All incubations were carried out at room temperature. Monoclonal antibody BrBm2 and the unrelated monoclonal antibody OC3 (5.8 ng/slide each) were incubated on the sections overnight at 4 °C. Biotinylated goat anti-mouse IgG was incubated on the tissue before streptavidin incubation. Peroxidase was detected by incubation with freshly prepared 0.05% 3,3′-diaminobenzidine tetrahydrochloride, containing 0.01% hydrogen peroxide in PBS. Slides were washed for 10 min in running tap water to stop the reaction and counterstained with hematoxylin.

2.6. Artificial feeding

Partially engorged *R. microplus* females were recovered manually from calves 20/21 days after the beginning of infestation. After, they were cleaned, weighed and fixed on expandable polystyrene plate (19 × 10 cm), with double face tape. Females with damaged mouthparts and/or weighing more than 60 mg or less than 25 mg were discarded. Then, microhematocrit capillary tubes (75 × 1.0 × 1.5 mm) without anticoagulant and filled with citrated bovine blood were placed over the ticks' mouthparts. Females were divided in experimental groups, each formed by 15 individuals, and fed for 28 h as follows: group not fed during the experiment; group fed with citrated bovine blood only; group fed with citrated bovine blood supplemented with PBS; groups fed with citrated bovine blood supplemented with the monoclonal antibodies against fully engorged female midgut extract (BrBm2) (Toro-Ortiz et al., 1997); groups fed with citrated bovine blood supplemented with the monoclonal antibodies against partially engorged female midgut extract (BrBm32) or non-related monoclonal antibody (OC3). After the feeding process, the females were weighed again to determine blood ingestion. After that, they were placed in Petri dishes

and maintained at 27 °C and 85% humidity, when their biological parameters were analyzed.

The biological parameters analyzed were those linked to oviposition: total weight, egg production index (EPI = (weight of eggs/initial weight of engorged tick) × 100) and females' weight loss and nutrient index (NI = [weight of eggs/(initial weight engorged tick − residual tick weight)] × 100) (Bennett, 1974). For results analysis, the biological parameters were examined independently. ANOVA was used for the statistical analysis.

3. Results and discussion

In this work, we standardized artificial feeding of the partially engorged *R. microplus* females through capillaries. Initial characterization comprised the analysis of factors that could influence the feeding process; for this reason, different capillaries, anticoagulants and blood types were tested for various weights and ages of females. In capillary feeding, *R. microplus* preferred bovine blood to rabbit blood, citrated blood to heparinized blood, and blood to either plasma or serum (data not shown). The standardization experiments afforded to observe that females above 25 mg and under 60 mg in weight performed better in the artificial feeding process. The females above 60 mg in weight did not accept the capillary (data not shown). It is possible that, when this average weight is reached, salivary glands degeneration happens faster after detachment from the host, making more difficult another blood meal. In a similar way, females under 25 mg did not take a great amount of blood meal (data not shown). Ixodid females only initiate the fast feeding period after mating (Sonenshine, 1991). It is probable that females weighing 25 mg on average had not mated yet, which made them unable to ingest a large amount of blood (Harris and Kaufman, 1984). In the present work, partially engorged *R. microplus* females fed artificially using capillaries had a significant increase in weight, from 94% to 168% (Table 1). As expected, the control group lost weight merely because no blood meal was offered during the experiment, and because ticks naturally went on consuming energy for survival.

Artificial feeding was conducted at 27 °C and in a relative humidity over 80%. Capillaries were replaced every 3 h. Twenty-eight hours appears to be the duration of the artificial feeding process, since after this time the females did not proceed feeding. After 28 h of artificial feeding, females presented rounded idiosoma visible even to the naked eye, and started to lay eggs. De la Vega et al. (2000) fed newly molted *R. microplus* females through micropipettes, observing that tick weight increases by 41.9%. In the present work, weight of artificially fed females increased by more than 100%. It is possible to hypothesize that these females had already mated and received the stimulation for fast feeding, which made these females more suitable for artificial feeding.

Parameters related to oviposition (EPI and NI index) were significantly different between females fed on blood (with or without monoclonal antibodies) and females without feeding (control group) ($p < 0.01$). These parameters in artificial feeding of females are similar to those observed for females fed on cattle (Da Silva Vaz et al., 1998; Leal et al., 2006; Parizi et al., 2011), indicating that artificial feeding can provide ticks with the amount of blood necessary for oviposition. Moreover, the capillary feeding technique allows the quantification of the blood meal ingested.

The capillary tube technique is useful (i) to investigate the ability of a host to receive, store and transmit pathogens or (ii) to follow the development of parasites inside the tick (Baldridge et al., 2007; Bouwknegt et al., 2010). In these pathogen-transmission experiments, the feeding period does not usually exceed 3 or 4 h, since full tick engorgement is not a strict requirement for parasite transmission. However, when the aim is the observation of physiologic effects caused by a microorganism, a drug (Fabres et al., 2010; Pohl et al., 2011), or even sera, full tick engorgement reveals its importance as an experimental aspect due to the correlation between the weight of engorged females and their egg-laying capacity (Santos and Furlong, 2002; Kaufman, 2004).

Table 1
Parameters associated to the feeding process of partially fed *Rhipicephalus* (*Boophilus*) *microplus* females by artificial feeding through capillaries, kept at 27 °C and in relative humidity over 80%.

Groups	Weight before artificial feeding (mg)	Weight after artificial feeding (mg)	Weight increase (mg)	Weight increase (%)
BrBm2	47.6 ± 8.3.6	78.5 ± 31.4*	30.9 ± 29.0*	78.8 ± 85.8*
BrBm32	41.4 ± 15.4	103.7 ± 22.5*	62.2 ± 20.1*	168.8 ± 82.2*
OC3	47.1 ± 7.2	85.7 ± 36.0*	38.6 ± 36.1*	94.8 ± 103.1*
Blood	45.6 ± 6.9	86.6 ± 32.0*	40.9 ± 34.9*	97.2 ± 106.2*
Control	44.7 ± 6.6	41.9 ± 6.5	−2.7 ± 0.5	−6.4 ± 1.4

Ticks of groups BrBm2; BrBm32 and OC3 received 100 μg of monoclonal antibodies.
* $P < 0.05$; compared to control (ANOVA).

We used two monoclonal antibodies against midgut proteins in the artificial feeding process. The BrBm2 monoclonal antibody recognized a 27-kDa protein, while the BrBm32 antibody reacted strongly with several bands of gut extract, ranging between 38 and 150 kDa (Fig. 1). In a previous work, BrBm2 was inoculated into fully engorged *R. microplus* females, decreasing ovoposition by approximately 70% (Toro-Ortiz et al., 1997).

The weight of female ticks fed on monoclonal antibodies (BrBm2: 78.5 ± 31.4 mg; BrBm32: 103.7 ± 22.5 mg; OC3: 85.7 ± 36.0 mg) was not below that of females fed on blood alone ($p > 0.4$) (Table 1), indicating that the ingestion of about 100 μg of antibodies as supplemented in the blood meal did not negatively interfere in the feeding behavior of the tick. The monoclonal antibody BrBm32 did not interfere with tick oviposition. However, in

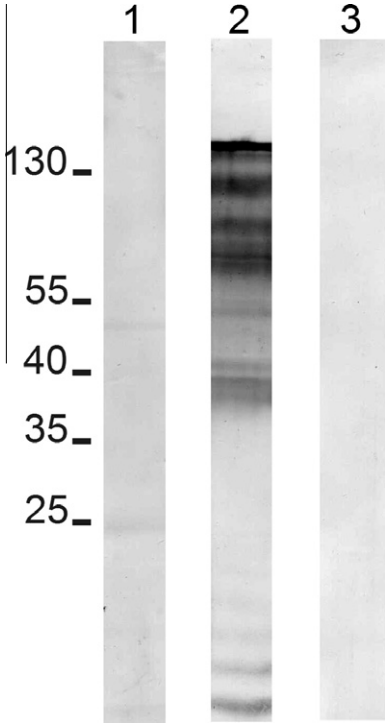

Fig. 1. Western blot with *Rhipicephalus* (*Boophilus*) *microplus* gut protein extract probed with monoclonal antibody. (1) BrBm2 MAb; (2) BrBm32 MAb; (3) OC MAb. Molecular weight markers X 103.

Table 2
Oviposition parameters of partially fed *Rhipicephalus* (*Boophilus*) *microplus* females after artificial feeding through capillaries, kept at 27 °C and in relative humidity over 80%.

Groups	Ovipositing female[a]	Total egg weight (mg)	EPI (%)[b]	NI (%)[c]
BrBm2	11/15	17.4 ± 04.2*	20.8 ± 03.2*	39.2 ± 05.6*
BrBm32	12/15	29.4 ± 10.4	25.7 ± 18.8	52.1 ± 24.6
OC3	15/15	27.3 ± 14.2	31.2 ± 13.3	53.6 ± 21.5
Blood	12/15	25.2 ± 12.6	25.7 ± 10.8	52.9 ± 21.3
Control	14/15	10.9 ± 8.9	23.2 ± 11.0	46.6 ± 14.8

Ticks of groups BrBm2; BrBm32 and OC3 received 100 μg of monoclonal antibodies.
[a] Number of ovipositing females/number of ticks incubated for oviposition.
[b] EPI = egg production index.
[c] NI = nutrient index.
* $P < 0.05$; compared to blood group (ANOVA).

the BrBm2-treated group, a reduction in oviposition (20%; $p < 0.05$) was observed (Table 2). This biological effect was confirmed based on the low EPI of the BrBm2-treated group, which was similar to the index recorded for the group that did not receive blood meal during the experiment. These data indicate that females that ingested BrBm2 antibody experienced greater difficulty to metabolize blood, as revealed by the low NI, suggesting that the BrBm2 antibody interferes with survival after oviposition and with the reproductive physiology of *R. microplus* females.

Immunological analysis of antigen in tick tissues are widely used to identify relevant antigenic components. Since the monoclonal antibody BrBm2 affected reproductive performance of female ticks, we used this antibody to localize the target protein in tissue sections. Immunohistochemistry and Western blot afforded to localize the expression site of the protein recognized by the monoclonal antibodies. BrBm2 identified a protein localized on digestive vesicles membranes of digestive cells of partially and fully engorged females (Fig. 2). These vesicles are involved in the endocitosis of hemoglobin from the luminal content. Digestive cells rest on the basement membrane of partially fed females. In fully engorged females, cells are completely detached from the epithelium and are free in the lumen of the midgut. They become

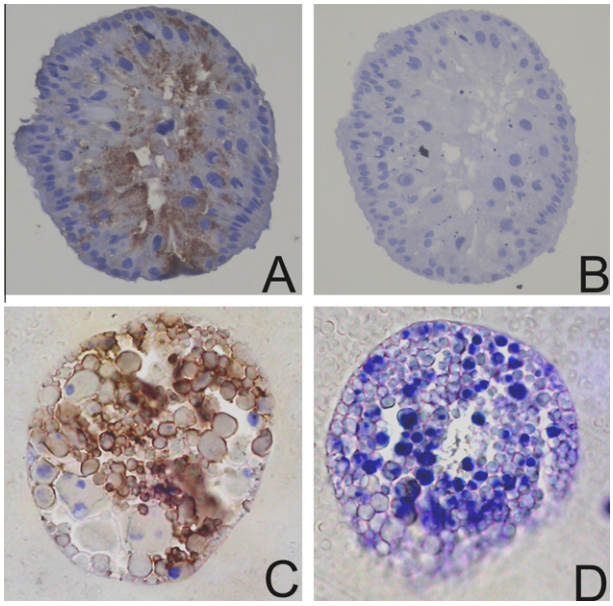

Fig. 2. Immunohistochemistry of *Rhipicephalus* (*Boophilus*) *microplus* females. Transversal section of midgut of partially engorged incubated with BrBm2 monoclonal antibody (A) or OC3 non-related monoclonal antibody (B) (20× magnification). Digestive cells of fully engorged incubated with BrBm2 monoclonal antibody (C) or OC3 non-related monoclonal antibody (40× magnification).

active in intracellular digestion and have a large amount of ingested blood. The immunolocalization of protein detected by BrBm2 in midgut cell suggests that the protein plays roles associated to feeding and nutrition, and that it is probably involved in blood digestion.

The protein expression profile in the tick tissues is coherent with the biological effects produced by BrBm2. The results suggest that the monoclonal antibody binding in gut cells interferes with the blood digestion and consequently with the ability to produce eggs. To confirm this hypothesis, it is necessary to identify and to isolate the protein recognized by BrBm2, so as to obtain more conclusive information about the physiological role of the protein recognized by this antibody.

In summary, our results demonstrate the anti-tick effect of the monoclonal antibody BrBm2 when administered to *R. microplus* through artificial feeding. The identification of the protein recognized by BrBm2 may pave the way to investigate the group of available antigens for an anti-tick vaccine in greater detail, since the antibody induced a deleterious effect on tick physiology. Moreover, our results showed that artificial feeding can be useful to identify proteins for the development of new tick control methods.

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