Ticks and Tick-borne Diseases xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition

Sandra Antunes[a,*], Octávio Merino[b], Joana Lérias[c], Nuno Domingues[a], Juan Mosqueda[d], José de la Fuente[b,e], Ana Domingos[a]

[a] *Institute of Hygiene and Tropical Medicine, New University of Lisbon, Rua da Junqueira 100, 1349-008 Lisboa, Portugal*
[b] *SaBio, Research Institute on Cinegetic Resources, IREC-CSIC-UCLM-JCCM, Ronda de Toledo s/n, 13005 Ciudad Real, Spain*
[c] *CIISA, College of Veterinary Medicine, Technical University of Lisbon, Av. da Universidade Técnica de Lisboa, 1300-477 Lisboa, Portugal*
[d] *Faculty of Natural Sciences, Autonomous University of Querétaro, Avenida de las Ciencias, Querétaro, Mexico*
[e] *Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA*

ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form 23 July 2014
Accepted 8 September 2014
Available online xxx

Keywords:
Ticks
Calreticulin
Artificial feeding
Babesiosis
Boophilus

ABSTRACT

Ticks are obligate haematophagous ectoparasites considered the principal vectors of disease among animals. *Rhipicephalus microplus* and *R. annulatus* ticks are the most important vectors for *Babesia bigemina* and *B. bovis*, two of the most important intraerythrocytic protozoan parasites species in cattle, responsible for babesiosis which together with anaplasmosis account for substantial economic losses in the livestock industry worldwide. Anti-tick vaccines are a proved alternative to traditional tick and tick borne diseases control methods but are still limited primarily due to the lack of effective antigens. Subsequently to the identification of antigens the validation is a laborious work often expensive. Tick artificial feeding, is a low cost alternative to test antigens allowing achieving critical data. Herein, *R. microplus* females were successfully artificially fed using capillary tubes. Calreticulin (CRT) protein, which in a previous study has been identified as being involved in *B. bigemina* infection in *R. annulatus* ticks, was expressed as recombinant protein (rCRT) in an *E. coli* expression system and antibodies raised against rCRT. Anti-rCRT serum was supplemented to a blood meal, offered to partially engorged *R. microplus* females and their effect in feeding process as well as infection by *B. bigemina* was analyzed. No significant reductions in tick and egg weight were observed when ticks fed with anti-rCRT serum. Furthermore, *B. bigemina* infection levels did not show a statistically significant decrease when ticks fed with anti-rCRT antibodies. Results suggest that CRT is not a suitable candidate for cattle vaccination trials.

Introduction

The "*One Health*" concept recognizes the need for joint work between veterinaries, physicians and environmental scientists, given the dynamic interface between people, animals and environment. This concept is becoming increasingly important for zoonotic diseases, such as animal host-dependent tick-borne diseases (TBDs) (Breitschwerdt, 2011). Ticks are resilient and constant arthropods in the environment, with a complex life-cycle. These haematophagous ectoparasites have a direct negative effect in the vertebrate host while feeding and act as vectors and reservoirs for several pathogens (Jongejan and Uilenberg, 2004).

Rhipicephalus microplus ticks are the most relevant ectoparasites in bovine cattle production, causing debilitation, anaemia, weight loss and ultimately death. *R. microplus* ticks are vectors of pathogens such as *Babesia bovis* and *B. bigemina* causing bovine babesiosis, also, these cattle ticks are vectors of *Anaplasma marginale*, etiologic agent of anaplasmosis (Almazan et al., 2010; Peter et al., 2009), which are the most prevalent and higher cost TBDs in cattle production (Suarez and Noh, 2011).

The use of chemical acaricides is currently the major method of tick control, however, their use is in most cases incorrect, leading to limited efficiency in terms of infestation reduction; besides, their use promotes selection of resistant ixodids as well as environmental, meat and milk contamination (George et al., 2004; Graf et al., 2004). Development of new improved acaricides could be a desirable perspective, but it is a long and expensive process, strengthening the need for alternatives. Developing anti-tick

* Corresponding author.
E-mail address: santunes@ihmt.unl.pt (S. Antunes).

http://dx.doi.org/10.1016/j.ttbdis.2014.09.003
1877-959X/

vaccines is therefore an alternative with clear advantages, allowing the combination of antigens for different pathogens and ticks, promoting control of both vectors and parasites (de la Fuente and Merino, 2013; Graf et al., 2004; Guerrero et al., 2012).

The main objective of an arthropod-directed vaccine is the control of vector infestation, causing reduction in vector-transmitted diseases (VTDs). The impact of vaccines on VTDs can be confirmed by (a) reduction in vector populations, which leads to reduced exposure of susceptible hosts to pathogens, (b) reduction of arthropod vector capacity and preferably and (c) a combination of both factors (de la Fuente et al., 2007; Merino et al., 2013). With the exception of the Bm86 antigen, directed against *R. microplus*, already included in a commercial vaccine (Canales et al., 2009; Rodriguez et al., 1994; Willadsen, 2006; Willadsen et al., 1989), identification of efficient antigens is still a major limitation in the development of new vaccines (Parizi et al., 2012) stressing the need for new experimental approaches or the combination of different approaches to develop effective vaccines (de la Fuente and Merino, 2013). A low cost alternative for testing new compounds directed to tick inoculation of medicines or antibodies against a specific protein, while not completely abolishing the need of cattle use, would significantly reduce it (Gonsioroski et al., 2012; Lew-Tabor et al., 2014). One approach to increase the possibilities of identifying tick protective antigens is to combine RNAi functional studies with *in vitro* tick feeding. RNAi allows screening of a relatively large number of genes involved in tick-pathogen interactions, while *in vitro* feeding with antibodies against selected candidate antigens should provide results more closely resembling vaccine protective capacity (Antunes et al., 2014; de la Fuente and Merino, 2013).

Tick artificial feeding is a broadly used technique for different purposes (Almazan et al., 2005; Antunes et al., 2014; Broadwater et al., 2002; Gonsioroski et al., 2012; Inokuma and Kemp, 1998; Matsuo et al., 2004), besides mimicking the natural process of feeding, allows blood meal manipulation once the polyclonal or monoclonal antibodies can be supplemented in the meal (Almazan et al., 2005; Antunes et al., 2014; Gonsioroski et al., 2012; Lew-Tabor et al., 2014), with their effects being therefore assessed.

Recently, functional genomics studies identified differentially expressed genes in *Babesia*-infected female *Rhipicephalus* sp. ticks (Antunes et al., 2012; Heekin et al., 2013; Rachinsky et al., 2007). These studies represent a step forward in the characterization of new antigens, potential vaccine candidates. Calreticulin (CRT) was identified as being involved in the transmission process of *B. bigemina* in *R. annulatus* ticks (Antunes et al., 2012). CRT is conserved in organisms with the exception of yeast and prokaryotes (Persson et al., 2002). This multifunctional protein is involved in Ca^{2+} storage, folding and quality control of newly synthesized glycoproteins (Michalak et al., 2009; Vitale, 2009). Phylogenetic analysis shows a high evolutionary relationship between tick CRTs and vertebrate CRTs; nevertheless, amino acid divergences among the full-length protein sequences of tick CRTs reveal a clear separation of tick CRTs (Parizi et al., 2009).

In ticks, CRT is expressed in salivary glands and saliva, presumably as a mechanism to divert host defensive responses: the protein becomes a target for both cell-mediated and innate immune responses and parasites might exploit the anti-thrombotic and complement-inhibiting characteristics of CRT to suppress host defense actions (Ferreira et al., 2004; Kovacs et al., 1998). Moreover, being secreted to the host it integrates the group of exposed antigens not requiring re-vaccination; thus suggesting that this protein can be integrated in an anti-tick vaccine (Kaewhom et al., 2008). CRT has been used in different immunization studies (Ferreira et al., 2002; Gao et al., 2008; Parizi et al., 2009; Rachinsky et al., 2008). Rabbits immunized with *Amblyomma americanum* CRT exhibited necrotic lesions in the tick bite sites, indicating that the immune reaction could disrupt the feeding cycle (Jaworski et al., 1995), vaccination of sheep with *Haemaphysalis qinghaiensis* rCRT conferred protective immunity against ticks (Gao et al., 2008) or anti-rBmCRT (*R. microplus*) and anti-rHlCRT (*H. longicornis*) bovine sera also recognized the native proteins in larvae extracts (Parizi et al., 2009). However, in immunization assays with rCRT in cattle against *R. microplus*, no effect in tick biological parameters was observed mainly due to its low immunogenic capacity in bovines (Ferreira et al., 2002).

Despite the various studies done with recombinant CRT, the evaluation of the direct effect of anti CRT antibodies on the tick and infection using artificial feeding was never conducted. The present study was designed in agreement to previous similar ones (Antunes et al., 2014; Gonsioroski et al., 2012) that used similar methodology. Herein, groups of adult female ticks (partially engorged *R. microplus*) were fed with serum enriched with anti-CRT antibodies supplemented-bovine blood characterizing CRT potential as antigen for the control of cattle tick infestations and infection with *B. bigemina*.

Materials and methods

Babesia bigemina non-infected and infected bovine blood

To obtain *B. bigemina* infected blood a six months old Holstein calf was splenectomized and after 2 weeks, intravenously inoculated with 2×10^8 cryopreserved *B. bigemina* (Chiapas Strain). Infection was monitored by measuring animal body temperature, observing hematocrit and peripheral-blood smears. Five days after inoculation, 500 ml of bovine blood were collected from the jugular vein. Non-infected blood was obtained in the same way, from a healthy babesiosis and anaplasmosis-free bovine. After collection blood was kept at 37 °C and used promptly. Calves were tested for antibodies to *Babesia* spp. and *Anaplasma* spp. infection using an IFA assay (Knowles et al., 1996; Shkap et al., 2005).

Rhipicephalus microplus ticks

R. microplus ticks (strain Media Joya, CENAPA, México), used in this study, were produced at the University of Querétaro, Mexico, being initially acquired from naturally infested cattle. For *R. microplus* feeding, a 3–4 months old Holstein calf free from babesiosis and anaplasmosis was used. The calf was infested with larvae resulting from 0.5 g of *R. microplus* eggs and 20–21 days after, partially engorged female adults were manually collected.

Expression and purification of rCRT from R. microplus ticks

CRT-corresponding coding sequence was obtained by PCR. Primers, forward 5′-CACC AT GCG GCT TCT CTG CAT TTT G-3 and reverse 5′-CAG TTCTTC GTG CTT GTG GTC-3′ were designed using as templates the sequences present in GenBank accession numbers AY395253 and AY395254 from *R. annulatus* and *R. microplus* respectively. To the forward primer a four base pair sequence (CACC) was included on the 5′ end necessary for directional cloning. Two hundred nanogram cDNA of *R. microplus* were used with Advantage® 2 PCR polymerase mix PCR Kit (Clontech, Mountain View, CA, USA). Positive PCR products were purified using illustra GFX™ PCR DNA Kit (GE Healthcare, Buckinghamshire, UK), following manufacturer instructions, sequenced (Stab Vida, Almada, Portugal) and analyzed. Protein sequences were aligned using the CLUSTAL 2.1 multiple sequence alignment tool (EMBL-EBI; http://www.ebi.ac.uk/Tools/).

Recombinant CRT with poly-histidine tail was obtained using Champion™ pET101 directional TOPO® Expression Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The PCR obtained sequence

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003

was inserted into the pET101/D-TOPO plasmid vector, used to transform *E. coli* OneShot® cells (Invitrogen Life Technologies, Carlsbad, CA, USA). The correct insertion of the target sequence was confirmed by sequencing and the same vector was used to transform BL21 Star™ (DE3) One Shot cells (Invitrogen Life Technologies, Carlsbad, CA, USA). Recombinant protein expression was induced by IPTG (isopropylthiogalactoside) at a concentration of 0.5 mM. The cell culture was lysed by sonication and recombinant proteins were purified from the soluble fraction of the extracts by affinity chromatography using HisTrap HP column (GE Healthcare, Buckinghamshire, UK). Sample purity was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and rCRT concentration was obtained by Bradford assay using BSA (bovine serum albumin) as standard.

Polyclonal serum production

Four to six weeks old BALB/c mice were immunized intraperitoneally with 20 μg of rCRT emulsified with incomplete Freund adjuvant (Sigma–Aldrich) with 3-week intervals. After 4 immunizations, blood was collected from the tail of each mouse and the antibody titer was determined by indirect ELISA (enzyme-linked immunosorbent assay). Briefly, 96-well ELISA plates were coated with 0.5 μg/well of rCRT, overnight at 4 °C and then blocked with 200 μL/well of 5% (w/v) skim milk in PBS (phosphate buffered saline) containing 0.05% (v/v) Tween-20 (PBS-T), for 1 hour at room temperature. The ELISA plates were washed three times with PBS-T and mice serum (1:500 and 1:1000 in 100 μL) incubated for 1 h at 37 °C following three times wash. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma–Aldrich, St Louis, MO, USA), diluted 1:3000 in PBS-T, was after added and incubated for 1 h at 37 °C. After a final five times wash with PBS-T, 100 μL of substrate solution BCIP (5-bromo-4-chloro-3-indolyl phosphate)-NBT (nitro blue tetrazolium) (Bio-Rad, CA, USA), in AP color development buffer (Bio-Rad, USA) was added. After 30 min of incubation at 37 °C, absorbance was read on an ELISA plate reader (Triad Series multimode detector, Dynex Technologies, Chantilly, VA, USA) at a wavelength of 405 nm. Mice were euthanized by cervical dislocation and total blood was collected. Blood was centrifuged at 1500 RPM (revolutions per minute) for 10 min and serum was collected. Serum against rCRT was used in Western blotting analysis, as well as in artificial feeding assays. Control serum from PBS immunized mice was obtained in similar way.

Immunolocalization assays by indirect immunofluorescence

Cattle tick *R. microplus* tissues (guts, salivary glands and ovaries) were dissected in ice-cold PBS buffer from individual engorged females. All tissues were washed in PBS giving special attention to gut samples from which the luminal contents were carefully removed and remaining tissues were gently washed from the host blood excess in the same buffer. Tissues were either used immediately after dissection or stored at −80 °C in RNAlater (Ambion, Austin, TX, USA). Samples were prepared for indirect fluorescent microscopy, as described previously in Antunes et al. (2014). To stain the internal cells, tissues were permeabilized with 0.5% (v/v) Triton X-100 (USB, Cleveland, OH, USA) in PBS for 30 min and washed three times with excess PBS before blocking overnight with 3% (w/v) BSA at 4 °C. After washing the slides again with excess PBS, the anti-rCRT serum diluted (1:100) in blocking solution, was applied and the slides incubated for 1 h at 37 °C. The slides were then washed three times with PBS and the appropriate secondary antibody, Alexa Fluor 488 (green)-conjugated anti-mouse (Molecular Probes), diluted (1:1000) in blocking solution was applied and slides incubated for 1 h at 37 °C. After a final PBS wash, a drop of ProLong® Gold Antifade Reagent with 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen Life Technologies, Carlsbad, CA, USA) was placed over the tissues and then slides were sealed with a coverslip. Slides were kept in a moist dark box until microscopic analysis to prevent drying and fluorescence fading. Tick tissues sections were visualized under a Nikon eclipse 80*i* fluorescence microscope using appropriate filters.

Artificial feeding by capillary tubes

Semi-engorged ticks were cleaned, weighted and fixed over a polystyrene tray (214 mm × 114 mm) with double-sided adhesive tape. Females with weight inferior to 20 mg and superior to 60 mg were discarded, as well as females showing mouth apparatus damaged. These assays were conducted under controlled environment, at 26–28 °C and a relative humidity of 85%. Experimental groups of 15 females/group were used (group fed with bovine blood, group fed with bovine blood supplemented with anti-rCRT and a final group fed with bovine blood supplemented with control serum). The same procedure was used for bovine blood infected with *B. bigemina*. Regarding groups fed with serum supplement roughly 15–20 μL of serum was offered to each tick. Microhematocrit capillary tubes (75 mm × ⌀1.5 mm) were filled with the blood meal and placed over the ticks' mouthparts. Tubes were replaced every 2–3 h, as described previously (Gonsioroski et al., 2012). After feeding, ticks were detached from the double-sided tape and weighed again to determine tick weight increase during feeding. Five ticks per group were then placed in Petri dishes and incubated at 27 °C and 85% relative humidity for oviposition. Weight increase during feeding (mg/tick) and oviposition (weight of eggs/tick in mg) were compared between ticks fed with blood supplemented with serum against rCRT and control ticks fed with bovine blood supplemented with control serum by Student's *t* test with unequal variance ($P = 0.05$).

Determination of Babesia bigemina DNA levels

In order to quantify pathogen DNA levels, 10 ticks per group were incubated in Petri dishes at 27 °C and 85% relative humidity for three days after feeding, then each tick was dissected and whole internal organs were placed in a 2 ml tube with 1 ml of Tri Reagent (Sigma–Aldrich, St. Louis, MO, USA). Total RNA and DNA were isolated according to the manufacturer's protocol. *B. bigemina* DNA levels were analyzed by real-time PCR using the oligonucleotide primers RTBbF 5′-AGCTTGCTTTCACAACTCGCC-3′/RTBbR 5′-TTGGTGCTTTGACCGACGACAT-3′ to amplify the 16S rDNA (HQ264118) gene. Real-time PCR was performed using the iScript SYBR Green RT-PCR Kit (BioRad, Hercules, CA, USA) in a BioRad IQ5 thermo-cycler following the manufacturer's recommendations. The *B. bigemina* 16S rDNA levels were normalized against tick 16S rDNA (L34310) using the comparative Ct method (Livak and Schmittgen, 2001; Schefe et al., 2006). Infection levels in ticks (arbitrary units) were compared between ticks fed with blood supplemented with serum anti rCRT and control ticks fed with bovine blood supplemented with control serum by Student's *t* test with unequal variance ($P = 0.05$).

Characterization of tick CRT mRNA levels

The *crt* mRNA levels were characterized in 10 ticks per group by real-time RT-PCR using the oligonucleotide primers5′-TGAGAGTCTTGTGGGGAAGG-3′ and 5′-CGTCATCCTCCTTCTTGCTC-3′, that amplify a 171 bp fragment (Antunes et al., 2012). Real-time RT-PCR was performed using the iScript One-Step RT-PCR Kit with SYBR Green and thermocycler BioRad IQ5 (Hercules, CA, USA) following the manufacturer's recommendations. The mRNA levels were normalized against tick 16S rRNA (Zivkovic et al., 2010) using

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003

the comparative Ct method (Livak and Schmittgen, 2001; Schefe et al., 2006). Normalized mRNA levels were compared between ticks fed with blood supplemented with anti rCRT serum and control ticks fed with bovine blood supplemented with control serum by Student's *t* test with unequal variance ($P = 0.05$).

Results and discussion

In a previous study calreticulin was shown to be up regulated in ticks infected with *B. bigemina* affecting tick infection when the expression of the protein was disrupted by RNA interference (Antunes et al., 2012). The present work reports the production of rCRT protein from *R. microplus* as a potential antigen to induce anti-tick immunity.

Expression of rCRT

First the *crt* open reading frame (ORF) was amplified by PCR using *R. microplus* cDNA. The obtained amplicon showed to have around 1250 bp. The amplicon was cloned in the pET101/D-TOPO plasmid vector, sequenced, analyzed and assembled sequence was deposited in GenBank with the accession number. This sequence was then compared to available sequences for *R. annulatus* (AAR29939), *R. microplus* (AGK88372), *R. sanguineus* (AY395275), *Ixodes scapularis* (AY690335), *Amblyomma americanum* (U07708) and *Dermacentor variabilis* (AY241957), revealing, as expected, a highly conserved sequence among ticks (Table 1). Additionally, the obtained sequence was translated to amino acid sequence and alignment reveals high conservation of CRT proteins among ticks (Fig. 1). As discussed in Ferreira et al. (2002) the tick calreticulin presents the N-terminal domain (N, residues 17–196), the internal domain (P, residues 197–306), and the C-terminal domain (C, residues 307–411) and it shares high conservation observed among the CRT sequences, mainly within the N and P-domains. The C-domain is more variable, but a high content of acidic residues is conserved in all sequences.

The recombinant CRT protein (rCRT) was obtained with the expected molecular weight of 46 kDa (Fliegel et al., 1989; Gao et al., 2008) and then purified by affinity chromatography (Fig. 2). The purified rCRT was used for the immunization of BALB/c mice, being conducted four immunizations and the immune response followed by ELISA (Fig. 3). As it can be perceived, phylogenetic

```
Putative_protein      MRLLCILLPLVGLISADPTVYFKEEFNDGDAWKDRWVESTK-GDNLGKFVLSAGKFYGDA
R.annulatus           MRLLCILLPLVGLISADPTVYFKEEFNDGDAWKDRWVESTK-GDNLGKFVLSAGKFYGDA
R.microplus           MRLLCILLPLVGLISADPTVYFKEEFNDGDAWKDRWVESTK-GDNLGKFVLSAGKFYGDA
D.variabilis          MRLLCILLPLVGLISADPTVHFKEEFNDGEAWKDRWVESTK-GDNLGKFVLTAGKFYGDE
I.scapularis          MRIVCLLLLLVGTVFADPKVYLKEEFADGDAWTSRWVHSTKKGSEQGTFKLSAGKFHGDP
                      **::*:** *** : ***.*::**** **:**..***.*** *.: *.* *:****:**

Putative_protein      EKSKGLQTSEDARFYGISAKFEPFSNEDKTLVIQFTVKHEQNIDCGGGYVKLFDCSLDQT
R.annulatus           EKSKGLQTSEDARFYGISAKFEPFSNEDKTLVIQFTVKHEQNIDCGGGYVKLFDCSLDQT
R.microplus           EKSKGLQTSEDAHFYGISAKFEPFSNEDKTLVIQFTVKHEQNIDCGGGYVKLFDCSLDQT
D.variabilis          EKSKGLQTSEDARFYGISAKFEPFSNEGKTLVVQFTVKHEQNIDCGGGYVKLFDCSLDQT
I.scapularis          EKDLGIQTSEDARFYGLSTKFEPFSNDGKTLVVQFTVKHEQNIDCGGGYVKLFDCSLDQK
                      **. *:******:***:*:*******:.****:*************************.

Putative_protein      QMHGESPYLIMFGPDICGPGTKKVHAIFNYKGKNHLINKEVRCKDDVFSHLYTLIVKPDN
R.annulatus           QMHGESPYLIMFGPDICGPGTKKVHAIFNYKGKNHLINQEVRCKDDVFSHLYTLIVKPDN
R.microplus           QMHGESPYLIMFGPDICGPGTKKVHAIFNYKGKNHLINKEVRCKDDVFSHLYTLIVKPDN
D.variabilis          QMHGESPYLIMFGPDICGPGTKKVHVIFNYKGKNHLINKEIRCKDDVFTHLYTLIVKPDN
I.scapularis          EMHGESPYHIMFGPDICGPGTKKVHVIFNYKGKNLLINKEIRCKDDVYTHLYTLIVKPDN
                      :******* ****************.******** ***:*:******::***********

Putative_protein      TYQIKIDNEVVEKGELEKDWSFLPPKKIKDPDAKKPEDWDDRAKIDDPDDKKPEDWDKPE
R.annulatus           TYQIKIDNEVVEKGELEKDWSFLPPKKIKDPDAKKPEDWDDRAKIDDPDDKKPEDWDKPE
R.microplus           TYQIKIDNEVVEKGELEKDWSFLPPKKIKDPDAKKPEDWDDRAKIDDPDDKKPEDWDKPE
D.variabilis          TYQVKIDNEVVEKGELEKDWSFLPPKKIKDPEAKKPEDWDDRAKIDDPDDKKPEDWDKPE
I.scapularis          TYTVKIDNEVVESGELEKDWSFLPPKKIKDPAAKKPEDWDDRAKIDDPEDTKPEDWDQPE
                      ** :********.****************** ****************:*.******:**

Putative_protein      YIPDPDATKPEDWDDDMDGEWEPPQINNPEYKGEWKPKQIDNPAYKGAWVHPEIDNPEYT
R.annulatus           YIPDPDATKPEDWDDDMDGEWEPPQINNPEYKGEWKPKQIDNPAYKGAWVHPEIDNPEYT
R.microplus           YIPDPDATKPEDWDDDMDGEWEPPQINNPEYKGEWKPKQIDNPAYKGAWVHPEIDNPEYT
D.variabilis          YIPDPDATKPEDWDDDMDGEWEPPQINNPEYKGEWKPKQIDNPAYKGAWVHPEIDNPEYT
I.scapularis          YIPDPDATKPEDWDDEMDGEWEPPQINNPAFKGEWKPKQIDNPAYKGAWVHPEIDNPEYS
                      ***************:************* :*****************************:

Putative_protein      PDPKLYRYKEICKIGFDLWQVKSGTIFDNILITDDEEYARVHGEETWAALKDEEKKMKDK
R.annulatus           PDPKLYRYKEICKIGFDLWQVKSGTIFDNILITDDEEYARVHGEETWAALKDEEKKMKDK
R.microplus           PDPKLYRYKEICKIGFDLWQVKSGTIFDNILITDDEEYARVHGEETWAALKDEEKKMKDK
D.variabilis          ADPKLYHYPEICKVGFDLWQVKSGTIFDNLLITDDEEYARVHGEETWAALKDAEKKMKDK
I.scapularis          PDPKLHSYKEICTLGFDLWQVKSGTIFDNILLTDDEEYARQYGEETWGATKDAEKKMKEQ
                      .****: * ***.:***************:*:******** :*****.* ** *****::

Putative_protein      QEEEEEAKSKKEDDAKDEDE-FEDDEDKDEDKKDEEETTPAPEDDEDHKHEEL
R.annulatus           QEEEEEAKSKKEDDAKDEDE-FEDDEDKDEDKKDEEETTPAPEDDEDHKHEEL
R.microplus           QEEEEEAKSKKDDDAKDEDE-FEDDEDKDEDKKDEEETTPAPEDDEDHKHEEL
D.variabilis          QEEEEEAKSKKEDDAKEEDE-FEDDEDKEE----KEEETTPAPDDEDHKHEEL
I.scapularis          QDEKEDKEAEKKDKEDEKDEDFEEEDDEDKKEDEEAPAGGDAEEEHKHDHEEL
                      *:*:*: :::*.*. .::** **:::*:::    :      . ::..*.****
```

Fig. 1. Alignment of CRT multiple amino acid sequences. Sequences aligned included part of the rCRT obtained in the present study (put), *Rhipicephalus* (*Boophilus*) *annulatus* (AAR29939), *R. microplus* (AGK88372), *R. sanguineus* (AY395275), *Ixodes scapularis* (AY690335), *Amblyomma americanum* (U07708) and *Dermacentor variabilis* (AY241957). Asterisks denote amino acids conserved in all sequences analyzed.

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003

Table 1
Comparison of the obtained nucleotide sequence with CRT nucleotide sequences available at *GenBank*. Similarity expressed as percentage.

	CRT sequence	*R. microplus* AY395254	*R. annulatus* AY395253	*R. sanguineus* AY395275	*I. scapularis* AY690335	*A. americanum* U07708	*D. variabilis* AY241957
CRT sequence	100	99.2	94.7	88.3	77.1	86.4	89
R. microplus AY395254	100	93.9	88.4	76.9	86.4	88.9	
R. annulatus AY395253	100	77.6	73.7	85.3	82.4		
R. sanguineus AY395275	100	72.3	84.6	82.3			
I. scapularis AY690335	100	78	75.7				
A. americanum U07708	100	87.4					
D. variabilis AY241957	100						

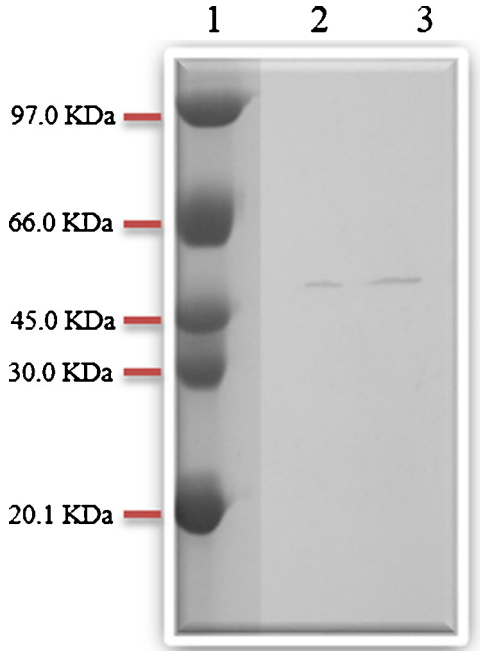

Fig. 2. Purified rCRT. SDS-PAGE gel at 12.5% stained with Coomassie-blue. 1 well: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); 2nd and 3rd wells: 1 μg of purified recombinant protein.

analysis show a high evolutionary relationship between tick CRTs and vertebrate CRTs; nevertheless, amino acid divergences among the full-length protein sequences of tick CRTs reveal a clear separation of tick CRTs (Parizi et al., 2009) indicating the existence of different immunogenic epitopes. In fact several studies point to

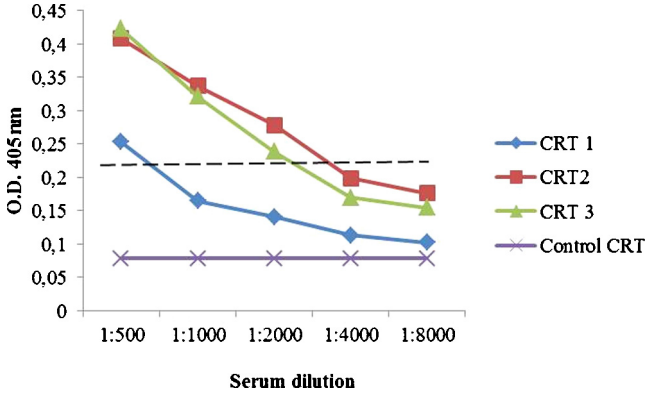

Fig. 3. Serum titer of each mouse after four rCRT immunizations. The dashed line represents three times the O.D. of the control serum.

the immunogenicity of tick CRT (Gao et al., 2008; Jaworski et al., 1995; Parizi et al., 2009; Xu et al., 2004) and, recently, antibodies against tick CRT were used as a biomarker to tick exposure (Alarcon-Chaidez et al., 2006; Vu Hai et al., 2013). Within the present study the rCRT showed to have a weak immunogenicity in mice since compared to control mice antibody titers were no greater than 1:4000 (Fig. 3). This appears in agreement with Ferreira et al. (2002) that found that bovines did not raise anti-tick CRT antibodies after vaccination with *R. microplus* rCRT. Differences in antigen production methodology and on adjuvant used, together with host natural genetic differences, may explain the discrepancies in results that have been obtained in different studies. As in Ferreira et al. (2002) study, the rCRT used for immunization in the present study did not have the native conformation therefore some epitopes may not have been accessible limiting the immunological response of the host. Both recombinant proteins were expressed using an affinity tag, which can influence protein folding/conformation (Smyth et al., 2003); (Klose et al., 2004). Since the capacity of an antigen to induce cross-reactive immunity in the host is essential when considering candidate vaccines to control infestation by multiple tick species, the CRT protein may not be an ideal candidate due to the high similarity to host CRT; more studies using anti-CRT antibodies and different adjuvants can clarify their effect in vector-tick-pathogen interactions.

Immunolocalization assays

The ability of the produced antibodies to recognize native CRT proteins was tested by immunofluorescence in different *R. microplus* tissues (Fig. 4) and compared with a negative control (Fig. 5). We used fixed paraffin-embedded tissue sections to detect the presence of CRT protein in tick tissues. Preliminary analysis showed that anti-CRT antibodies recognized native tick protein in all tissues (Fig. 4). IFA assays revealed that, as expected, CRT is dispersed in salivary glands cells (Gao et al., 2008; Jaworski et al., 1995; Kaewhom et al., 2008). CRT was also found in ovaries and, in this tissue, a predominantly cytoplasmic localization is clear in immature eggs. Anti-rCRT antibodies detected native CRT in tick midgut. Due to the many cellular functions, such as Ca^{2+} storage and signaling, it was expected that this protein would be found in the midgut (Michalak et al., 1999; Rachinsky et al., 2007).

Artificial feeding

CRT is an attractive candidate to the development of an anti-tick vaccine since it is highly conserved between ticks and as it is secreted by the tick to the host integrating the group of exposed antigens not requiring re-vaccination: feeding of ticks would *per se* re-boost the host immune system. The low immunogenicity

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003

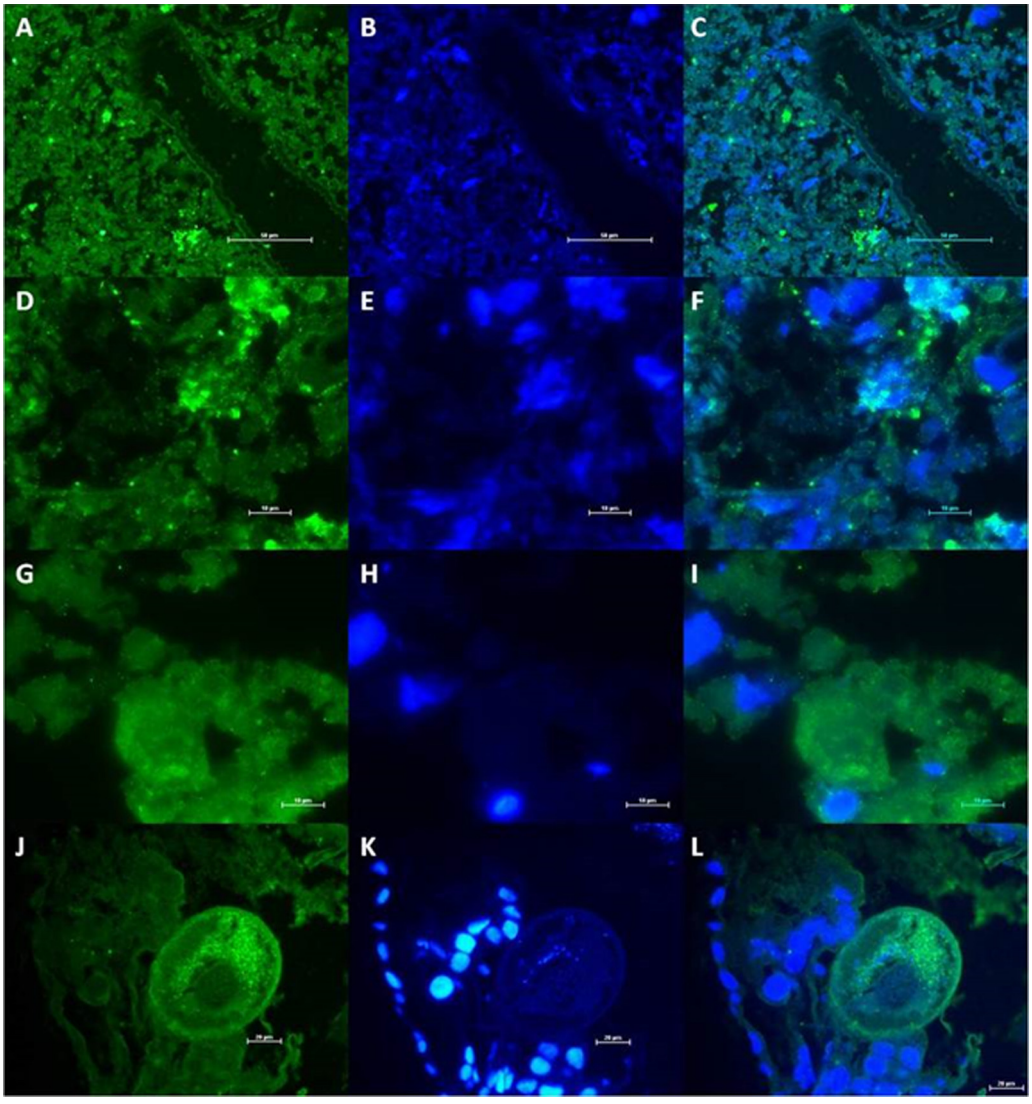

Fig. 4. Immunofluorescence analysis of cattle tick *R. microplus* tissues probed with anti-CRT antibodies. Sections of 2–3 μm of different tick tissues were made and subsequently incubated with anti-CRT serum (1:100). Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Nikon fluorescence microscope. Nuclear DNA was stained with DAPI. (A) Salivary glands section under a fluorescein filter at 400× amplification; (B) salivary glands section with DAPI staining; (C) merge of both staining in salivary glands; (D) salivary glands section under a fluorescein filter at 1000× amplification; (E) salivary glands section with DAPI staining; (F) merge of both staining in salivary glands; (G) midgut section under a fluorescein filter at 1000× amplification; (H) midgut section with DAPI staining; (I) merge of both staining in midgut section; (J) ovaries section under a fluorescein filter at 400× amplification; (K) ovaries section with DAPI staining; (L) merge of both staining in ovaries section. Bars, 50 μm (A–C); 10 μm (D–I) and 20 μm (G–H).

observed within the present study is in fact a drawback that can affect the outcome of the present study. Having this in mind, 15–20 μL of anti-CRT serum was presented to each tick during capillary feeding assays in an attempt to surpass the low antibodies titers. Semi-engorged females were used in accordance with Gonsioroski et al. (2012) and Sonenshine (1991) that demonstrate that females within a weight range accept artificial feeding better. Table 2 summarizes the results acquired relatively to parameters associated with artificial feeding. A significant weight increment in all groups was observed. Statistically, no significant differences

Table 2
Tick weight of capillary fed *R. microplus* females.

Groups	Weight (mg) avg ± SD		Weight increment	
	Before AF	After AF	(mg) avg ± sd	(%) avg ± sd
A – bovine blood	32.7 ± 6.8	116.6 ± 19.9	83.8 ± 20.7	134.1 ± 48.1
B – infected blood (*B. bigemina*)	32.9 ± 9.4	126.2 ± 41.6	93.3 ± 36	195.2 ± 45.6
C – blood + anti-CRT serum	29.7 ± 2.7	110.9 ± 20.8	81.2 ± 21.7	139.1 ± 47.8
D – blood + control serum	30.1 ± 4.6	127.3 ± 29.2	97.2 ± 27.1	162.1 ± 41.7
E – infected blood + control serum	30.3 ± 8.3	91.4 ± 29.2	61.1 ± 26.1	104.5 ± 43.3
F – infected blood + anti-CRT serum	36.7 ± 7.6	125.7 ± 37.6	89.1 ± 33.6	122.8 ± 42.6

Fifteen females were fed artificially by capillary tubes. Blood infected with *B. bigemina* at 0.7% parasitaemia or non-infected blood, both supplemented or not with antibodies, was offered to ticks. Around 15–20 μL of serum was presented to each tick. After 28 h ticks were weighted and placed into an incubator for 2–3 days to promote blood digestion. (AF, artificial feeding; avg, average; sd, standard deviation).

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003

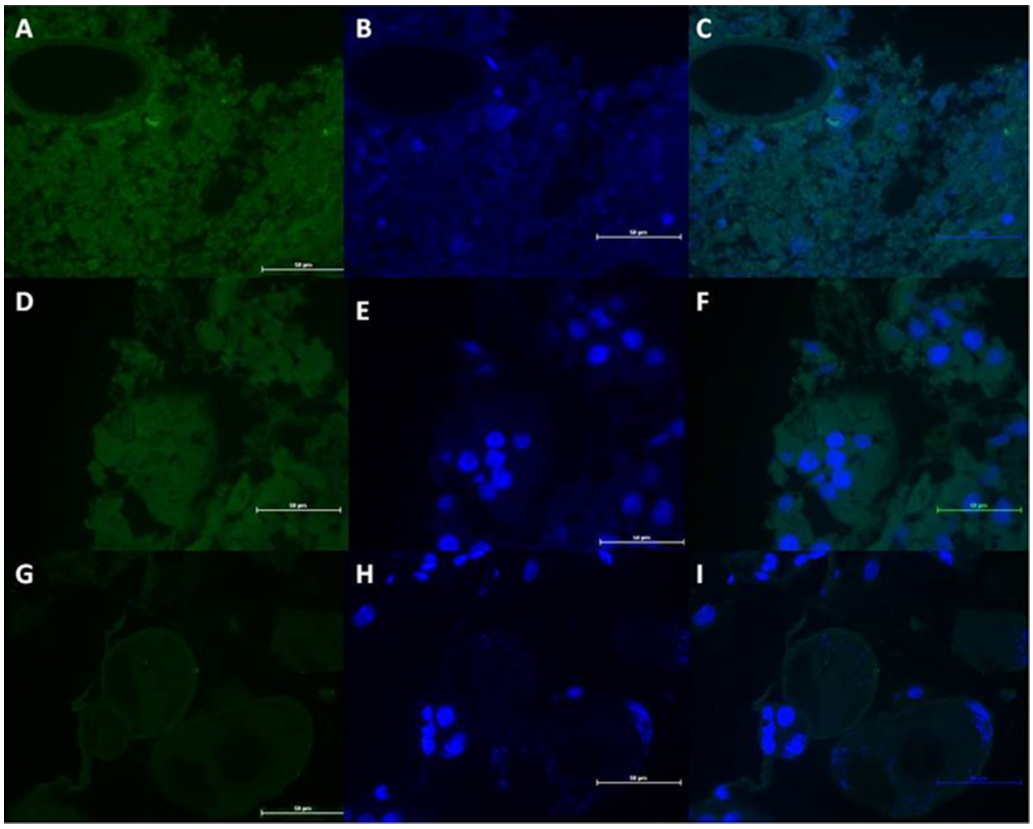

Fig. 5. Immunofluorescence analysis of cattle tick tissues probed with pre-immune serum (1:100). Sections of 2–3 μm of different tick tissues were made and subsequently incubated with pre-immune serum (1:100). Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Nikon fluorescence microscope at 400× amplification. Nuclear DNA was stained with DAPI. (A) Salivary glands section under a fluorescein filter; (B) salivary glands section with DAPI staining; (C) merge of both staining in salivary glands; (D) midgut section with under a fluorescein filter; (E) midgut section with DAPI staining; (F) merge of both staining in midgut section; (G) ovaries section under a fluorescein filter; (H) ovaries section with DAPI staining; (I) merge of both staining in ovaries section. Bars 50 μm.

were observed in weight increment between the group fed with bovine blood and the group fed with infected blood ($P > 0.05$), which is in accordance with previous studies that demonstrate the lack of impact of infection on blood ingestion by *R. microplus* (Antunes et al., 2014; Cen-Aguilar et al., 1998; Guglielmone et al., 1989).

Anti CRT serum did not influenced the increment of weight when compared to the control group (*t*-Student's test $P > 0.05$). Given the described putative functions to the CRT protein, which appears to be essential for feeding and pathogen transmission (Antunes et al., 2012; Gao et al., 2008; Jaworski et al., 1995; Michalak et al., 1999), these results were not expected. Instead, specimens fed with anti-CRT antibodies should have suffered weight loss. Concerning reproductive parameters analyzed here as the oviposition success by the weight of the laid eggs (Fig. 6), results demonstrate that infection does not influence the reproductive capacity of ticks (*t*-Student's test, $P > 0.05$) and also anti CRT serum did not influenced these tick parameters (*t*-Student's test, $P > 0.05$).

Regarding infection levels by *B. bigemina*, there was an apparent reduction in ticks fed with infected blood supplemented with anti-rCRT serum, both when compared with the group fed with infected blood (±65%) and with the group fed with control serum (±37%) (Table 3). However, these differences were not statistically significant (*t*-Student's test, $P > 0.05$).

As for CRT gene expression, a significant difference exists between the group fed with infected blood and the group fed with healthy blood (*t*-Student's test, $P < 0.05$) (data not shown), as observed previously by Antunes et al. (2012), who identified higher expression in the infected blood-fed population.

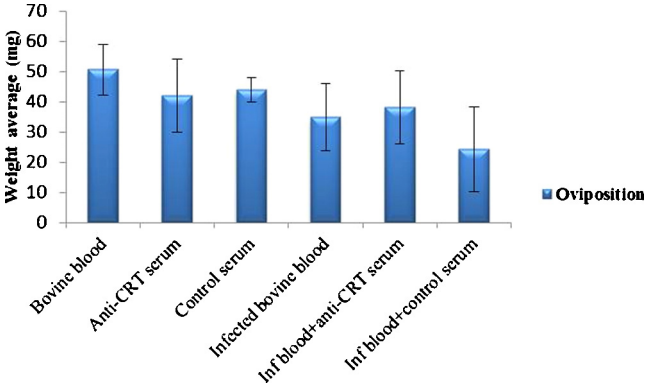

Fig. 6. Oviposition average in tick groups fed by capillary tubes. Five females of each group were randomly chosen and oviposition was allowed. Each specimen was placed into a 2 ml Eppendorf tube and incubated at 28 °C with an 85% relative humidity, which promotes oviposition.

Table 3
Babesia bigemina infection levels in partially engorged females after artificial feeding by capillary tubes.

	Infection levels by *B. bigemina* (avg ± sd)
Infected blood *B. bigemina*	7.89E−03 ± 2.25E−02
Infected blood + control serum	4.36E−03 ± 9.07E−03
Infected blood + anti-CRT serum	2.74E−03 ± 7.96E−03

Infection levels of 10 females fed artificially by capillary tubes with blood infected by *B. bigemina* at 0.7% parasitaemia, supplemented or not with antibodies, were determined by Real Time PCR using the delta delta *Ct* method and 16S rDNA tick gene as reference. (avg, average; sd, standard deviation).

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003

Tick to tick variation may have masked the real effect of specific antibodies and probably a larger sample number would improve the discussion of the present results (Antunes et al., 2014). Moreover, as CRT is a ubiquitous protein (Michalak et al., 1999) quantity of antibodies presented to ticks may have not been enough to promote visible effects: the number of offered antibodies as to be higher to ensure that a significant number of CRT proteins are blocked in the tick in order to affect tick metabolism. As reported in Antunes et al. (2014) other factors that could lead to this lack of effect on infection acquisition were the levels of parasitaemia in blood used for capillary feeding (0.7% infected erythrocytes in the present study) that may need to be higher to evidence the effect of the antibodies on pathogen infection and differences in pathogen infection mechanisms between *in vivo* tick feeding and *in vitro* capillary feeding. Although Inokuma and Kemp (1998) showed that it is possible to infect cattle ticks with *B. bigemina* using capillary feeding with infected blood, Kocan et al. (2005) demonstrated that the *in vitro* capillary feeding system does not reproduce *in vivo A. marginale* infection in ticks. Despite all these possibilities, under the conditions this study was undertaken, CRT shows not to be a good candidate for vaccine development since ingested antibodies did not affect any of the analyzed parameters. Also, the results obtained in the present study suggest that using serum, while raising the number of available antibodies in the meal, limited the amount of specific antibodies ingested by the specimens.

Conclusions

Herein recombinant CRT protein was expressed in an *E. coli* expression system. For being such a conserved protein, mice immunization resulted in a low immunogenic response and as a result high titers of anti-CRT antibody were not obtained. The central objective of this study was to evaluate the CRT antigen using *in vitro* tick feeding with antibodies directed against tick CRT involved in tick-pathogen interactions, as determined by systems biology and RNAi functional studies. *R. microplus* females were successfully artificially fed with infected and healthy bovine blood supplemented with anti-CRT serum with remarkable gain weight in all experimental groups. Also, after artificial feeding with infected and uninfected blood, the *crt* levels were found to be statistically different between groups in accordance to previous studies showing that this feeding method in fact mimics natural feeding. No significant reductions in tick body and egg weight were observed when ticks were fed with anti-rCRT serum. Regarding *B. bigemina* infection levels, in groups fed with infected blood containing anti-rCRT antibodies did not show a statistically significant decrease. The results reported here show that using *in vitro* tick capillary the recombinant CRT is not a good candidate for anti-tick vaccine development. A possible alternative to obtain different results could be the use of monoclonal antibody directed to protective epitopes. More studies focused in these protective epitopes can contribute not only to the characterization of this CRT protein but also to understand if surpassing the low immunogenic capacity of this protein would suffice to obtain different outcomes.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

JF and AD designed the study. JF and SA performed data analysis; SA, OM and JM performed the tick work. SA, OM, and JL performed the laboratory work. SA and ND wrote the manuscript and all authors edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Mario Hidalgo Ruiz (University of Queretaro, Mexico) for technical assistance and also the technical support in the histological sections given by Jorge Correia and Maria do Rosário (Faculdade de Medicina Veterinária). This research was supported by the project PTDC/CVT/112050/2009, subsidized by the Fundação para a Ciência e Tecnologia (FCT) and the EU FP7 ANTIGONE project number 278976. S. Antunes is a recipient of PhD grant supported by FCT (SFRH/BD/48251/2008). O. Merino is an Early Stage Researcher supported by the POSTICK ITN (Post-graduate training network for capacity building to control ticks and tick-borne diseases) within the FP7-PEOPLE-ITN programme (EU Grant No. 238511).

References

Alarcon-Chaidez, F., Ryan, R., Wikel, S., Dardick, K., Lawler, C., Foppa, I.M., Tomas, P., Cushman, A., Hsieh, A., Spielman, A., Bouchard, K.R., Dias, F., Aslanzadeh, J., Krause, P.J., 2006. Confirmation of tick bite by detection of antibody to *Ixodes* calreticulin. Clin. Vaccine Immunol. 13, 1217–1222.

Almazan, C., Blas-Machado, U., Kocan, K.M., Yoshioka, J.H., Blouin, E.F., Mangold, A.J., de la Fuente, J., 2005. Characterization of three *Ixodes scapularis* cDNAs protective against tick infestations. Vaccine 23, 4403–4416.

Almazan, C., Lagunes, R., Villar, M., Canales, M., Rosario-Cruz, R., Jongejan, F., de la Fuente, J., 2010. Identification and characterization of *Rhipicephalus* (*Boophilus*) *microplus candidate* protective antigens for the control of cattle tick infestations. Parasitol. Res. 106, 471–479.

Antunes, S., Galindo, R.C., Almazan, C., Rudenko, N., Golovchenko, M., Grubhoffer, L., Shkap, V., do Rosario, V., de la Fuente, J., Domingos, A., 2012. Functional genomics studies of *Rhipicephalus* (*Boophilus*) *annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*. Int. J. Parasitol. 42, 187–195.

Antunes, S., Merino, O., Mosqueda, J., Moreno-Cid, J.A., Bell-Sakyi, L., Fragkoudis, R., Weisheit, S., Perez de la Lastra, J.M., Alberdi, P., Domingos, A., de la Fuente, J., 2014. Tick capillary feeding for the study of proteins involved in tick–pathogen interactions as potential antigens for the control of tick infestation and pathogen infection. Parasites Vectors 7, 42.

Breitschwerdt, E.B., 2011. Comparative medical importance of a One Health approach to emerging tick-borne diseases. In: Press, T.N.A. (Ed.), Critical Needs and Gaps in Understanding Prevention, Amelioration, and Resolution of Lyme and Other Tick-Borne Diseases. The National Academies Press, Washington, DC, pp. 49–53.

Broadwater, A.H., Sonenshine, D.E., Hynes, W.L., Ceraul, S., De Silva, A.M., 2002. Glass capillary tube feeding: a method for infecting nymphal *Ixodes scapularis* (Acari: Ixodidae) with the Lyme disease spirochete *Borrelia burgdorferi*. J. Med. Entomol. 39, 285–292.

Canales, M., Almazan, C., Naranjo, V., Jongejan, F., de la Fuente, J., 2009. Vaccination with recombinant *Boophilus annulatus* Bm86 ortholog protein. Ba86, protects cattle against *B. annulatus* and *B. microplus* infestations. BMC Biotechnol., 9.

Cen-Aguilar, J.F., Rodriguez-Vivas, R.I., Dominguez-Alpizar, J.L., Wagner, G.G., 1998. Studies on the effect of infection by *Babesia* sp. on oviposition of *Boophilus microplus* engorged females naturally infected in the Mexican tropics. Vet. Parasitol. 78, 253–257.

de la Fuente, J., Kocan, K.M., Blouin, E.F., 2007. Tick vaccines and the transmission of tick-borne pathogens. Vet. Res. Commun. 31, 85–90.

de la Fuente, J., Merino, O., 2013. Vaccinomics, the new road to tick vaccines. Vaccine 31, 5923–5929.

Ferreira, C.A.S., Vaz, I.D., da Silva, S.S., Haag, K.L., Valenzuela, J.G., Masuda, A., 2002. Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) calreticulin. Exp. Parasitol. 101, 25–34.

Ferreira, V., Molina, M.C., Valck, C., Rojas, A., Aguilar, L., Ramirez, G., Schwaeble, W., Ferreira, A., 2004. Role of calreticulin from parasites in its interaction with vertebrate hosts. Mol. Immunol. 40, 1279–1291.

Fliegel, L., Burns, K., Maclennan, D.H., Reithmeier, R.A.F., Michalak, M., 1989. Molecular clonning of the high affinity calcium binding protein (calreticulin) of the skeletal-muscle sarcoplasmic reticulum. J. Biol. Chem. 264, 21522–21528.

Gao, J., Luo, J., Fan, R., Fingerle, V., Guan, G., Liu, Z., Li, Y., Zhao, H., Ma, M., Liu, J., Liu, A., Ren, Q., Dang, Z., Sugimoto, C., Yin, H., 2008. Cloning and characterization of a cDNA clone encoding calreticulin from *Haemaphysalis qinghaiensis* (Acari: Ixodidae). Parasitol. Res. 102, 737–746.

George, J.E., Pound, J.M., Davey, R.B., 2004. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. Parasitology 129, S353–S366.

Gonsioroski, A.V., Bezerra, I.A., Utiumi, K.U., Driemeier, D., Farias, S.E., Vaz Jr., I.d.S., Masuda, A., 2012. Anti-tick monoclonal antibody applied by artificial capillary feeding in *Rhipicephalus* (*Boophilus*) *microplus females*. Exp. Parasitol. 130, 359–363.

Graf, J.F., Gogolewski, R., Leach-Bing, N., Sabatini, G.A., Molento, M.B., Bordin, E.L., Arantes, G.J., 2004. Tick control: an industry point of view. Parasitology 129, S427–S442.

Guerrero, F.D., Miller, R.J., de Leon, A.A.P., 2012. Cattle tick vaccines: many candidate antigens, but will a commercially viable product emerge? Int. J. Parasitol. 42, 421–427.

Guglielmone, A.A., Mangold, A.J., Aguirre, D.H., Gaido, A.B., Deolsen, A.A., 1989. The effect of infection by *Babesia* sp. on some biological parameters of engorged females *Boophilus microplus*. Folia Parasitol. 36, 1–6.

Heekin, A.M., Guerrero, F.D., Bendele, K.G., Saldivar, L., Scoles, G.A., Dowd, S.E., Gondro, C., Nene, V., Djikeng, A., Brayton, K.A., 2013. Gut transcriptome of replete adult female cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*, feeding upon a *Babesia bovis*-infected bovine host. Parasitol. Res. 112, 3075–3090.

Inokuma, H., Kemp, D.H., 1998. Establishment of *Boophilus microplus* infected with *Babesia bigemina* by using in vitro tube feeding technique. J. Vet. Med. Sci. 60, 509–512.

Jaworski, D.C., Simmen, F.A., Lamoreaux, W., Coons, L.B., Muller, M.T., Needham, G.R., 1995. A secreted calreticulin protein in Ixodid tick (*Amblyomma americanum*) saliva. J. Insect Physiol. 41, 369–375.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129, S3–S14.

Kaewhom, P., Stich, R.W., Needham, G.R., Jittapalapong, S., 2008. Molecular analysis of calreticulin expressed in salivary glands of *Rhipicephalus* (*Boophilus*) *microplus* indigenous to Thailand. Ann. N. Y. Acad. Sci. 1149, 53–57.

Klose, J., Wendt, N., Kubald, S., Krause, E., Fechner, K., Beyermann, M., Bienert, M., Rudolph, R., Rothemund, S., 2004. Hexa-histidin tag position influences disulfide structure but not binding behavior of in vitro folded N-terminal domain of rat corticotropin-releasing factor receptor type 2a. Protein Sci. 13, 2470–2475.

Knowles, D., deEchaide, S.T., Palmer, G., McGuire, T., Stiller, D., McElwain, T., 1996. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. J. Clin. Microbiol. 34, 2225–2230.

Kocan, K.M., Yoshioka, J., Sonenshine, D.E., De la Fuente, J., Ceraul, S.M., Blouin, E.F., Almazan, C., 2005. Capillary tube feeding system for studying tick-pathogen interactions of *Dermacentor variabilis* (Acari: Ixodidae) and *Anaplasma marginale* (Rickettsiales: Anaplasmataceae). J. Med. Entomol. 42, 864–874.

Kovacs, H., Campbell, I.D., Strong, P., Johnson, S., Ward, F.J., Reid, K.B.M., Eggleton, P., 1998. Evidence that Clq binds specifically to C(H)2-like immunoglobulin gamma motifs present in the autoantigen calreticulin and interferes with complement activation. Biochemistry 37, 17865–17874.

Lew-Tabor, A.E., Bruyeres, A.G., Zhang, B., Rodriguez Valle, M., 2014. *Rhipicephalus* (*Boophilus*) *microplus* tick in vitro feeding methods for functional (dsRNA) and vaccine candidate (antibody) screening. Ticks Tick Borne Dis. 5, 500–510.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(*T*)(−Delta Delta *C*) method. Methods 25, 402–408.

Matsuo, T., Inoue, N., Ruheta, M.R., Taylor, D., Fujisaki, K., 2004. Tickcidal effect of monoclonal antibodies against hemocytes, Om21, in an adult female tick, *Ornithodoros moubata* (Acari: Argasidae). J. Parasitol. 90, 715–720.

Merino, O., Alberdi, P., Pérez de la Lastra, J., de la Fuente, J., 2013. Tick vaccines and the control of tick-borne pathogens. Front. Cell. Infect. Microbiol., 3.

Michalak, M., Corbett, E.F., Mesaeli, N., Nakamura, K., Opas, M., 1999. Calreticulin: one protein, one gene, many functions. Biochem. J. 344, 281–292.

Michalak, M., Groenendyk, J., Szabo, E., Gold, L.I., Opas, M., 2009. Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. Biochem. J. 417, 651–666.

Parizi, L.F., Githaka, N.W., Logullo, C., Konnai, S., Masuda, A., Ohashi, K., Vaz Jr., I.d.S., 2012. The quest for a universal vaccine against ticks: cross-immunity insights. Vet. J. 194, 158–165.

Parizi, L.F., Rech, H., Sanchez Ferreira, C.A., Imamura, S., Ohashi, K., Onuma, M., Masuda, A., Vaz Jr., I.d.S., 2009. Comparative immunogenicity of *Haemaphysalis longicornis* and *Rhipicephalus* (*Boophilus*) *microplus* calreticulins. Vet. Parasitol. 164, 282–290.

Persson, S., Rosenquist, M., Sommarin, M., 2002. Identification of a novel calreticulin isoform (Crt2) in human and mouse. Gene 297, 151–158.

Peter, R.J., Van den Bossche, P., Penzhorn, B.L., Sharp, B., 2009. Tick, fly, and mosquito control – lessons from the past, solutions for the future. J. S. Afr. Vet. Assoc. 80, 121.

Rachinsky, A., Guerrero, F.D., Scoles, G.A., 2007. Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem. Mol. Biol. 37, 1291–1308.

Rachinsky, A., Guerrero, F.D., Scoles, G.A., 2008. Proteomic profiling of *Rhipicephalus* (*Boophilus*) *microplus* midgut responses to infection with *Babesia bovis*. Vet. Parasitol. 152, 294–313.

Rodriguez, M., Rubiera, R., Penichet, M., Montesinos, R., Cremata, J., Falcon, V., Sanchez, G., Bringas, R., Cordoves, C., Valdes, M., Lleonart, R., Herrera, L., Delafuente, J., 1994. High level expression of the *B. microplus* BM86 antigen in the yeast *Pichia pastoris* forming highly immunogenic particles for cattle. J. Biotechnol. 33, 135–146.

Schefe, J.H., Lehmann, K.E., Buschmann, I.R., Unger, T., Funke-Kaiser, H., 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel gene expression's C-T difference formula. J. Mol. Med. 84, 901–910.

Shkap, V., Leibovitz, B., Krigel, Y., Hammerschlag, J., Marcovics, A., Fish, L., Molad, T., Savitsky, I., Mazuz, M., 2005. Vaccination of older *Bos taurus* bulls against bovine babesiosis. Vet. Parasitol. 129, 235–242.

Smyth, D.R., Mrozkiewicz, M.K., McGrath, W.J., Listwan, P., Kobe, B., 2003. Crystal structures of fusion proteins with large-affinity tags. Protein Sci. 12, 1313–1322.

Sonenshine, D.E., 1991. Biology of Ticks. Oxford University Press, New York.

Suarez, C.E., Noh, S., 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. Vet. Parasitol. 180, 109–125.

Vitale, A., 2009. Calreticulins are not all the same. Proc. Natl. Acad. Sci. U. S. A. 106, 13151–13152.

Vu Hai, V., Pages, F., Boulanger, N., Audebert, S., Parola, P., Almeras, L., 2013. Immunoproteomic identification of antigenic salivary biomarkers detected by *Ixodes ricinus*-exposed rabbit sera. Ticks Tick Borne Dis. 4, 459–468.

Willadsen, P., 2006. Tick control: thoughts on a research agenda. Vet. Parasitol. 138, 161–168.

Willadsen, P., Riding, G.A., McKenna, R.V., Kemp, D.H., Tellam, R.L., Nielsen, J.N., Lahnstein, J., Cobon, G.S., Gough, J.M., 1989. Immunological control of a parasitic arthropod: Identification of a protective antigen from *Boophilus microplus*. J. Immunol. 143, 1346–1351.

Xu, G.A., Fang, Q.Q., Keirans, J.E., Durden, L.A., 2004. Cloning and sequencing of putative calreticulin complementary DNAs from four hard tick species. J. Parasitol. 90, 73–78.

Zivkovic, Z., Torina, A., Mitra, R., Alongi, A., Scimeca, S., Kocan, K.M., Galindo, R.C., Almazan, C., Blouin, E.F., Villar, M., Nijhof, A.M., Mani, R., La Barbera, G., Caracappa, S., Jongejan, F., de la Fuente, J., 2010. Subolesin expression in response to pathogen infection in ticks. BMC Immunol. 11, 12.

Please cite this article in press as: Antunes, S., et al., Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick-borne Dis. (2014), http://dx.doi.org/10.1016/j.ttbdis.2014.09.003