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Comparative immunogenicity of *Haemaphysalis longicornis* and *Rhipicephalus* (*Boophilus*) *microplus* calreticulins

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ABSTRACT

The ticks *Rhipicephalus* (*Boophilus*) *microplus* and *Haemaphysalis longicornis* are blood-sucking ectoparasites of bovines, causing serious damages to the livestock production. The main control method for these ticks is based on acaricides. However, the use of vaccines has been studied as a promising control strategy. Calreticulin (CRT) is a multifunctional, predominantly intracellular protein present in almost all cells of animals. The secretion of CRT during feeding might be linked to the modulation of the parasite–host interaction. In the present study, recombinant CRTs of *R. microplus* (rBmCRT) and *H. longicornis* (rHlCRT) were expressed in *Escherichia coli* and purified by ion exchange chromatography and used for the immunization of bovines and mouse. ELISA demonstrated that both rCRTs are recognized by the sera of immunized bovines. *In silico*, despite the difference in amino acid sequences, antigenic index analysis of HlCRT and BmCRT using the Jameson–Wolf algorithm indicated that both proteins were very similar in antigenicity index, although six different epitopes between the tick CRTs have been inferred. These data were corroborated by competitive ELISA analyses, which suggest the presence of different epitopes within the proteins. Western blot analyses showed that anti-rBmCRT and anti-rHlCRT bovine sera also recognized the native proteins in larvae extracts and, moreover, sera of bovines immunized with saliva and extract of salivary glands recognized both recombinant CRTs. Thus, mouse and bovine immune system recognized rCRTs, resulting in the production of antibodies with similar specificity for both recombinant proteins, although different epitopes could be distinguished between rBmCRT and rHlCRT.

1. Introduction

The ticks *Rhipicephalus* (*Boophilus*) *microplus* and *Haemaphysalis longicornis* are blood-sucking ectoparasites of bovines, causing serious damages to the livestock production. The tick *H. longicornis* is distributed mainly in East Asia and Australia, where it transmits a wide range of pathogens, including *Theileria* spp., *Babesia ovata*, *Babesia gibsoni* and *Rickettsia japonica* (Fujisaki et al., 1994;

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Jongejan and Uilenberg, 2004). *R. microplus* is a one-host tick that usually infests cattle and is responsible for economic losses that range over hundreds of millions of dollars per year (Guerrero et al., 2006). However, despite the importance of ticks as parasites and vectors of diseases, very little is known about these parasitic relationships and their basic biology. Also, the mechanisms by which cellular and humoral immune responses of the hosts damage ticks are not fully understood. This knowledge is important for the determination of host–pathogen interactions that allows parasite survival and facilitate or limit disease transmission (Jaworski et al., 1995; Willadsen, 2006a; Gao et al., 2007). Host immune responses to ixodidae ticks are acquired through either tick infestation or artificial immunization with tick antigens. These immune responses affect feeding, reproduction, and survival of the ticks (Szabo et al., 1995; Szabo and Bechara, 1997; Kotsyfakis et al., 2007). In the development of host resistance, antibody responses have been shown to be important in this mechanism (Kashino et al., 2005; Willadsen, 2006a; Wikel and Allen, 1976). An anti-tick vaccine is probably the most promising method to control the parasite, but the success of this strategy is dependent on the characterization of the roles of essential tick molecules in the arthropod physiology. Due to the importance of saliva in blood feeding, host immunity and pathogen transmission, the characterization of saliva molecules may improve our knowledge on the mechanisms involved in tick–host interaction and the way through which these mechanisms could be inhibited, and therefore configure potential vaccine candidates. Salivary secretions are well recognized to perform such modulatory events in the tick–host relationship (Ribeiro, 1989; Wikel, 1999; Ciprandi et al., 2003; Steen et al., 2006), but the purification and further characterization of these activities are often unfeasible, due to the small amounts of saliva that are available from ticks. An alternative approach is the identification of tick salivary genes in bacterial expression systems and the search for their biological functions using recombinant proteins. Calreticulin (CRT) is a calcium-binding protein present in almost all cells of animals and known to perform several functions in mammals (Michalak et al., 1992, 1999; Gold et al., 2006). CRT is also secreted by ticks into their hosts (Jaworski et al., 1995; Ferreira et al., 2002). The presence of calreticulin in tick saliva (Jaworski et al., 1995; Ferreira et al., 2002) and other parasite secretions (Kasper et al., 2001; Suchitra and Joshi, 2005; Cabezon et al., 2008) suggests a role for this protein in feeding, host immunosuppression and antihemostasis. As CRTs show a high degree of conservation, we hypothesized that CRT of *H. longicornis* (HlCRT) could stimulate a humoral response that would also be able to recognize the CRT of *R. microplus* (BmCRT), and vice versa. This cross-species immunity could be useful for the development of a vaccine against multi-species tick infestations. The characterization of the recombinant forms of BmCRT (rBmCRT) and HlCRT (rHlCRT), like the studies of immunogenic potential of these proteins in bovines, could provide insights into the potential of CRTs as multi-species protective antigen. In this work, we report the isolation and characterization of the *H. longicornis* cDNA sequence, as well as the cloning, expression and purification of the calreticulins from *H. longicornis* and *R. microplus*, and the analyses of the immunogenic potential of the recombinant proteins as multi-species cross-reactive antigen candidates.

2. Materials and methods

2.1. Cloning of HlCRT and BmCRT

The HlCRT coding region was amplified by PCR from a *H. longicornis* cDNA library (Mulenga et al., 1999) and cloned into the pGEM-T vector (Promega). The primers used in the process (sense, aaaaacatatggatcccaacgtttacttcaa; antisense, aaaaaaagcttttagtggtggtggtggtgcaactcttcgtgcttgtgg) were based on conserved regions of the *R. microplus* calreticulin gene (accession number AF420211). The amplied cDNA product was separated by agarose gel electrophoresis and puried by Glassmilk DNA purication kit (BIO 101 Systems). The HlCRT cDNA was ligated into the pGEM-T vector, and transformed into DH5α strain of *Escherichia coli*. The plasmids were puried and the nucleotide sequences of the inserts were determined on an 8-capillary Beckman CEQ2000 automated sequencer. The *H. longicornis* calreticulin sequence was deposited into GenBank (accession number FJ536258).

The coding sequence of mature HlCRT was then cloned into pET-43a expression vector (Novagen). PCR was performed using pGEM/HlCRT and primers containing restriction sites of NdeI and HindIII (sense, aaaaacatatggatcccaacgtttacttcaa; antisense, aaaaaaagcttttagtggtggtggtggtggtgcaactcttcgtgcttgtgg). Following cleavage and precipitation, amplicon and vector were ligated with T4 DNA ligase (Invitrogen) to give pET-43a/HlCRT plasmid.

The BmCRT gene was isolated previously (Ferreira et al., 2002) and cloned in the pBluescript II (Stratagene) plasmid. This plasmid was used to amplify the coding sequence of mature BmCRT by PCR using primers containing restriction sites of NdeI and HindIII (sense, aaaaacatatggacccgaccgtatacttca; antisense, aaaaaaagcttttagtggtggtggtggtggtgcaactcttcgtgcttgtgg). Following cleavage and precipitation, amplicon and vector were ligated with T4 DNA ligase (Invitrogen) to give pET-5b/BmCRT plasmid.

The resulting plasmids were transformed by electroporation into XL1 Blue *E. coli* cells (Novagen) and selected in SOC medium containing ampicillin (100 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). The pET-43a/HlCRT and pET-5b/BmCRT integrities were confirmed by DNA sequencing.

2.2. Phylogenetic analysis

Calreticulin sequences were aligned with the BmCRT and HlCRT deduced protein sequences using BioEdit version 5.0.6 software program (Hall, 1999). An unrooted neighbor-joining phylogenetic tree was created using the MEGA version 4 (Tamura et al., 2007). Bootstrap support was assessed using 1000 replicates. The GenBank accession numbers for the species used in analysis were as follow: *H. longicornis*, FJ536258; *Homo sapiens*, AAH025; *Macaca mulatta*, XP001110174; *Mus musculus*, AAH03453;

Rattus norvegicus, AAH62395; *Bos taurus*, BAB86913; *Oryctolagus cuniculus*, AAB20096; *Gallus gallus*, AAS49610; *Amblyomma americanum*, AAR29932; *Dermacentor variabilis*, AAR29944; *R. microplus*, AAN03709; *Rhipicephalus sanguineus*, AAR29961; *Amblyomma scutatum*, AAR29938; *H. leporispalustris*, AAR29947; *Ixodes pararicinus*, AAR29956; *I. ricinus*, AAR29958; *I. scapularis*, AAQ18696; *Necator americanus*, CAA07254 and *Haemonchus contortus*, AAR99585.

2.3. Jameson–Wolf analysis

The antigenic index analysis of HlCRT and BmCRT was performed with the Jameson–Wolf algorithm by software LASERGENE version 8.0.2 to predict potential antigenic determinants by combining existing methods for protein structural predictions (Jameson and Wolf, 1988).

2.4. Expression of recombinant HlCRT and BmCRT proteins

The recombinant plasmids pET-5b/BmCRT and pET-43a/HlCRT were transformed into *E. coli* AD494 (DES) pLysS strain (Novagen). Recombinant *E. coli* were induced to grow in SOB medium containing ampicillin (100 μg ml^{-1}). Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (at OD = 0.6) for 16 h at 23 °C (rHlCRT) and 30 °C (rBmCRT). After, cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C and resuspended in 50 ml of phosphate lysis buffer (10 mM sodium phosphate, 300 mM NaCl, pH 6.5). For cell lysis, the suspension was frozen, thawed out three times and sonicated five times for 30 s at 40 MHz on ice. The soluble and insoluble fractions were separated by centrifugation at 10,000 × *g* for 10 min at 4 °C and the soluble fractions were stored at −20 °C prior to use.

2.5. Purification of rHlCRT and rBmCRT

The soluble fractions containing the rBmCRT or rHlCRT were purified by HiTrap Mono Q ion-exchange column chromatography following the manufacturer's instructions (Amersham Biosciences). Briefly, the soluble fractions were ltered in 0.45-μm filters (Millipore) and then applied onto the columns previously equilibrated with phosphate lysis buffer. Proteins of interest were eluted with phosphate buffer with two concentrations of NaCl (10 mM sodium phosphate, 400 or 500 mM NaCl, pH 6.5) at room temperature with a ow rate of 0.5 ml/min. The purified protein fractions were dialyzed against phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.2). Eluted fractions were concentrated (Centricon YM10—10,000 MW cut-off, Millipore) and analyzed following 12% SDS-PAGE (Laemmli, 1970) stained with Coomassie blue G-250.

2.6. Animals

Hereford (*Bos taurus taurus*) cattle were acquired from a tick-free area, housed in individual tick-proof pens on slatted oors and maintained at the Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. BALB/c mice used for immunizations were maintained at the Animal Facility of Centro de Biotecnologia, UFRGS. Animal care was in accordance with institutional guidelines.

2.7. Larvae extract preparation

R. microplus were maintained on experimentally infested Hereford cattle and engorged adult female ticks were kept in Petri dishes at 28 °C and 85% relative humidity until completion of oviposition and, then, eggs were collected and incubated under the same conditions. Twenty-day-old larvae were disrupted and homogenized using a mortar and pestle on ice bath with 10 mM phosphate buffer, pH 7.2. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4 °C to remove the insoluble material and the soluble supernatant fraction was collected. The protein extracts were prepared *according* to the method *previously described* (de Lima et al., 2002). The protein concentrations of the extracts were measured using the *modified Bradford method* with bovine serum albumin as standard.

2.8. Immunization of bovines and mice with rBmCRT and rHlCRT

Groups of two mice were immunized individually with rHlCRT or rBmCRT eluted with phosphate buffer plus NaCl 500 mM or phosphate buffer plus NaCl 400 mM. Immunizations were performed intraperitoneally with three doses of 100 μg of protein emulsified in Freund's incomplete adjuvant at 14-day intervals. Fourteen days after the last booster sera were collected.

Three bovines were immunized subcutaneously: bovine 1 (control) received an emulsion composed of 1 ml PBS plus 1 ml of oil adjuvant (Montanide 888 and Marcol 52) per dose; bovines 2 and 3 received emulsions composed of 1 ml of rBmCRT or rHlCRT (fractions eluted with phosphate buffer plus NaCl 500 mM) solution in PBS (100 μg/dose) plus 1 ml adjuvant, respectively. The bovines received three doses, at 14-day intervals and sera were collected 14 days after each inoculation. The antibody kinetics of the sera was estimated by ELISA and the sera after the last immunization were used in all subsequent experiments.

2.9. Immunization with saliva and extract of salivary glands

Salivary glands extracts were obtained from partially engorged female ticks according to Vaz et al. (1994). Briey, the dorsal surface was dissected and salivary glands were separated and washed in PBS. The tissues were sonicated and solubilized in 0.5% sodium deoxycolate, 0.1% pepstatin A, 0.1% leupeptin and 0.1 mM TPCK in 10 mM tris buffer (pH 8.2). The material was centrifuged and supernatants were stored at −70 °C.

One bovine was immunized with one dose of 200 μg of partially engorged extract of salivary glands in 0.5 ml of PBS and mixed with an equal volume of Freund's complete adjuvant, followed by 7 further immunizations of 300–400 μg of partially engorged extract of salivary glands in 0.5 ml of PBS mixed with an equal volume of Freund's incomplete adjuvant. One bovine was immunized with one dose of 400 μl of partially engorged pilocarpine induced saliva mixed with an equal volume of Freund's complete

adjuvant, followed by 6 further immunizations of 400–600 μl of partially engorged or fully engorged pilocarpine induced saliva mixed with an equal volume of Freund's incomplete adjuvant. Sera were collected 15 days after the last booster.

2.10. Serological analysis by ELISA

The bovine and mouse humoral responses were verified by ELISA and Western blot using rBmCRT or rHlCRT as antigen. All sera were incubated for 1 h with *E. coli* extract to allow the absorption of anti-*E. coli* protein antibodies (Ferreira et al., 2002). For IgG titration, by ELISA, microplates were coated with 100 ng/well of rBmCRT or rHlCRT (fractions eluted with phosphate buffer plus NaCl 500 mM) for 16 h at 4 °C with 50 mM carbonate–bicarbonate buffer pH 9.6. The two antigens were probed with sera of bovines immunized with rBmCRT or rHlCRT and detected with rabbit anti-IgG bovine peroxidase conjugate. Development was performed with 10 ml of phosphate–citrate buffer containing 5 μl of H_2O_2 and 3.2 mg of OPD. After 20 min, the reaction was stopped by the addition of H_2SO_4 12.5% and the absorbance was measured at 490 nm. Sera were considered positive in ELISA when an OD value higher than the mean plus 2 SD of the OD showed from pre-immunization sera (control sera) was obtained.

Bovine sera and rBmCRT or rHlCRT were used in competition ELISA. Microplates were coated with rBmCRT or rHlCRT as described above, and increasing rBmCRT or rHlCRT quantities (from 50 to 800 ng) were added. Sera diluted at 1:200 were added and incubated for 1 h. Positive signals were detected with bovine anti-IgG peroxidase conjugate and development was performed as described above. Each analysis was performed twice in duplicate.

2.11. SDS-PAGE and Western blot analyses

For Western blot analyses, proteins were separated by SDS-PAGE 12% and transferred onto a nitrocellulose membrane. The membrane was then cut into 5-mm-wide strips. The strips containing recombinant proteins were probed with sera of bovine or mouse immunized with the recombinant proteins.

Strips containing *R. microplus* larval extract were incubated with sera from bovines immunized with rHlCRT or rBmCRT in order to test the recognition of native CRT. Also, strips containing the recombinant proteins were probed with sera from bovines immunized with saliva or extract of salivary glands of *R. microplus*.

After the serum incubations, the anti-IgG species-specific alkaline phosphatase conjugates were used as secondary antibodies and revelations were performed with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma).

3. Results

3.1. Sequences and phylogenetic analyses of CRTs

Sequences from *R. microplus* and *H. longicornis* calreticulins have 88.1% identity in the coding region. The identities comparing CRT sequences of the ticks *R. sanguineus* and *A. americanum* for HlCRT were, respectively, 87.9% and 97.8%, and for BmCRT were 88.3% and 90.3%. Sequence comparisons between other highly divergent parasite species such as *N. americanus* and *H. contortus* show 62.9% and 59.2% sequence identity to HlCRT and 62.1% and 57.5% to BmCRT. Also, confirming the high conservation between CRT sequences of various species the comparison between the sequences of HlCRT or BmCRT and tick host species (mammalian CRTs: mouse, rat, human, rabbit and bovine) show similar identities (around 62%).

A neighbor-joining tree constructed with CRTs sequences is shown in Fig. 1. The branches of the tree regarding the sequences of CRTs of ticks showed high bootstrap values. The HlCRT and BmCRT sequences group with other tick sequences, while the host tick sequences *clearly group* outside the tick sequences. Interestingly, tick sequences group primarily with mammalian than with other invertebrate sequences.

3.2. Cloning, expression and purification of rCRTs

The cloning of the coding sequences of mature CRTs from *H. longicornis* and *R. microplus* were performed in pET (Novagen) expression vectors. The apparent molecular masses of rHlCRT and rBmCRT were of approximately 55–60 kDa in SDS-PAGE (Fig. 2a). The bands visualized in SDS-PAGE were confirmed as rHlCRT and rBmCRT proteins by Western blot with bovine anti-rHlCRT serum (Fig. 2b).

The purifications of the rHlCRT or rBmCRT were carried out by ion-exchange chromatography. Both proteins were eluted with phosphate buffer 10 mM containing 400 or 500 mM of NaCl. However, rHlCRT or rBmCRT eluted with phosphate buffer plus NaCl 500 mM were shown to present a smaller number of bacterial protein bands in SDS-PAGE 12% stained with Coomassie Blue G-250, under denaturing conditions (data not shown). The recombinant proteins were further purified by gel filtration chromato-

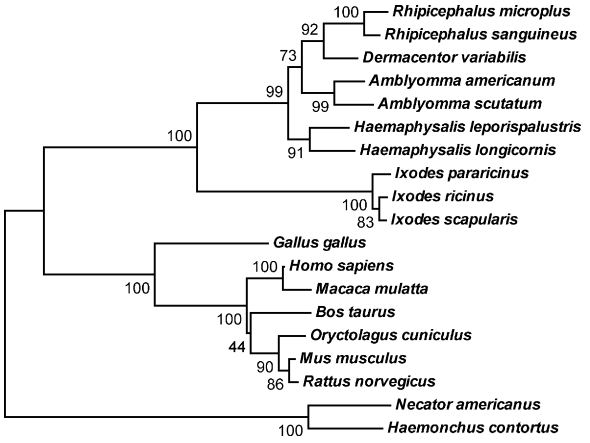

Fig. 1. Dendrogram based on CRT of *H. longicornis*, *R. microplus* and other selected eukaryotes. The tree is based on aminoacid divergences between the full-length protein sequences. GenBank accession numbers for the sequences are in material and methods. Bootstrap values of 1000 simulations are shown at the branches.

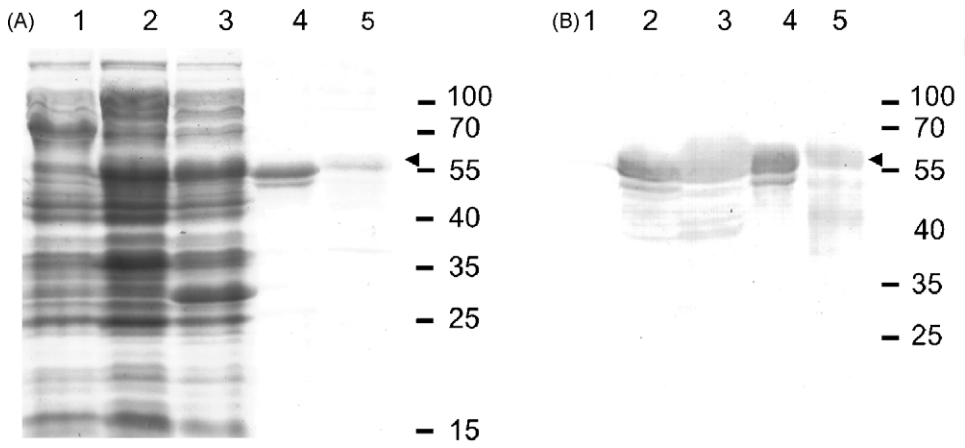

Fig. 2. SDS-PAGE 12% stained with Coomassie blue G-250 (A) and Western blot (B) of recombinant HlCRT and BmCRT in bacterial extract and purified form. Lane 1, supernatant of pET-43a bacterial extract; lane 2, supernatant of pET-43a/HlCRT bacterial extract; lane 3, supernatant of pET-5b/BmCRT bacterial extract; lane 4, purified rHlCRT; lane 5, purified rBmCRT. Western blot probed with bovine rHlCRT antiserum. Arrow indicates the 55–60 kDa bands representative of rHlCRT and rBmCRT. Molecular mass standards are expressed in kDa.

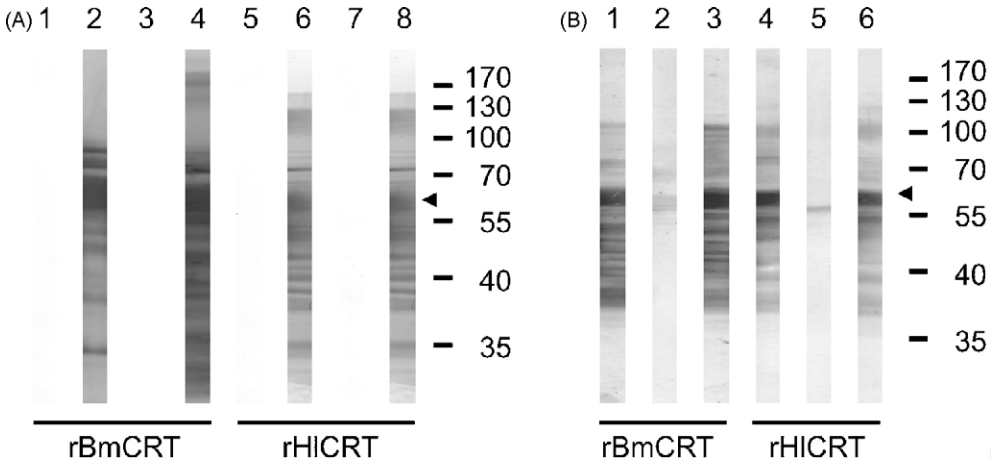

Fig. 3. Western blot analysis using sera from mice (A) and bovines (B) immunized with rBmCRT or rHlCRT. (A) 1, mouse 1 pre-immune serum; 2, serum of mouse 1 immunized with rBmCRT eluted with 400 mM NaCl; 3, mouse 2 pre-immune serum; 4, serum of mouse 2 immunized with rBmCRT eluted with NaCl 500 mM; 5, mouse 3 pre-immune serum; 6, serum of mouse 3 immunized with rHlCRT eluted with NaCl 400 mM; 7, mouse 4 pre-immune serum; 8, serum of mouse 4 immunized with rHlCRT eluted with NaCl 500 mM. (B) 1 and 4, sera of bovines immunized with rHlCRT; 2 and 5, sera of bovines inoculated with PBS; 3 and 6, sera of bovines immunized with rBmCRT. Molecular mass standards are expressed in kDa.

graphy and the collected fractions were analyzed by SDS-PAGE 12% (Fig. 2a) and Western blot using bovine anti-rHlCRT serum (Fig. 2b), demonstrating the presence of molecules with compatible molecular masses for rHlCRT and rBmCRT.

3.3. Immunogenicity of rHlCRT and rBmCRT in mice and bovines

Sera of mice and bovines immunized with rHlCRT or rBmCRT were used in Western blot analyses. Both NaCl-eluted (400 mM and 500 mM) fractions of both CRT proteins were recognized by sera from immunized bovines or mice (Fig. 3). In addition, sera from bovines immunized with rHlCRT or rBmCRT also recognized BmCRT in extract of larvae of *R. microplus* (Fig. 4), which confirms that antibodies directed to recombinant proteins also recognize the native BmCRT.

A comparative recognition analysis was performed by ELISA, as can be seen in Fig. 5. The sera of bovines vaccinated with rHlCRT or rBmCRT recognized both recombinant proteins. The titer of the bovine immunized with rBmCRT was 1500 for both proteins, while titers of 6000 and 1500 were obtained for the bovine immunized with rHlCRT and tested against rHlCRT and rBmCRT, respectively. The sera from the bovine inoculated with PBS during the immunization period did not recognize rBmCRT or rHlCRT (data not shown).

3.4. HlCRT and BmCRT display different epitopes

In silico analysis of HlCRT and BmCRT by Jameson–Wolf index demonstrated various common areas and six different areas with antigenic index larger than 1 between the proteins HlCRT and BmCRT (Fig. 6a). These data indicate that the two proteins present similar immuno-

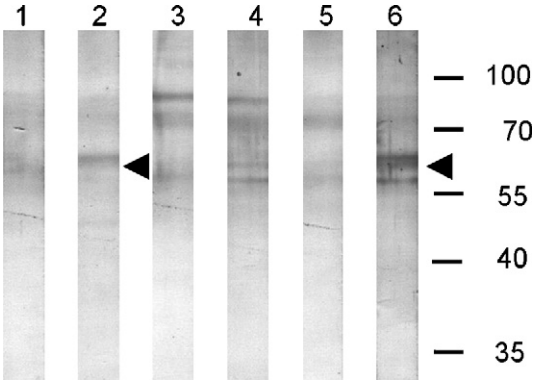

Fig. 4. Western blot analysis using sera of bovines immunized with rHlCRT or rBmCRT against 20 days old larvae extract. (1) Pre-immune serum of bovine immunized with rHlCRT; (2) serum of bovine immunized with rHlCRT; (3) pre-immune serum of bovine mock immunized; (4) serum of bovine mock immunized; (5) pre-immune serum of bovine immunized with rBmCRT; (6) serum of bovine immunized with rBmCRT. Arrow indicates the 55–60 kDa bands representative of rHlCRT and rBmCRT. Molecular mass standards are expressed in kDa.

genic potentials, but may also show different epitopes, which is corroborated by competition ELISA analysis, as seen in Fig. 6b. The competition of rBmCRT incubated with the serum of the bovine immunized with rHlCRT occurs with less intensity than that of rHlCRT incubated with the serum of the bovine immunized with rBmCRT, indicating the presence of different epitopes within the proteins (Fig. 6b).

3.5. Immunogenicity of native BmCRT

Extract of salivary glands and saliva of *R. microplus* were used to immunize bovines. The sera of the immunized bovines used in Western blot analyses, showed that the immunizations of native and secreted BmCRT in the presence of adjuvant are able to induce the production of antibodies. Therefore, antibodies raised against native proteins recognize epitopes present in the recombinant forms (Fig. 7). On the other hand, sera from bovines naturally infested with *R. microplus* did not recognize the native or recombinant CRTs (data not shown).

4. Discussion

New tick control strategies have been widely promoted in response to concerns triggered by the emergence and spread of worldwide acaricide resistance. Experimental protection against tick infestation has been demonstrated in several host species; however, a limited immunity against tick infestation has been achieved with commercial vaccines (Imamura et al., 2005; Seixas et al., 2008). Notwithstanding, vaccination is one of the most promising methods of tick control (Willadsen, 2006b, 2008). Still, the success of this strategy depends on the identification and characterization of essential tick molecules whose impairment could be accomplished by antibody responses. Some tick saliva components are responsible for the inhibition and/or inactivation of specific functions of the host immune system (Lawrie et al., 2005) and homeostasis (Imamura et al., 2005; Ciprandi et al., 2006). In this context, it has been proposed that the secretion of CRT into the hosts may perform functions enabling the feed of blood-sucking parasites. The secreted CRT may prevent blood clotting by binding to Ca^{2+} and clotting factors (Suchitra and Joshi, 2005; Suchitra et al., 2008), and Ca^{2+} and its associated proteins are suggested to play important roles in the bovine anti-tick naturally acquired resistance (Bagnall et al., 2009). Data obtained indicate that some parasite CRTs bind to C1q (Kasper et al., 2001; Suchitra and Joshi, 2005), modulating the host's immune response and

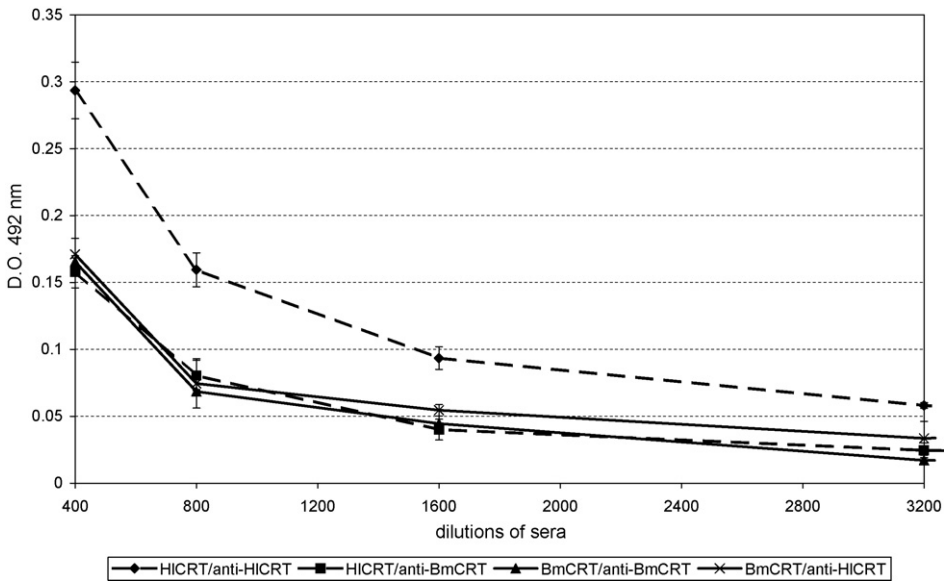

Fig. 5. Sera titration of immunized bovine with rHlCRT or rBmCRT tested against rHlCRT or rBmCRT coated plates by indirect ELISA (antigen coated in the plate/antiserum tested). The means of negative serum plus 2 SD were used as cut off. The bovine inoculated with PBS showed similar values of pre-immune serum (data not shown). The results were expressed as means of two experiments. Error bars represent the mean ± SD.

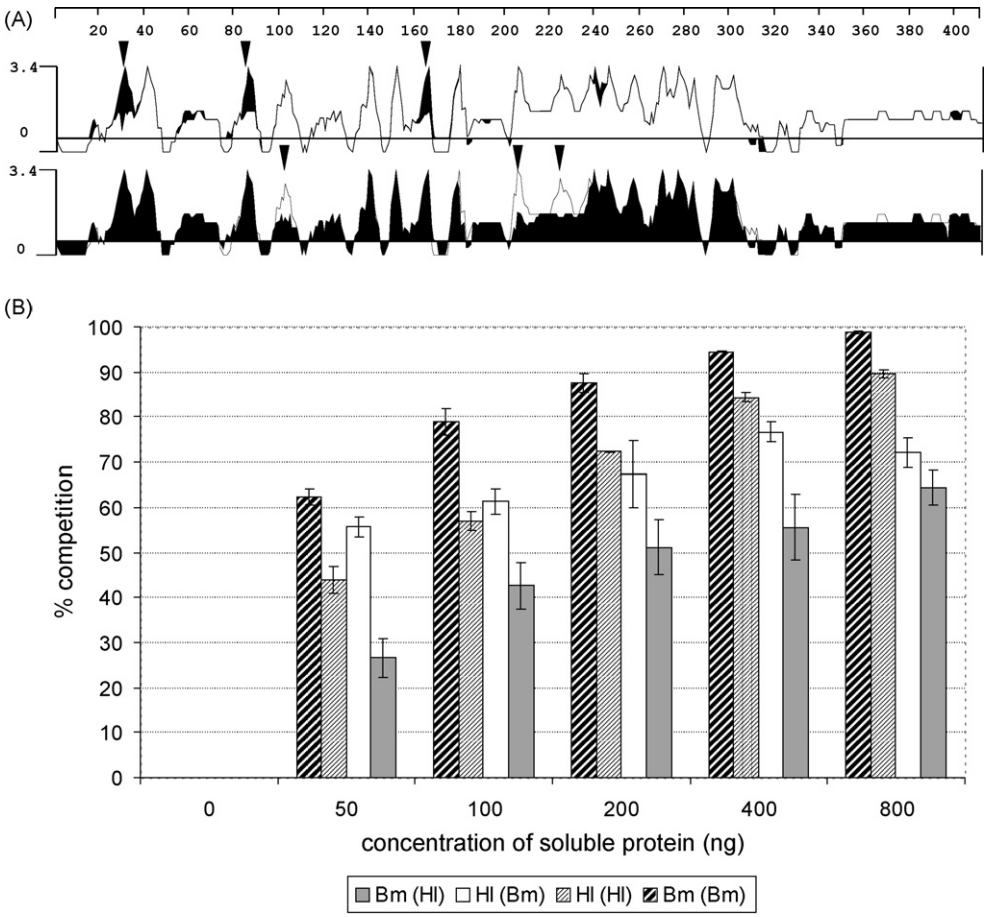

Fig. 6. (A) Antigenic index plot of *H. longicornis* and *R. microplus* CRTs predicted using the Jameson–Wolf algorithm. Increased positivity based on scalar number on left side of plot is predictive of antigenic site. The plot 1 is HlCRT (black) sequence overlapped by BmCRT (white) sequence and the plot 2 is BmCRT (white) sequence overlapped by HlCRT (black) sequence. The arrows indicated regions with *difference* in *immunogenic potential*. (B) Competition ELISA of rHlCRT or rBmCRT with sera from bovines immunized with rHlCRT or rBmCRT. The abbreviations Bm and Hl out of parenthesis indicate the immunized bovines sera and the same abbreviations within parenthesis indicate the soluble proteins incubated for competition. Abscissa axis indicates the concentration of soluble antigen and orderly axis indicates percentage of competition. The results were expressed as means of two experiments. Error bars represent the mean ± SD.

facilitating parasite's survival. It was demonstrated that vaccination of sheep with *Haemaphysalis qinghaiensis* recombinant CRT (Gao et al., 2007) and rabbit with *A. americanum* recombinant CRT (Jaworski et al., 1995), conferred a moderate protective immunity against these ticks.

The phylogenetic tree of 18 selected species shown in Fig. 1 indicates that tick CRT genes are monophyletic. Tick sequences grouped closer to host sequences when compared to CRT sequences of other parasite species, as already described (Ferreira et al., 2002; Gao et al., 2008).

The coding region of HlCRT and BmCRT genes were cloned and expressed in *E. coli*. The migrations of rHlCRT and rBmCRT in SDS-PAGE are consistent with molecular masses of approximately 55 kDa (Fig. 2a), although the predicted molecular weights lie around 46 kDa, which is derived from the highly acidic C-domain (Coppolino and Dedhar, 1998).

Humoral immune responses were induced after the immunization with both recombinant proteins. The *in silico* (Fig. 6a) and *in vitro* (Figs. 3a and 6b) analyses showed the presence of similar epitopes in both proteins. The bovines immunized with rHlCRT or rBmCRT developed antibodies that react against both rCRTs (Fig. 2b). The immunogenicity of rCRTs observed in this work are different from immunogenicity previously described (Ferreira et al., 2002), which revealed, using a different not fully mature recombinant protein and a different immunization protocol, the absence of humoral immune response in a bovine immunized with the recombinant form of CRT from *R. microplus*. Differences in methodology, as antigen production and adjuvant, as well as genetic differences, may explain the discrepancy in the results of these two CRT immunizations. In this sense, Rodriguez et al. (1995) demonstrated that 6% of bovines, in a group of 98 animals, did not develop immune response against the recombinant protein Bm86. Bak et al. (2008) demonstrated that the presence of additional exogenous adjuvant is essential for CRT-associated peptides to be accessed from antigen-presenting cells and elicit a CTL response. On the

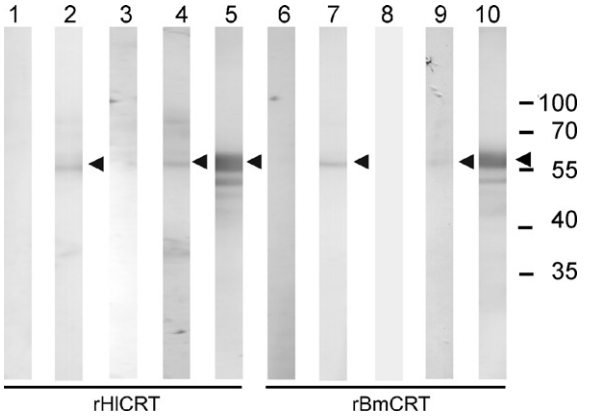

Fig. 7. Western blot analysis of sera from bovines immunized with protein extract of salivary glands or saliva against rHlCRT or rBmCRT. 1 and 6, pre-immune serum from bovines immunized with protein extract of salivary glands; 2 and 7, serum from bovine immunized with protein extract of salivary glands; 3 and 8, pre-immune serum from bovine immunized with saliva; 4 and 9, serum from bovine immunized with saliva; 5, serum from bovine immunized with rHlCRT; 10, serum from bovine immunized with rBmCRT. Arrows indicate the 55–60 kDa bands representative of rHlCRT and rBmCRT. Molecular mass standards are expressed in kDa.

other hand, this work, as well as that by Ferreira et al. (2002), did not find anti-CRT antibodies in sera from naturally *R. microplus* infested bovines. This result was not expected, as other ticks (Ferreira et al., 2002; Alarcon-Chaidez et al., 2006) as well as other parasites (Ribeiro et al., 2009; Rzepecka et al., 2009) induce the synthesis of antibodies against secreted calreticulin in their hosts. The lack of specific antibodies anti-calreticulin in the bovine sera used in these analyses may indicate that the *R. microplus* calreticulin represents a silent antigen for bovines, as suggested for sialostatin L2 (Kotsyfakis et al., 2008). An alternative explanation is that the repertoire of antibodies present in the sera used could be influenced by the specific immune modulatory interplay between *R. microplus* and *Bos taurus taurus* bovines, similar to what was described by Cruz et al. (2008), as different levels of infestation influence the antigens recognized by bovines.

It is important to emphasize that the antibodies raised by bovines immunized with rHlCRT or rBmCRT recognized, by Western blot, the native BmCRT (Fig. 4) in extract of 20-day-old larvae of *R. microplus*. This result demonstrates that the immunogenicity was maintained in the rHlCRT and rBmCRT and this observation is important for the use of recombinant proteins in a vaccine. In similar manner, the calreticulin of *H. qinghaiensis* was detected using sera of sheep immunized with recombinant HqCRT (Gao et al., 2007). These results are corroborated by the recognition of rCRTs by the sera of bovines immunized with *R. microplus* saliva and salivary gland extracts, which has been shown to contain BmCRT (Ferreira et al., 2002).

At same time that the ELISA and *in silico* analyses suggest that HlCRT and BmCRT display different epitopes despite their high conservation, the degree of cross-reactivity observed by immunological analyses suggested the presence of immunodominant epitopes conserved in both CRTs.

In conclusion, we found that bovines immunized with extract of salivary glands or saliva developed antibodies against rHlCRT and rBmCRT (Fig. 7). The conservation of immunodominant epitopes during evolution in ticks, as well as its presence in saliva, makes calreticulin an interesting candidate for inclusion in an anti-tick vaccine, as already demonstrated (Gao et al., 2008), what could possibly become protective against multiple species, as already described for Bm86/Ba86 (Canales et al., 2009) and 64TRP proteins (Trimnell et al., 2005). Further studies are required to address the capacity of natural infestations to keep anti-BmCRT and HlCRT antibody levels in animals previously immunized and to investigate the possibility that this circumstance abolish the need for repeated immunizations.

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