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EXPRESSION AND CHARACTERIZATION OF *CALRETICULIN* GENE ISOLATED FROM *RHIPICEPHALUS ANNULATUS* AFTER *BABESIA BIGEMINA* INFECTION.

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DISSERTAÇÃO DE MESTRADO EM MEDICINA VETERINÁRIA.

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Expression and characterization of *calreticulin* gene isolated from *Rhipicephalus (Boophilus) annulatus* after *Babesia bigemina* infection.

Abstract

Ticks are obligate parasites in a large variety of hosts and are considered to be, after mosquitoes, the second worldwide vector of human and animal diseases.

Bovine babesiosis causes substantial economic losses due to animal mortality, abortions and anaemia, among other effects. Calreticulin protein that has been identified in several species including ticks and previous experiments showed that *calreticulin* gene was up-regulated in *R. annulatus* ticks infected with *B. bigemina* and that its knockdown by RNAi technique leads to a reduction of both pathogen transmission and ticks weight.

This Master thesis was developed within a project on "Differential expression and functional characterization of tick (*Rhipicephalus annulatus*) genes in response to pathogen infection (*Babesia bigemina*)", financed by the Science and Technology Foundation (FCT), with the project number of PTDC/CVT/112050/2009.

The aim of this study was the isolation of *calreticulin* gene, purification of calreticulin protein and its further use to produce antibodies for the purpose of immunolocalization studies and vaccination tests. In this Master thesis, *calreticulin* gene was amplified by PCR technique, sequenced and compared with *calreticulin* from the *R. annulatus* sequence, showing 98% identity. Afterwards, an *Escherichia coli* recombinant system was used in order to produce a calreticulin protein. Finally, recombinant proteins were purified using IMAC technique, due to the affinity of expressed calreticulin protein histidine tail to nickel ions. After specific elution and a final sample concentration, a unique protein was achieved in the purified sample, corresponding to recombinant calreticulin.

The results of this study were optimistic and represent one more step to improve ticks control, as we showed in this study that CRT can be produced and purified without contaminants, though further vaccination and immunolocalization studies will be the key to understand CRT future use.

Keywords: *Babesia bigemina*/ *Rhipicephalus* (*Boophilus*) *annulatus*/ Calreticulin/ *Escherichia coli*/ Immobilized metal ion affinity chromatography.

Expressão e caracterização do gene *calreticulina* isolado de *Rhipicephalus (Boophilus) annulatus* após infecção com *Babesia bigemina*.

Resumo

As carraças são parasitas obrigatórios de uma grande variedade de hospedeiros, sendo consideradas, depois dos mosquitos, os mais importantes vectores de doenças em humanos e animais. A babesiose bovina conduz a elevados prejuízos económicos, devido ao aumento da mortalidade animal, abortos e anemia, entre outros. A calreticulina é uma proteína já identificada em várias espécies, incluindo carraças e estudos anteriores demonstraram que o gene *calreticulina* estava sobreexpresso em carraças *R. annulatus* infectadas com *B. bigemina* e, após o seu silenciamento através da técnica de RNAi, ocorria uma redução tanto na transmissão do agente patogénico, como no peso das carraças.

Esta tese de Mestrado foi desenvolvida em paralelo com o projecto "Expressão diferencial e caracterização de genes de carraça (Rhipicephalus annulatus) em resposta à infecção por agente patogénico (Babesia bigemina)", financiado pela Fundação para a Ciência e Tecnologia, sendo o número do projecto PTDC/CVT/112050/2009.

A finalidade deste trabalho consistiu no isolamento do gene *calreticulina* e purificação da correspondente proteína, para posteriormente ser usada na produção de anticorpos destinados a estudos de imunolocalização e de vacinação. Nesta tese de mestrado, amplificou-se o gene da *calreticulina* pela técnica de PCR, e sequenciou-se esse gene e comparou-se com a sequência da *calreticulina* da carraça *R. annulatus*, obtendo-se uma identidade de 98%. Posteriormente, o gene foi expresso em *Escherichia coli* de modo a produzir-se calreticulina. Finalmente, as proteínas recombinantes foram purificadas através do método IMAC, dado a calreticulina expressa ter uma cauda de histina com afinidade para iões níquel. Após a eluição específica e a concentração das amostras finais, verificou-se que uma única proteína, correspondente à calreticulina recombinante, se encontrava presente na amostra purificada.

Os resultados deste estudo foram positivos e representam mais um passo para melhorar o controlo das carraças, uma vez que este estudo demonstrou que a CRT pode ser produzida e purificada sem contaminantes, apesar de estudos posteriores de vacinação e imunolocalização serão essenciais para perceber qual o futuro da CRT.

Palavras-chave: *Babesia bigemina*/ *Rhipicephalus* (*Boophilus*) *annulatus*/ Calreticulina/ *Escherichia coli*/ Cromatografia de afinidade por iões metálicos imobilizados.

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Abbreviations and Symbols

µg	Microgram
µl	Microliter
µm	Micrometer
A, C, G, T	Adenine, cytosine, guanine, thymine
Abs	Absorbance
A.D.	*Anno Domini*
ArgE	Acetylornithinase
AMA-1	Apical membrane antigen 1
BbAMA-1	*Babesia bovis* apical membrane antigen 1
Bbo-MIC1	Recombinant microneme protein from *B. bovis*
B.C.	Before Christ
Bp	Base pair
cDNA	Complementary DNA
CRP	cAMP regulatory protein
CRT	Calreticulin
Cu/Zn-SODM	Cu/Zn-superoxide dismutase
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
EF-1a	Elongation factor-1 alpha
e.g.	*exempli gratia*
EgCRT	Recombinant CRT protein from *Echinococcus granulosus*
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
F2	Second generation after cross-breeding
FPLC	Fast protein liquid chromatography
GST	Glutathione-S transferase
Hz	Hertz
i.e.	*id est*
IFAT	Indirect immunofluorescent antibody test
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
kHz	Kilohertz
LAMP	Loop-mediated isothermal PCR
LB-agar	Luria Bertani-agar
M	Mole
mA	Milliampere
ml	Millilitre
min	Minute
mM	Millimole
mRNA	Messenger RNA

MSA	Merozoite surface antigen
nm	Nanometer
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PVDF	Polyvinylidene fluoride
rDNA	Ribossomal DNA
rBmCRT	Recombinant calreticulin protein from *R. annulatus*
rCRT	Recombinant calreticulin
rHlCRT	Recombinant calreticulin protein from *Haemaphysalis longicornis*
rHqCRT	Recombinant calreticulin protein from *H. qinghaiensis*
RLB	Reverse line blot
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotations per minute
TAE	Tris acetate buffer
TBE	Tickborne encephalitis virus
TBV	Transmission blocking vaccine
TEMED	Tetramethylethylenediamine
Tris-HCl	Tris hydrochloride
TTBS	Tween-Tris buffer saline
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEL	Selenoprotein W
SlyD	Peptidoylproline cis-trans isomerase
SPA	Soluble parasite antigens
spp.	Species (plural)
S.O.C.	Super-optimal catabolite repression medium
UBQ	Ubiquitin
V	Volt
v/v	Volume per volume
w/v	Weight per volume
YodA	Metal-binding lipocalin

Chapter 1. Preamble

This Master thesis was carried out in the Center for Malaria and other Tropical Diseases (CMDT), Institute of Hygiene and Tropical Medicine (IHMT) of New University of Lisbon (UNL), from September 2011 to February 2012. The main objectives of this work plan were the isolation of *calreticulin* (CRT) gene and the production and purification of the CRT protein in a recombinant system; this purified protein will be further used to generate antibodies to be applied in immunolocalization studies and in vaccination trials.

Among several possible themes, it was decided to study this parasite, because Babesiosis is probably one of the most relevant and expensive cattle affecting diseases in Portugal and, it may also become a public health problem, further enhancing the need to control this disease. It is already well known that the use of chemoprophylaxis to fight babesiosis is difficult and increases tick-resistance, the development of new vaccines being urgently needed.

This Master project was developed within a project on "Differential expression and functional characterization of tick (*Rhipicephalus annulatus*) genes in response to pathogen infection (*Babesia bigemina*)", financed by the portuguese Science and Technology Foundation (FCT) and developed in IHMT in collaboration with other institutions, namely the Institute for Cinegetic Resources Research of Castilla-La Mancha University (IREC-UCLM-JCCM) in Spain and the Kimron Veterinary Institute (KVI) in Israel. The aim of this project is the functional characterization of the *R. annulatus* genes differentially expressed after pathogen infection, thus constituting candidate protective antigens for the development of vaccines to control ticks infestations and the transmission of tick-borne pathogens. During this project, genes differentially expressed in a *R. annulatus* infected with *B. bigemina*, but not in non-infected population, were selected by suppression-subtractive hybridization library. A total of 96 contigs were obtained and 16 candidates with putative functions in tick-pathogen interactions were further selected for expression validation by real-time PCR, CRT being one of the differentially expressed genes in *B. bigemina* infected ticks. Gene silencing of selected genes (including CRT), using RNA interference (RNAi) technique was performed and results revealed that CRT knockdown induced a lower *B. bigemina* infection levels and reduced ticks weight, when compared to controls, suggesting that CRT could contribute to the development of novel vaccines designed to reduce ticks infestations and prevent pathogens infection in

ticks and, consequently, in vertebrate hosts. So, based on these results, CRT was chosen for gene isolation and recombinant protein production, being the subject of this Master project.

Chapter 2. Literature Review

2.1. Ticks

2.1.1. Introduction

Throughout history, ticks have been condemned for their activity as pathogen vectors. Dating back to 16th Century B.C., a reference to a possible 'tick fever' was found on a papyrus scroll (Krantz, 1978) and even in Anazasi culture (400-1300 A.D.) it was described ticks presence (Mcllwain, 1984).

Ticks are obligate ectoparasites with a worldwide distribution and a major importance in animal and human health, only matched by mosquitoes as vectors of disease (Heyman et al., 2010). Over the past two decades, ticks have become a great problem in animal production (Randolph, 2004; Pattnaik, 2006) due to their direct and indirect damage capacity and particularly their vector ability to transmit important diseases (Graf et al., 2004). Ticks parasitize terrestrial vertebrates, including amphibians, reptiles, birds and mammals (Barker & Murrel, 2004) and their prevalence differs depending on the locals, as it happens, for instance, on Europe where species on its North or South areas are different (table 1). Theobald Smith and Frederíck Kilbourne first demonstrated (1889 - 1893) that ticks were responsible for spreading diseases from their experiments on transmission by *Rhipicephalus* (*Boophilus*) *annulatus* of *B. bigemina* in cattle (Assadian & Stanek, 2002).

Ticks transmit pathogens such as *Borrelia burgdorferi* (agent of Lyme disease), *Anaplasma* spp., *Babesia* spp. (agent of tick fever), *Coxiella burnetti* (agent of Q fever), *Francisella* spp., *Rickettsia* spp. (Peter, Van den Bosseche, Penzhom & Sharp, 2005), *Theileria* spp., *Ehrlichia* spp. (Dumler et al., 2001; de la Fuente, Estrada-Pena, Venzal, Kocan & Sonenshine, 2008) and viruses such as tickborne encephalitis virus (TBE) (Peter et al., 2005), conveying some of the most important diseases transmitted by ticks.

2.1.2. Characterization, identification and morphology

Ticks belong to phylum Arthropoda, subphylum Chelicerata, class Arachnida, subclass Acari, superorder Parasitiformes, order Ixodida and superfamily Ixodoidea. There are three families: Argasidae or soft ticks with 185 species, Ixodidae or hard ticks with 713 species and Nuttalliellidae with only one species (Barker & Murrell, 2004). The Ixodidae family has a chitin cover that extends throughout all dorsal surface of the male and over a small area behind the head in case of larvae, nymphs and female ticks. The mouth pieces are anterior, there are a series of wrinkles in the cover and body, genital orifice is in ventral medial line, anus is posterior and adults have spiracles behind their fourth legs pair. In the case of Argasidae family, the difference lies in the fact that these ticks do not have a chitin cover (Urquhart, Armour, Duncan, Dunn & Jennings, 1996). As mentioned above, Nuttalliellidae comprises one single species, *Nuttalliella namaqua*, found in Southern Africa from Tanzania to Namibia (Aragão, 1936) and it is considered the most basal lineage of ticks (Klompen, Lekveishvili & Black, 2007; Klompen, 2010).

Table 1 Prevalent ticks species in Europe

North Europe	**South Europe**
- Dermacentor reticulatus	*- Rhipicephalus annulatus*
- Dermacentor marginatus	*- Dermacentor marginatus*
-Haemaphysalis concinna	*- Haemaphysalis leachi*
- Hyalinella punctata	*- Haemaphysalis parva*
- Ixodes canisuga	*- H. punctata*
- Ixodes hexagonus	*- Hyalomma anatolicum anatolicum*
	- Hyalomma marginatum marginatum *- I. canisuga* *- I. hexagonus* *- I. ricinus* *- Rhipicephalus bursa* *- Rhipicephalus sanguineus* *- Rhipicephalus turanicus*

Adapted from Heyman et al. (2010).

2.1.3. Life cycle

The life cycle of a tick can be classified into 4 stages. Ticks begin as eggs (stage 1) that hatch into larvae (stage 2). Larvae live and feed on animal hosts before detaching and molting (shedding) anywhere. The larvae molt to nymph (stage 3), which feed on animals, engorge, detach and molt. Once nymph molts, it becomes an adult tick (male or female). To distinguish larvae from nymphs and adults, the first have 6 legs and adults/nymphs has 8 legs (Kohls,

Sonenshine & Clifford, 1965). Adults climb up grass and plants to hold their prey and when a warm-blooded animal walks on the pasture, tick can crawl onto them and begin feeding. Ticks insert their mouths, attach to their prey and engorge themselves with a blood meal (stage 4). During feeding, ticks saliva can get into the host body and blood stream.

Usually *Ixodes* adult ticks take 4 to 10 days to get full engorgement and feed themselves by forcing their hypostome through the host skin and sucking blood and fluids that are drained from the resulting wound (Sonenshine, 1991). Ticks from Ixodidae family may have between one to three vertebral hosts. The coupling occurs in the host, where the adult female tick feeds itself for several days; then it falls into the ground, where the oviposition of millions of eggs occurs, culminating with tick's death. The male ticks can couple many times without dying, feeding themselves intermitting. In the Argasidae family, females feed themselves frequently, coupling happens outside the host and oviposition occurs more than once in ticks life (Urquhart et al., 1996).

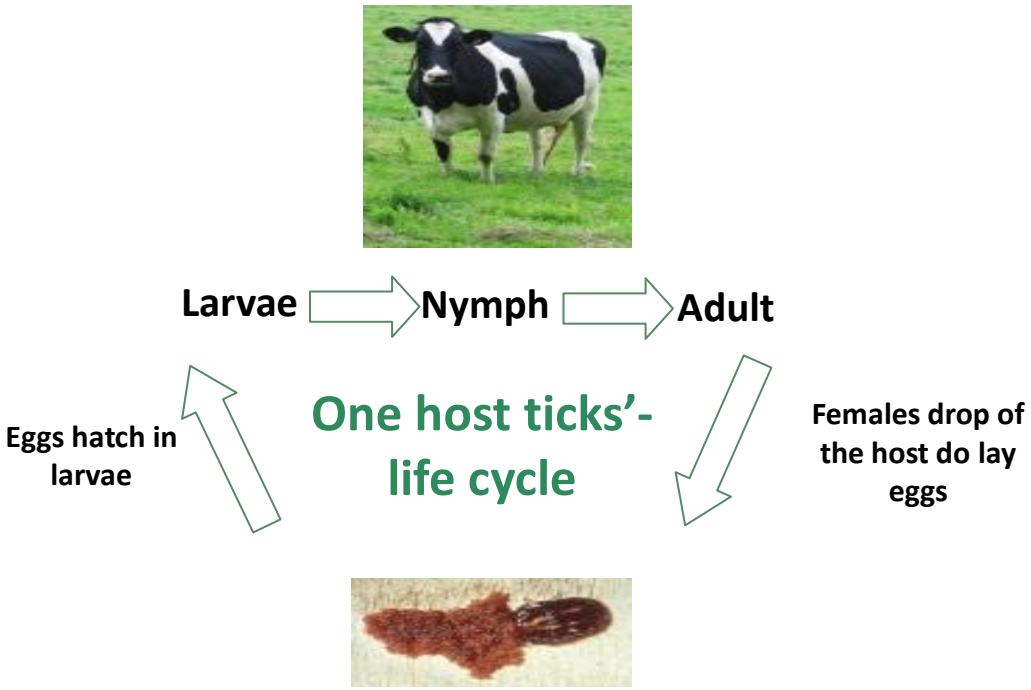


Figure 1 One host ticks life cycle.

2.1.4. Tick control

2.1.4.1. Introduction

Ticks control is essential to diminish tick-borne diseases prevalence and, therefore, reduce ticks impact on livestock productivity. Nowadays, ticks control is mainly based in the application of acaricides, despite their disadvantages and limitations, although other control methods as vaccination or biocontrol agents are available (Willadsen, 2006). Probably, vaccination will be one of the most important methods for ticks control and its transmited pathogens, because several different vaccines have already been used against *B. bovis* and *B. bigemina* in Australia (Bock & de Vos, 2001) or against *Theileria annulata* in Israel (Shkap & Pipano, 2000) and China (Gu et al., 1997; Shirong, 1997) with good results.

2.1.4.2. Chemoprophylaxis

There are many acaricides that can be used against ticks: pyrethroids as flumethrin and deltamethrin; organochlorines as dichlorodiphenyltrichloroethane (DDT); organophosphates as diazinon and coumaphos; carbamates as carbaril; formamidines as amitraz; cicloamidines as clenpirin and macrocyclic lactones (avermectins and milbemycins), among others (Botana, Landoni & Martín-Jiménez, 2002). Synthetic flumethrin in pour-on protects cattle from ticks for 2 weeks and, in case of deltamethrin, for 3 weeks; this approach, in the case of babesiosis control, led to a significant decay in clinical disease (Zintl, Mulcahy, Skerrett, Taylor & Gray, 2003). The application of acaricides has some drawbacks: the appearance of residues in milk and meat products, the environmental contamination and, most important nowadays, the development of ticks' resistance against acaricides (Botana et al., 2002). This resistance is mainly due to mutations in genes encoding detoxificating enzymes, as esterases, glutathione-S-transferases and monooxidases and due to genetic drift (Rosario-Cruz et al., 2009). Resistance to compounds such as organophosphorous, pyrethroids and amitraz has been described for *R. microplus* (Ortiz et al., 1995; Soberanes, Santamaría, Fragoso & García, 2002). Pesticides rotation is used in crop agriculture to minimize resistance, but its application in ticks control is not widely used yet. The association of acaricides with vaccines acts in a synergetic manner, because, in the case of *R. microplus*, efficacy of macrocyclic lactone acaricides is deeply enhanced in cattle vaccinated against those ticks (Kemp, McKenna, Thullner & Willadsen, 1999).

2.1.4.3. Biocontrol

Currently, an appeal is being made to biocontrol and to the use of more "environmental friendly" acaricide products. Like other parasites, ticks carry some microorganisms in their bodies, which, along with ticks, are essential for the survival of each other (endosymbiosis). Since endosymbionts are essential for ticks' survival, elimination of those microorganisms would be deleterious for the continued existence, growth and development of ticks. Those endosymbionts could be either destroyed by a chemical approach or used to animals' immunization, in order to interfere in ticks' nutrition (Noda, Munderloh & Kuffi, 1997; Benson, Grawronski, Eveleigh & Benson, 2004). The use of other organisms pathogenic to ticks, like fungi of genera *Beauveria* and *Metarhizium* (Frazzon, Vaz Junior, Masuda, Scharank & Vainstein, 2000; Gindin, Samish, Zangi, Mishoutchenko & Glazer, 2002) or herbal acaricides (Khudrathulla & Jagannath, 2000; Lundh, Wiktelius & Chirico, 2005) are an alternative and important possibility of achieve the control of ticks.

2.1.4.4. Genetic resistance

The genetic resistance to ticks and tick-borne diseases is complex, but, facing other control methods and their problems, breeds resistance has become an important parameter in some regions.

Bos indicus cattle is more resistant to *R. microplus* ticks and babesiosis than *Bos taurus*, this acquire resistance being heritable, though those animals remain susceptible to *A. marginale* (Bock et al., 1997, 1999). This additional resistance is possibly due to the fact that zebuine cattle breeds have more dermal mast cells than taurine breeds, as F2 crossbreed cattle has a higher developed resistance against ticks correlated with an increased number of mast cells in dermis (Engracia Filho, Bechara & Teodoro, 2006).

2.1.4.5. Vaccines

2.1.4.5.1. Introduction

Major alternatives to conventional acaricide treatments have been developed in recent years and vaccines are among the most important developments. It is now a decade since the first commercial vaccine against *R. microplus* based on the recombinant antigen Bm86 was released (Willadsen, Bird, Cobon & Hungerford, 1995; de la Fuente et al., 1999).

In the market there are two vaccines based on recombinant *R. microplus* Bm86 gut antigen:

Gavac™ vaccine (Heber Biotec S.A., Havana, Cuba) and TickGARD (Hoechst Animal Health, Australia), which confirming the advantages of tick control by this method, because it is cost-effective, it reduces environmental contamination, it prevents acaricide-resistant ticks selection and it can reduce pathogens transmission, by decreasing ticks number and/or changing their vector capacity. This control scheme also has another advantage, as vaccines can protect the animal-host against both pathogens and vector, especially if antigens are conserved in both species (de la Fuente et al., 1998; de la Fuente & Kocan, 2003; de la Fuente, Kocan & Blouin, 2007).

Nevertheless, there is a problem with vaccines formulation because many of tick-protective antigens studied for propose of a future application are cytoplasmic and highly conserved, which can favour host tolerance to them and a total inefficacy of vaccine; yet this is one of the most promisor methods to fight these ectoparasites (Almazán et al., 2010).

There are two different kinds of antigens, the exposed and the concealed antigens (Willadsen, 1980). The first type is the most used in vaccines production, even though vaccines with concealed antigens may inhibit other parasites development, such as *Babesia* spp., by altering gut homeostasis and preventing pathogen transmission (Rachinsky, Guerrero & Scoles, 2007). Another kind of vaccines are the transmission blocking vaccines (TBV), which are supposed to block pathogen development in arthropod vectors through targeting pathogen or arthropod molecules as transmission blocking targets (Carter, 2001).

2.1.4.5.2. Recombinant vaccines

These vaccines use recombinant proteins as antigens to immunize animals and have several advantages, such as prevention or reduction of pathogens transmission (de la Fuente et al., 1998), environmental safety, low cost production (Odongo et al., 2007), prevention of drug-resistant ticks selection and inclusion of multiple antigens that could target several tick species (de la Fuente & Kocan, 2006), among others.

Canales, Almazán, Naranjo, Jongejan & de la Fuente (2009) have cloned ortholog genes (Ba86 and Bm86 genes) from *R. annulatus* and *R. microplus*, respectively, which were used in vaccine trials later on. Vaccination of cattle with Ba86 reduced, respectively for *R. annulatus* and *R. microplus*, ticks infestations (71% and 40%), ticks weight (8% and 15%), oviposition (22% and 5%) and eggs fertility (25% and 50%). For *R. decoloratus*, Odongo et al. (2007), using a Bm86 based-vaccine, found a reduction of 46% on engorged adult female ticks number, 56% on ticks weight and 61% on eggs weight after cattle immunization and de

Vos, Zeinstra, Taoufik, Willadsen & Jongejan (2001) described a reduction of 70% in *R. decoloratus* reproductive capacity after feeding on vaccinated calves and 50% in engorged *H. anatolicum anatolicum* nymphs total weight. Despite these results, vaccine had no action against *A. variegatum* and *R. appendiculatus* nymphs. Pipano et al. (2003) tested the efficacy of a Bm86 vaccine in protection against ticks and pathogens (*B. bovis* and *B. bigemina*) transmitted by those ectoparasites. The results showed that immunized cattle, when challenged with *B. bovis*-infected ticks, continued to become infected, but in the case of *B. bigemina*, Bm86-immunized animals remained protected against infection, probably due to the fact that larvae didn't molt to nymph. Still related to the Bm86 gene, Bastos, Ueti, Knowles & Scoles (2010) studied the effect of Bm86 gene silencing on fitness of *R. microplus* ticks fed in *B. bovis* infected cattle, showing that this procedure decreased survival engorged ticks rate and eggs weight. A gene from *R. microplus* (strain A) called Bm95, homologue to Bm86, was used to immunize cattle, after it has been discovered that some *R. microplus* ticks (strain A) had a moderate low susceptibly to Bm86 vaccine, possibly due to a genetic variation in ticks (Freeman, Davey, Kappmeyer, Kammlah & Olafson, 2010). Bm95 immunized cattle showed a higher protection efficacy to both susceptible and strain A than Bm86 vaccine (García-García et al., 2000). Other authors showed that Bm95 antigen based vaccine presents better responses than Bm86 vaccine to low susceptibility ticks populations (Jittapalapong et al., 2010).

Immunization trials in cattle with recombinant subolesin, a conserved protein among vertebrates and insects, decreased *R. microplus* survival and reproduction rates (Merino et al., 2011), weight and oviposition (de la Fuente et al., 2011) and, moreover, protein knockdown led to degeneration of ticks parts as embryos, salivary glands and reproductive tissues (Merino et al., 2011). Further studies with this antigen showed that immunization of rats decreased vector capacity of *I. scapularis* nymphs for *A. phagocytophilum* (de la Fuente & Kocan, 2006).

The recombinant antigen 64P from *R. appendiculatus* was found to be involved in ticks attachment and feeding and was used to immunize guinea pigs, reducing, respectively, 48% and 70% nymph and adult infestation rates; additionally, response of animals immunized with 64P was similar to those observed during development of natural resistance in guinea pigs infested with *R. appendiculatus*, showing the typical local cutaneous inflammatory immune response (Trimnell, Hails & Nuttall, 2002). The recombinant protein from *R. sanguineus* and *I. ricinus* was again tested by Trimnell et al. (2002), respectively in guinea pigs and hamsters, demonstrating damage on ticks post-challenge. Other study indicated that there is an antigenic cross-reactivity between tick extracts of *R. sanguineus*, *I. ricinus*, *A. variegatum* and *R.*

microplus, showing the potential of this protein to develop a large-spectrum anti-tick vaccine (Havlíková et al., 2009).

2.1.4.5.3. Antigens selection

Molecular tools are important experimental components for the study of tick gene functions. RNAi technique allows silencing of gene expression, contributing to the characterization of gene function and its phenotypic effect (Fire et al., 1998). The first report related to the use of RNAi in ticks belongs to Aljamali, Sauer & Esseberg (2002) and quickly that technique has become universally adopted for gene-silencing in ticks (de la Fuente & Kocan, 2006).

Kocan, Manzano-Roman & de la Fuente (2007) showed that subolesin knockdown in *I. scapularis*, *D. variabilis* and *A. americanum* also affected oviposition, eggs embryogenesis, larval hatching and fertility (reduction of 93% and 71% for *D. variabilis* and *I. scapularis*, respectively). Subolesin expression is activated when ticks are infected with *A. marginale* and *B. bigemina*, suggesting a connection between tick gene expression and pathogen-infection (Merino et al., 2011). Zivkovic et al. (2010) obtained the opposite result, showing that *D. variabilis, D. andersoni, D. reticulatus, R. sanguineus, R. microplus* and *R. annulatus* had equal or even lower mRNAs level than control groups.

Almazán et al. (2010) studied the knockdown effect of several *R. microplus* genes, such as glutathione-S transferase (GST), ubiquitin (UBQ), selenoprotein W (SEL), elongation factor-1 alpha (EF-1a) and subolesin, and found out that GST and SEL genes knockdown lead to a lower ticks attachment when compared to control group, but did not influence ticks mortality or oviposition, though the other three genes results showed an increase on *R. microplus* mortality and reduction of oviposition.

2.2. Babesia

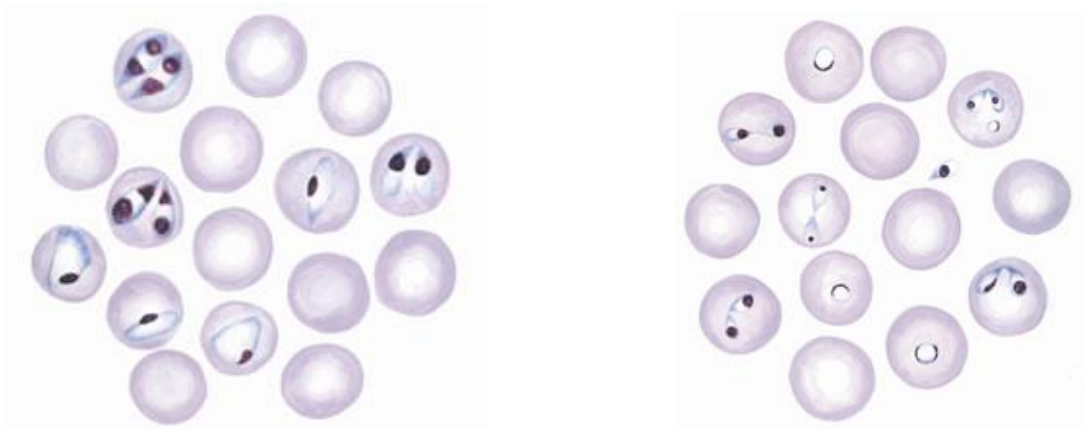
2.2.1. Introduction

Babesia spp. was first discovered in the 19th century in association to bovine hemoglobinuria (Babes, 1888). In 1893, Smith and Kilbourne recognized *B. bigemina* as the causative agent of Texas Cattle Fever (Smith & Kilbourne, 1893).

All over the world, there are some 100 *Babesia* species or even more (Homer, Aguilar-Delfin, Telford 3rd, Krause & Persing, 2000), *Rhipicephalus* spp. being one of its main tick vector (Urquhart et al., 1996). Bovine babesiosis are generally caused by *B. bovis*, *B. bigemina* and *B. divergens* which are the most important *Babesia* species for cattle babesiosis; however *B. major* can also cause disease in cattle (Zintl et al., 2003) and in the case of human babesiosis, *B. divergens* and *B. microti* are the most dangerous species (Antunes, 2008). The different *Babesia* species infect a large variety of animals (Bock, Jackson, de Vos & Jorgensen, 2004; de Vos & Geysen, 2004) and in cattle they can induce animal mortality, abortions, reduction of milk/meat production, and, sometimes, neurological symptoms (Saegerman et al., 2003).

2.2.2. Characterization and life cycle

Babesia spp. belongs to phylum Apicomplexa, class Aconoidasida, family Babesiidae and genus Babesia. In blood smears stained by Giemsa method, *Babesia* parasites usually appear in pairs, with a pear-shape, an elongated or a cigar form with a red nucleus and a blue cytoplasm (figure 2). Concerning to its size, *Babesia* species can be divided in small and big *Babesia*, with 1.0-2.5 µm and 2.5-5.0 µm of length, respectively (Urquhart et al., 1996). These morphological categorizations are usually consistent with the phylogenetic characterization based on nuclear small subunit-ribossomal RNA gene (18S rDNA) sequences, showing that small babesias are divided in two different phylogenetic clusters (Homer et al., 2000).

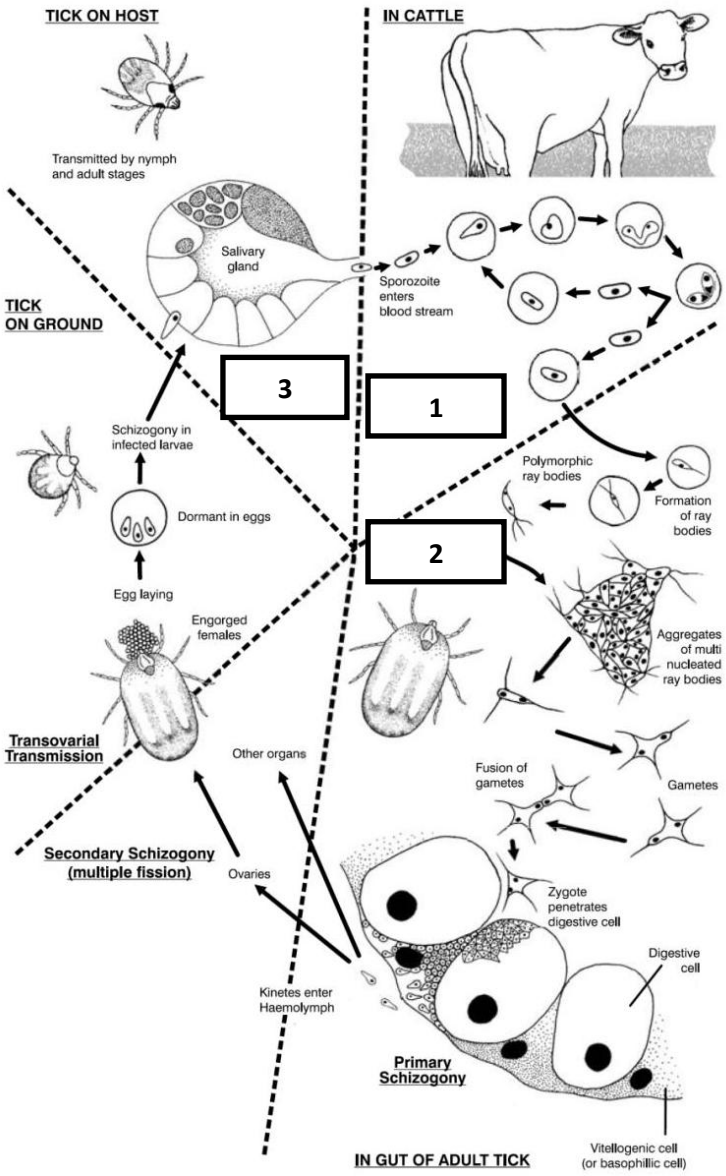


A B

Taken from DPI- Queensland (EUA) http://www.dpi.qld.gov.au

Figure 2 Giemsa staining of *B. bigemina* (A) and *B. bovis* (B) infected red blood cells.

The life cycles of all *Babesia* parasites are very similar and all species are transmitted by infected ticks' bites. The main difference among these life cycles is the presence of transovarial transmission in some species and not in others (Hunfeld, Hildebrandt & Gray, 2008). The *Babesia* spp. life cycle includes three phases, merogony, gamogony and sporogony, and two hosts, one vertebrate (mammal) and one invertebrate (tick), being *R. annulatus* and *R. microplus* the main vectors for both *B. bovis* and *B. bigemina* (Walker et al., 2003; Taylor, Coop & Wall, 2007). The parasite come into vertebrate hosts blood stream through infected ticks saliva, allowing sporozoites to invade erythrocytes. In these cells, parasite asexually divides itself and becomes a merozoite, with a typical pear-shape. This multiplication leads to erythrocytes lysis, with merozoites release, which infects other red blood cells, becoming trophozoites. These new-forms can divide themselves producing a new pair of merozoites, perpetuating merogony phase (**1**). Some merozoites turn to gametocytes and, when tick feeds in a vertebrate host, it becomes infected. In ticks' midgut, gametocytes suffer a sexual phase involving the formation of macro and micro gametes, culminating in zygote production (gamogony). The zygote invades midgut digestive cells and then transforms into kinetes, which can access the hemolyimph in the haemocoel of tick (**2**). After this phase, organisms can be transferred either transtadial (between stages) or transovariac (from female to offspring via the egg). Once in larvae, kinetes migrate to salivary glands cells, where they become sporozoites and multiply themselves (sporogony (**3**)). When ticks feed again, they transmit the sporozoites parasites to vertebrate host (Riek, 1966; Melhorn & Schein, 1984).



Adapted from Bock et al. (2004).

Figure 3 The life cycle of *Babesia bigemina* in cattle and in the ixodid tick vector *R. microplus.*

2.2.3. Symptoms of bovine babesiosis

Most cases of *Babesia* infection are symptomatic. Depending on affected species, clinical signals of babesiosis are different, occasionally culminating to death in few days, as the globular volume may decrease to less than 20% (Urquhart et al., 1996; Melo, Passos, Facury-Filho, Saturnino & Ribeiro, 2001). Some of the clinical symptoms in acute disease are similar to malaria and include high fever, hemolytic anaemia, lethargy, hemoglobinuria, icterus liver (figure 3-B), kidneys with congestion (figure 4-A), abortion, weight loss, splenomegaly and decreased milk/meat production (Bock et al., 2004). Cerebral babesiosis (figure 3-A), caused

by *B. bovis*, is characterized by convulsions, hyperaesthesia and paralysis, due to sequestrations of parasites in the brains capillaries, resulting in low parasitaemia level (less than 1%) in circulating blood. *B. bigemina* infection usually leads to a less pathogenic disease, even though parasitaemia often exceeds 10% (Ristic, 1981; Bock et al., 2004). At necropsy, animal skin and mucous membranes are pale and icteric, bile is granular and dense (Urquhart et al., 1996), bladder is distended and there is hematuria (figure 4-B) (Howard, Rozza, Graça & Fighera, 2001).

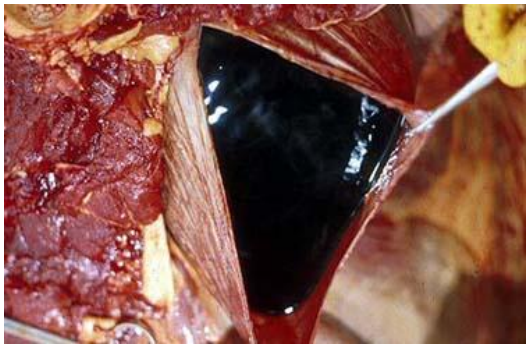
A

B

Taken from Howard et al. (2001). Bovine Babesiosis. http://www.vet.uga.edu/vpp/archives/NSEP/babesia/PORT/necropsy_findings.htm

Figure 4 Cerebral form of babesiosis (A); icterus liver (B)

A



B

Taken from Howard et al. (2001). Bovine Babesiosis. http://www.vet.uga.edu/vpp/archives/NSEP/babesia/PORT/necropsy_findings.htm

Figure 5 Kidneys with edema and congestion (A); hematuria (“Red water”) (B)

2.2.4. Diagnosis

Diagnosis of clinical cases of babesiosis is most frequently made by examination of blood smears stained with Giemsa or acridine orange. Blood films from *B. bovis* are prepared from capillary blood, as blood of general circulation may contain up to 20 times fewer parasites due to sequestration of infected erythrocytes in capillaries of brain and other organs (Böse, Jorgensen, Dalgliesh, Friedhoff & de Vos, 1995). In *B. bigemina* infections, parasitized cells are evenly distributed throughout blood circulation. These techniques are inexpensive and reasonably portable, though accuracy of diagnosis relies on training and skill of microscopist (Papadopoulos, Brossard & Perié, 1996). Low parasitaemias and the presence of different, but morphologically similar parasites (*e.g.* other *Babesia* spp. and also *Theileria* spp.) may adversely affect the proper classification of infections (Homer et al., 2000).

Another test is the cultivation *in vitro*, which can be used to detect infection in animals with low parasitaemia and has the advantage of being very sensitive, yet a long period of time needed for parasites to grow (Thomford, Conrad, Boyce, Holman & Jessup, 1993). Indirect immunofluorescent antibody tests (IFATs) have been used as standard diagnosis test for babesiosis; nevertheless, sensibility, specificity and subjective interpretation are the major problems with this technique. IFATs and enzyme-linked immunosorbent assay (ELISA) can reveal animals that were in contact with parasites, but do not have an active infection. However, long-term carriers are frequently sera-negative and, moreover, serological tests are often cross-reactive among different piroplasm species (Burridge, Young, Stagg, Kanhai & Kimber, 1974; Papadopoulos et al., 1996).

In cases whose diagnosis is difficult by means of blood smear or serology, or when detection of carrier animals with very low parasitaemias is required, direct recognition of parasites by polymerase chain reaction (PCR) based assays can be used. With the evolution of more sensitive PCR based techniques, several methods for the detection and differentiation of bovine babesiosis infections have been described, including nested PCR (Figueroa, Alvarez, Ramos, Vega & Buening, 1993), reverse line blot (RLB) hybridization (Gubbels et al., 1999), LAMP (Loop-Mediated Isothermal PCR) (Iseki et al., 2007) and real time PCR (Buling et al., 2007). Currently, none of these methods is used globally, because some have not been validated to worldwide use, others require complicated post-PCR detection methods to further enhance sensitivity or differentiation, or require special equipment and also some may be prone to amplicon contamination issues (Martins, 2009).

2.2.5. Human babesiosis

A risk factor for being infected with *Babesia* spp. *sensu stricto* is splenectomy (Telford 3rd & Maguire, 2006). *B. microti* is recognized as a diverse species complex, parasitizing a variety of hosts, including rodents, insectivores and carnivores, but the majority of zoonotic strains utilize microtine rodents as reservoir hosts. The reservoir host for *B. divergens sensu stricto*, which is implicated in most cases of human babesiosis in Europe, is cattle and the vector for this parasite is *I. ricinus* (Duh, Petrovec & Avsic-Zupanc, 2001). In United States of America, white-footed mouse *Peromyscus leucopus* is the main reservoir host and *I. scapularis* the invertebrate vector, also known as the deer or black-legged tick. Symptoms and signs can appear one to nine weeks post infection and include hemolytic anaemia, fever, myalgia, headache, drenching sweats, malaise and chills (Hunfeld et al. 2008) or even disseminated intravascular coagulation and respiratory distress syndrome in fulminant cases (Homer et al, 2000). Diagnose involves the same techniques used for animals, namely the presence of the parasite in blood smears (Healy & Ruebush, 1980), PCR (Brandt, Healy & Welch, 1977) and serologic studies (Krause et al., 1994). In human babesiosis treatment, there are different approaches, such as the clindamycin plus quinine (Wittner et al., 1982) or the atovaquone plus azithromycin (Krause et al., 2000). Recently, randomized trials in humans infected with *B. microti* showed that atovaquone plus azithromycin therapy achieved the same results of standard quinine/clindamycin combination, but causing fewer side effects (15% versus 72%) (Hunfeld et al., 2008).

2.2.6. Babesiosis control

2.2.6.1. Introduction

Babesiosis control is essential, due to its huge implications in livestock production and its relations to public health issues (Bock et al., 2004), infecting a large variety of animals and humans. Nowadays, with the introduction of exotic breeds, babesiosis control is even more important, because those breeds usually do not have natural immunity against *Babesia* spp. (Graf et al., 2004). Several approaches, as ticks-vectors control (see chapter 2.1.4.), chemoprophylaxis and vaccination can be applied.

2.2.6.2. Chemoprophylaxis

One way to control this parasite is to control its vector, using acaricides, vaccines against ticks, among others (see chapter 2.1.4.). There are some drugs used against these parasites, as quinuronium sulfate, amicarbilide, diminazene and imidocarb dipropionate. The anti-babesia drugs, when used inappropriately, result in the drug-resistant *Babesia* strains emergence (Zintl et al., 2003).

2.2.6.3. Genetic resistance

Babesiosis control can also be achieved by introducing hosts genetically resistant to hemoparasite infections. The nonspecific immune response against *Babesia* infection is attributed to age or breed related factors since, in general, young cattle is less susceptible to *Babesia* spp. infections than adult cattle, possibly due to the effect of passive immunity conferred by colostrum antibodies (Rogers et al., 2005). *Bos indicus* shows more resistance than *Bos taurus* and animals resulting from crosses between breeds are more resistant to *Babesia* spp. infection and to tick infestation (Bock et al., 1997).

2.2.6.4. Vaccines

There are many types of vaccines against *Babesia* spp. parasites, including attenuated (calf-derived and culture-derived), recombinant and subunit vaccines.

Attenuated vaccines can be produced by parasites multiple passages *in vivo* in splenectomized calves, the calf-derived vaccines (Bock et al., 2004; De Waal & Combrink, 2006), or by parasites growth *in vitro*, the culture-derived vaccines (Jorgensen, de Vos & Dalgliesh, 1989; Echaide, de Echaide & Guglielmone, 1993; Shkap & Pipano, 2000).

Calf-derived vaccines have several associated concerns, such as the possible spread of silent pathogens, difficulties in standardizing vaccine dose, risk of virulence reversion, maintenance of carrier animals, which might serve as reservoirs for pathogens transmission, quality of vaccine production (Shkap et al., 2007), short shelf-life (Bock et al., 2004), vaccines maintenance and transportation (Shkap et al., 2007), limitations of use in animals older than 8-9 months, adverse effects (De Waal & Combrink, 2006) and potential risk of parasite transmission, since vaccinated cattle remains persistently infected for several months (Pipano, 1995).

Culture-derived vaccines do not have entail the risk of pathogens spreading, however, for *Babesia* spp. culture, the main disadvantages remain in the need to fresh bovine erythrocytes and serum from specific donors, which have to be maintained in highly strict conditions and in the fact that vaccines can lose their immunogenicity and virulence in a long-term cultivation (de Vos, 1978). Despite some attenuated vaccines disadvantages, live attenuated strains of *B. bovis* and *B. bigemina* have been used for many years, because they offer a long-lasting protection (Benavides & Sacco, 2007).

There are several studies reporting the use of these attenuated vaccines. An *in vitro* derived attenuated live vaccine (*B. bovis-B. bigemina*) was used in endemic areas to protect cattle against these parasites, conferring an effective level of protection of 93%, showing a very favorable way to protect animals against this disease (Ojeda et al., 2010).

Fish, Leiboyich, Krigel, McElwains & Shkap (2008) studied the efficacy of calf-derived *B. bovis* vaccine. Immunized cattle developed a good immunity, though very susceptible animals had fever, low parasitaemia and a decrease of hematocrit. There was a 65.3% inhibition of *B. bovis* dissemination and a solid protection against babesiosis was acquired in vaccinated animals.

Shkap et al. (2007) studied two different vaccines against *B. bigemina*, a culture-derived and a calf-derived. It was shown in that study that, attenuated vaccines, whether produced from splenectomized calf or from cultures, offered a total protection against clinical babesiosis upon challenge with virulent homologous parasites.

Cysteine peptidases are molecules with a huge importance to many parasites, including *Babesia* spp., as specific inhibitors of these enzymes can stop *B. bovis* merozoites growth *in vitro* (Okubo, Yokoyama, Govind, Alhassan & Igarashi, 2007). Mesplet et al. (2010) showed that gene coding to *B. bovis* cysteine peptidases - bovipaine-1 and bovipaine-2 - were transcripted only into the infected erythrocytes and that animals vaccinated with bovipaine-2 protein strongly reacted with the formation of antibodies. Furthermore, the anti-bovipaine-2 antibodies cross-reacted with erythrocytes infected with *B. bigemina*. Martins et al. (2010, 2011) also showed that cysteine proteases from *B. bigemina* are potential vaccine candidates.

Silva et al. (2010) analyzed a recombinant microneme protein from *B. bovis* (Bbo-MIC1), secreted on parasite surface, demonstrating that antibodies against Bbo-MIC1 inhibit erythrocyte invasion in *B. bovis in vitro* cultures and this protein was recognized by antibodies in serum of *B. bovis* infected cattle, showing the immunogenicity of Bbo-MIC1 and its use as potential vaccine.

Carcy, Précigout, Schetters & Gorenflot (2006) recognized that recombinant merozoite surface antigens (MSA) were expressed in the merozoites and sporozoites and that antibodies

anti-MSA-1 and MSA-2c inhibit, *in vitro*, the invasion of erythrocytes by sporozoites, suggesting this may be used *in vivo* to block erythrocytes invasion. The same authors used a *B. bigemina* protein, gp 45, similar to MSA-1 of *B. bovis*, to immunize cattle, which became protected against the parasite.

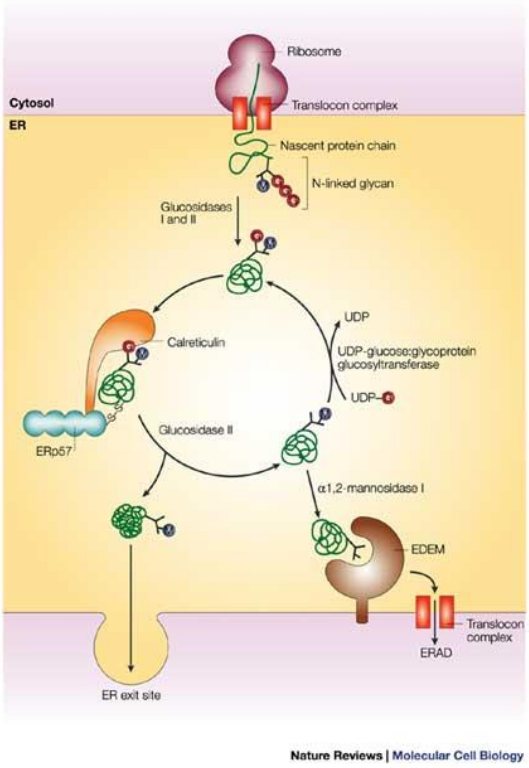
The apical membrane antigen 1 (AMA-1), has been evaluated as a possible subunit vaccine. Antibodies against *B. bovis* AMA-1 (BbAMA-1) reduced the invasion efficiency *in vitro*; moreover, this molecule is highly similar to another AMA-1 from *B. bigemina*, indicating that this vaccine could, possibly, have a cross reactivity with other *Babesia* species (Torina et al., 2009).

2.3. Calreticulin

2.3.1. Introduction

CRT was first isolated by Ostwald and Maclennan in 1974 and further cloned by Fliegel, Burns, MacLennan, Reithmeier & Michalak in 1989 (Fliegel et al., 1989**).**

CRT has been identified in several species, but there is no CRT genes identified in yeasts and prokaryotes, whose genomes were totally sequenced (Persson, Rosenquist & Sommarin, 2002). CRT importance to cells is relevant, given that the absence of CRT gene is embryonically lethal (Mesaeli et al., 1999). Since its first detection, CRT has been identified in other cellular structures, as cytoplasm, cell membranes and extracellular matrix (Burns, Atkinson, Bleackley & Michalak, 1994); several functions have been attributed to CRT, such as acting as a chaperone to help other proteins to fold correctly (Nauseef, McCormick & Clark, 1995). This activity was demonstrated by immunoprecipitation experiments, which showed that CRT associates itself transiently to several cellular proteins immediately after their synthesis (Peterson, Ora, Van & Helenius, 1995). When a polypeptide is incorrectly folded, it bounds to one or more chaperones (including CRT) and is retained in endoplasmatic reticulum (ER), mechanism that prevents expression, aggregation and secretion of misfolded proteins (Nauseef et al., 1995).



Adapted from Mendlovic & Conconi (2011).

Figure 6 CRT action as a chaperone.

Besides the essential functions that CRT performs in ER lumen, this multifaceted protein has also been implicated in many unexpected roles that occur at cells surface, cytosol, nucleus and extracellular matrix. Indeed, since discovery of CRT chaperone and calcium-regulating functions, scientists have learned that CRT has many other duties in the cell. This protein has been implicated in diverse cellular processes including signaling (Mitra & Schlaepfer, 2006), regulation of gene expression (Gardai et al., 2005), wound healing (Pallero, Elzie, Chen, Mosher & Murphy-Ullrich, 2008), removal of cancer cells (Tesniere et al., 2008) and autoimmunity (Eggleton & Llewellyn, 1999).

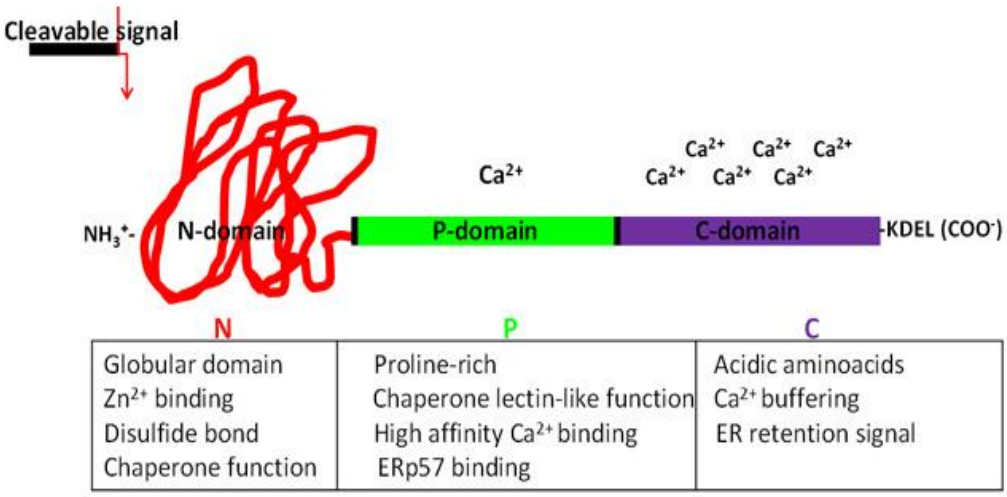
2.3.2. Calreticulin structure

CRT has two codifying genes, CRT-1 and CRT-2 (Persson et al., 2002), and is highly conservative with a 96% amino acid identity for CRT from human, rabbit, rat and mouse (Waser, Mesaeli, Spencer & Michalak, 1997). The CRT gene has nine exons and is localized in chromosomes 19 and 8, respectively for human and mouse genes. These sequences have more than 70% identity, with the exception of introns 3 and 6, showing a great gene

evolutionary conservation (McCauliffe, Yang, Wilson, Sontheimer & Capra, 1992). CRT gene from ticks has two exons and one intron, the last one with a conserved position, but variable size and nucleotide sequence. In *R. annulatus* CRT gene, intron is localized between nucleotides 88 and 412 and has 1559 base pairs (bp) (Xu, Fang, Sun, Keirans & Durden, 2005).

CRT proteins have a molecular weight around 46 kDa (Fliegel et al., 1989), an N-terminal cleavable amino acid signal sequence and a KDEL ER retrieval signal in the C-terminal domain. These amino acids are responsible for CRT targeting and retaining in ER lumen (Michalak et al., 1999). The protein has three cysteine residues, all located in N-domain of the protein. Two of those cysteine residues form a disulphide bridge (Matsuoka et al., 1994), which probably is responsible for correct folding of CRT N-terminal region. Structural studies show that CRT has three domains (Bedard, Szabo, Michalak & Opas, 2005), as illustrated in figure 7:

- The N-terminal domain (amino acid residues 1 to 180) is the most conserved domain, contains a binding site to monoglycosylated oligosaccharides (Schrag et al., 2001), several phosphorylation sites and is anticipated to have a folded globular structure with eight anti-parallel β-strands connected by protein loops (Michalak et al., 1999);
- The P-domain (amino acid residues 181 to 290) forms an β-stranded hairpin configuration extended curved-arm structure rich in prolin, which binds to Ca^{2+} with high affinity and low capacity (Ellgaard et al., 2001) and interacts with ER chaperones (Michalak, Groenendyk, Szabo, Gold & Opas, 2009);
- The C-terminal region or (C-domain) (amino acids residues 291-400) (Giraldo et al., 2010), characterized by a helix form (Del Cid et al., 2010), mainly constituted by aspartic acid and glutamic acid amino acids (Michalak et al., 1999), shows low affinity to Ca^{2+}, but a high capacity, important for Ca^{2+} storage in ER (Castãneda-Patlán, Razo-Paredes, Carrisoza-Gaytán, González-Mariscal & Robles-Flores, 2010). The structure of CRT differs at C-domain considering Ca^{2+} level, since this domain has a disordered structure with a low calcium concentration, but when this level rises, protein becomes more rigid and compact (Giraldo et al., 2010).



Adapted from Mendlovic & Conconi (2011).

Figure 7 Structure and functions of the CRT domains.

2.3.3. Calreticulin in ticks and other parasites

CRT is a protein that exists in ticks salivary glands and saliva; probably, this protein is essential to ticks feeding and pathogen transmission, through its anti-thrombotic and complement inhibition functions. All these facts reveal the possibility of CRT being used as an antigen in a vaccine against cattle ticks (Kaewhom, Stich, Needham & Jittapalapong, 2008).

Immunization assays with CRT showed its importance to immune reaction against ticks and, consequently, its relevance as a possible global anti-ticks vaccine component. Immunization of rabbits with CRT from *A. americanum*, followed by rabbits' infestation with those ticks led to necrotic lesions in ticks' local bite, demonstrating an immune reaction capable to interfering with ticks feeding (Jaworsky et al., 1995).

Gao et al. (2008) used a recombinant CRT (rCRT) protein from *H. qinghaiensis* (rHqCRT) to immunize sheep and results showed a reduced ticks weight and oviposition and a higher mortality comparing to control group.

There are various evidences of cross-reactivity between different anti-CRT antibodies, probably due to the fact that CRT is a highly conserved protein. Parizi et al. (2009) used rCRT from *H. longicornis* (rHlCRT) to immunize cattle and their sera reacted to both rHlCRT and rCRT from *R. annulatus* (rBmCRT). Besides, sera from cattle, whether immunized with rHlCRT or with rBmCRT, recognized native BmCRT.

Immunolocalization experiments using polyclonal antibodies anti-CRT from *A. americanum* and *D. variabilis*, revealed a specific protein from salivary glands homologous to CRT, which appears to be secreted during tick feeding (Jaworsky et al., 1995).

Finally, as CRT is a conserved protein, its use in a vaccine could protect animals not only from ticks, but also from tick-borne diseases. Concerning this aspect, Rachinsky et al. (2007) associated CRT with *R. microplus* infection by *B. bovis*, when they tested proteins up and down-regulated in infected and uninfected ticks and discovered that, among up-regulated proteins, there was CRT. Antunes et al. (2012) studied differentially expressed genes in *R. microplus* and *R. annulatus* after infection by *B. bigemina*, showing that gene encoding CRT was overexpressed. Knockdown of CRT in *R. microplus* reduced 73% the pathogen transmission as well as ticks weight.

As described previously with respect to ticks, there are several studies referring the use of CRT to protect animals from other parasites. Winter et al. (2005) used a *N. americanus*-CRT based vaccine to immunize mice, resulting in a significant reduction of worm number in lungs (43-49%) comparing with control group. In another study, hamsters were immunized with *Taenia solium*-CRT and, besides reduction of parasite numbers, worms were unable to mature in vaccinated animals (Mendlovic et al., 2004).

Immunolocalization studies using *Haemonchus contortus*-CRT antibodies showed that this protein is localized in external openings, such as the buccal cavity, vaginal tipi of female and bursa of male worms (Suchitra & Joshi, 2005). CRT from *Echinococcus granulosus* (EgCRT) is expressed in external tegument and cellular region of hydatic cysts germinal layer, possibly with the intent to inhibit classical complement pathway or to lead to an antiangiogenic effect in cysts periphery (Cabezón, Cabrera, Paredes, Ferreira & Galanti, 2008).

Debrabant, Lee, Pogue, Dwyer & Nakhasi (2002) evaluated the effect of *Leishmania donovani*-CRT P-domain overexpression in transfected parasites, which resulted in reduction of acid phosphatase-secretion and in a survival decrease in human macrophages, showing that changes in CRT expression may affect the “virulence” of the parasite.

Table 2 CRT functions in parasites that cause disease in humans and animals

Parasite	Disease	CRT localization	CRT function
Entamoeba histolytica	Amibiosis	ER, cell surface, uropod	Phagocytosis
Trypanossoma cruzi	Chagas disease	ER, cell surface	Lectin-like chaperone, interaction with C1q, recognized by iGg from patients
L. donovani	Visceral leishmaniosis	Somatic and secreted forms	Involved in secretion of acid phosphatases
N. americanus	Hookworm infection	Somatic and secreted forms	Allergen, induces basophil histamine release, interaction with C1q
Schistosoma mansoni	Schistosomiosis	Somatic and secreted forms, penetration glands	Regulation of Ca^{2+} dependent proteases involved in skin penetration and migration, T and B cell immunogen
T. solium	Taeniosis and neurocysticercosis	Somatic and secreted forms, gametes and developing embryos	Possible role in egg development

Adapted from Mendlovic & Conconi (2011).

Chapter 3. Material and Methods

3.1. *Rhipicephalus annulatus* ticks

Total RNA was extracted from *R. annulatus* female ticks as described in Antunes et al. (2012). Briefly, ticks were rinsed in distilled water and 75% (v/v) ethanol, dissected and the whole of the internal organs were placed in a 2 ml tube with 1 ml of Tri Reagent (Sigma-Aldrich, St. Louis, Missouri, USA). Total RNA was used in the synthesis of approximately 1 µg of cDNA using the iScript™ cDNA synthesis (Bio-Rad, Hercules, California, USA).

3.2. Amplification of the *calreticulin* gene

The CRT sequence was amplified by PCR technique. Primers were designed based on the CRT gene sequence of *R. annulatus* (accession number AY395253), ensuring full coding region coverage. The primers used were: forward 5'-CACC AT GCG GCT TCT CTG CAT TTT G -3' and reverse 5'- CAG TTC TTC GTG CTT GTG GTC -3'.

PCR was performed using Kit GoTaq® (Promega, Madison, Wisconsin, USA) under the following conditions: one amplification round in a final volume of 25 µl, including 1 µl of template cDNA and 1 µl of each primer (95ºC for 2 min, then 40 cycles: 30 s at 94ºC, 45 s at 55ºC and 2 min at 72ºC; final extension 2 min at 72ºC). PCR assays were performed in MJ Research PTC-200 Thermo Cycler (GMI Biotech, Minnesota, USA).

The positive PCR products were purified using the illustra GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions. Purified samples were sequenced at Stab Vida (Almada, Portugal) and further analysed.

3.3. Expression of recombinant calreticulin

For rCRT expression in *Escherichia coli* system, Champion^{TM} pET101 directional TOPO® Expression Kit (Invitrogen Life Technologies, Carlsbad, California, USA) was used. This

expression kit uses a highly efficient 5-min cloning strategy to insert a blunt end PCR product into a vector with no requirement of post-PCR procedures or restriction enzymes (Invitrogen, 2010). The recombinant proteins produced, using this expression kit, have a histidine tail attached.

3.3.1. Cloning and transformation

The purified PCR products, previously obtained (chapter 3.2.), were initially cloned into plasmids pET101/D-TOPO vectors using the above described kit according to manufacturer's instructions. 10 µl of pET TOPO® (Invitrogen Life Technologies, Carlsbad, California, USA) construct, previously obtained, was mixed with 50 µl of the *E. coli* OneShot® cells (Invitrogen Life Technologies, Carlsbad, California, USA), and then incubated in ice during 30 min, followed by incubation for 30 s at 42ºC and further incubated in ice. Afterwards, 250 µl S.O.C. medium were added to the previous sample and incubated at 37ºC, 200 rpm during 1 hour. After that period, 200 µl of the sample were used to seed 4 LB-agar/ampicillin (100 µg/ml) Petri-dishes, followed by overnight incubation at 37ºC.

3.3.1.1. Screening of the transformed colonies

Cell colonies were analyzed by PCR to confirm plasmids incorporation. Six colonies were picked up and individually suspended into 12 µl of water. Kit GoTaq® (Promega, Madison, Wisconsin, USA) was used. The PCR conditions were as followed: 94ºC for 10 min to lyse cells and inactivate nucleases, then 35 cycles: 94ºC for 3 min, 53ºC for 30 s and 72ºC for 1 min, followed by 72ºC for 10 min, in MJ Research PTC-200 Thermo Cycler (GMI Biotech, Minnesota, USA).

3.3.1.2. Plasmid purification for sequencing

For plasmids purification, illustra plasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) was used, since it applies a simple plasmid DNA purification protocol involving a modified alkaline lysis procedure and a silica-based membrane to achieve highly efficient plasmid DNA purification (GE Healthcare, 2008).
The colonies, previously obtained (chapter 3.3.1.), were picked and suspended in 5 ml of LB/ampicillin (100 µg/ml) and incubated overnight at 37ºC and 200 rpm. Samples were then centrifuged at 16000 rpm during 10 min using a Heraeuspico 17 centrifuge (Thermo Electron

Corporation, Marietta, Ohio, USA) and supernatant were discarded. The obtained pellet was further purified using the above described kit according to manufacturer's instructions and samples were then sequenced in Stab Vida (Almada, Portugal).

3.3.2. Expression

For rCRT expression, BL21 Star™ (DE3) One Shot *E. coli* cells were used. Plasmids, previously purified (chapter 3.3.1.2.), were inserted in BL21 Star™ (DE3) One Shot *E. coli* cells according to manufacturer's instructions. The entire transformation was used to inoculate 10 ml of LB/ampicillin (100 µg/ml) and was incubated overnight at 37ºC and 200 rpm. 850 µl of cells culture were mixed with 150 µl of glycerol and stored at -80ºC maintaining, therefore, a stock of cells with plasmid incorporated, and 500 µl were used to, once again, inoculate 10 ml of LB/ampicillin (100 µg/ml), in order to perform a pilot expression assay. Cells were incubated at 37ºC and 200 rpm, split into two 5 ml cultures and one was induced at an Abs 600 nm (optical density) of 0.5-0.8 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). To determine the optimal induction time, 500 µl aliquot from each culture have been removed every hour for 5 hours. Samples were centrifuged at 16000 rpm for 1 min, supernatant discarded and pellets frozen at -20ºC.

3.3.3. SDS-PAGE

A common method for proteins separation by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature proteins. According to Laemmli method, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the previously obtained cellular lysate (chapter 3.3.2.) and confirm the presence of rCRT.

Cellular lysates were mixed with 1x SDS-PAGE sample buffer, denatured by heat and separated in a 12.5% SDS-PAGE polyacrylamide gel using the SDS-PAGE apparatus Mini-protean Tetra Cell (Bio-Rad, Hercules, California, USA). Electrophoresis was performed at 120 V using a running buffer and a low molecular marker (Amersham^{TM} LMW calibration kit for SDS electrophoresis, GE Healthcare, Buckinghamshire, UK) was added. Gel was stained using Coomassie Brilliant Blue dye solution.

3.3.4. Western-Blot

Western-Blot is a technique that identifies proteins which have been separated according to their size by gel electrophoresis, using specific antibodies. The blotting membrane, usually made of nitrocellulose or PVDF (polyvinylidene fluoride), binds to proteins. With this method, the polyacrylamide gel is placed over the membrane and the application of electrical current forces proteins to move from gel to membrane, where they adhere and can be subsequently linked to a specific antibody. This binding can then be visualized using a second antibody which recognizes the first one, developing a visual signal in the presence of the appropriate substrate (Abcam, 2012).

To detect the rCRT using Western-Blot technique, a SDS-PAGE gel was prepared without Coomassie Brilliant Blue staining, as previously described. Briefly, a cassette was constructed with sponge, filter papers, SDS-PAGE gel and nitrocellulose membrane (Trans-Blot® Transfer Medium pure cellulose membrane (0.45 µm), Bio-Rad, Hercules, California, USA) and was applied on a Western-Blot apparatus (Mini Trans-blot® Cell, Bio-Rad, Hercules, California, USA) with transfer buffer running overnight at 90 mA. Nitrocellulose membrane was then placed in a container with Ponceau Red 0.2% (w/v) diluted in acetic acid 3% (v/v) allowing bands visualization. Membrane was cutted into strips, which were placed in 1.5 ml of blockage solution for 1 hour. Subsequently, the strips were washed 3 times with 2 ml of TTBS for 15 min. Strips were incubated in 1 ml of anti-histidine antibodies solution (1:5000) (Anti-His (C-term) antibody, Invitrogen Life Technologies, Carlsbad, California, USA) for 1 hour and washed again as described above. Then, the strips were incubated in 1 ml of polyvalent anti-mouse antibody solution (1:10000) (Anti-Mouse Polyvalent Immunoglobulins (G, A, M) - Alkaline Phosphatase, Sigma-Aldrich, St. Louis, Missouri, USA) for 1 hour and the wash procedure was repeated. 1.5 ml of revelation buffer (AP Color Development Buffer, Bio-Rad, Hercules, California, USA) was used to develop the visual signal. The reaction was stopped by adding water and keeping the membrane in the dark.

3.4. Production of soluble calreticulin

The previously produced stock of BL21 Star™ (DE3) One Shot *E. coli* cells (chapter 3.3.2.) was used to seed a LB/ampicillin (100 µg/ml) Petri-dish and was incubated overnight at 37ºC. Then, six colonies were used to perform a PCR in the same conditions previously described in chapter 3.3.1.1.. Afterwards, four colonies were used to inoculate 200 ml of LB/ampicillin (100 µg/ml). Cells were induced with IPTG and were grown for two hours at 37ºC and 200

rpm as described in chapter 3.3.2.. Then, pellet was suspended in 20 ml lysis buffer and divided into 2 fractions; in one of the samples a sonicator (Ultrasonic Homogenizer Power Supply 4710 Series, Cole Parmer, Illinois, USA) with an amplitude of 80%, a frequency of 20 kHz and cycles of 10 s/min for a total of 5 min were applied and the other fraction was thawed at 42ºC and frozen at -80ºC for three times. Subsequently, samples were centrifuged for 15 min at 16000 rpm, pellet was suspended in 1 ml of PBS and supernatant stored. These samples were used to perform a SDS-PAGE and Western-Blot, as described in chapters 3.3.3. and 3.3.4., respectively, in order to determine whether the protein was present in the soluble or insoluble fractions of lysate.

3.5. Purification of recombinant calreticulin

To perform the purification of rCRT, HisTrap HP column (GE Healthcare, Buckinghamshire, UK) associated with fast performance liquid chromatography (System Pump-500, GE Healthcare, Buckinghamshire, UK) was used. Prior to purification, samples were diluted in binding buffer. Purification protocol, according to manufacturer's instructions, was as follows:

- Column was washed with 15 ml of distilled water and equilibrated with 25 ml of binding buffer at a flow-rate of 5 ml/min;
- Samples were applied with a pump and column washed with 75 ml of binding buffer;
- 25 ml of elution buffer were added.

The above described protocol was also performed with some adjustments in order to select optimal purification conditions:

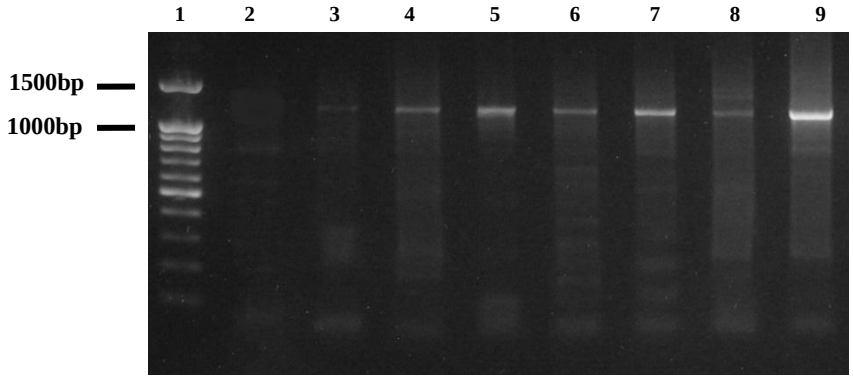
- Elution buffer without imidazole and with a pH of 4.5;
- Different binding buffer imidazole concentrations (20 mM, 30 mM and 40 mM) with two washes;
- Concentration of purified samples (1.5 ml Microcon® YM-3 tube, membrane: regenerated cellulose MCO 3000).

The obtained samples (non-purified, purified and samples resulting from washes) were loaded into the SDS-PAGE, as described in chapter 3.3.3..

Chapter 4. Results

4.1. Amplification of *calreticulin* cDNA fragment

Reverse-transcriptase PCR technique was used to amplify the fragment correspondent to CRT, in order to allow the posterior rCRT protein production. This procedure was performed several times using different PCR condictions (different cycles number, elongation time and annealing temperature) and different samples in order to improve RNA quality and obtain a unique band with 1235 bp, which is the length of CRT without introns. This result (figure 8) was obtained with the specific PCR condictions: 95ºC for 2 min, then 40 cycles of 30 s at 94ºC, 45 s at 55ºC and 2 min at 72ºC; final extension 2 min at 72ºC.



Lane 1. Ladder 100bp
Lane 2. Negative PCR control
Lanes 3-9. Different cDNA samples

Figure 8 PCR results. Samples were electrophoresed in a 1% Agarose/CYBRsafe gel, 1x TAE.

PCR amplified fragments were further purified and sequenced (StabVida, Almada). The obtained fragment sequence had only 1100 bp (figure 9) instead of 1235 bp and, when compared with annotated *R. annulatus*-CRT, had an overall identity of 98%. When compared with CRT from different ticks, the sequence showed a identity from 83% to 98% (table 3), confirming the fact that this protein is highly conserved among ticks.

CNNNCNNNNTCGCTATATCGGCCGACCCGACCGTATACTTCAAAGAGGAGTTCAACGATGGAGACGCGTGGAAGGAC
CGGTGGGTGGAGTCTACGAAAGGCGACAACCTCGGAAAGTTCGTTCTAAGCGCTGGCAAGTTCTACGGTGACGCGGA
GAAGAGCAAAGGACTGCAGACCTCTGAAGACGCCCGCTTCTACGGCATCTCCGCCAAGTTCGAACCCTTCTCCAACG
AAGACAAGACCCTGGTCATCCAATTCGCGGTGAAGCACGAGCAGAACATCGACTGCGGCGGTGGCTACGTCAAGCTG
TTCGACTGCAGCCTAGACCAGACTCAAATGCACGGCGAATCTCCCTACCTCATCATGTTCGGCCCTGACATCTGCGGT
CCCGGCACCAAAAAAGTGCACGCCATCTTCAACTACAAGGGCAAGAACCACCTTATCAACAAGGAGGTGCGCTGCAA
AGACGACGTCTTCAGCCACCTGTACACCCTGATCGTTAAGCCCGATAACACATACCAGATCAAGATCGACAACGAAG
TGGTCGAGAAGGGCGAACTCGAGAAGGACTGGTCCTTCCTGCCCCCCAAGAAGATCAAGGACCCCGACGCCAAGAAG
CCCGAGGACTGGGACGACCGGGCCAAGATCGACGACCCCGACGACAAGAAGCCCGAGGACTGGGACAAGCCCGAGT
ACATTCCCGACCCGGACGCCACCAAGCCCGAGGACTGGGACGACGACATGGACGGCGAGTGGGAACCCCCGCAGAT
CAACAACCCCGAGTACAAGGGCGAGTGGAAACCCAAGCAGATCGACAACCCGGCCTACAAGGGTGCCTGGGTACAC
CCGGAGATCGACAACCCCGAGTACACGCCGGACCCCAAGCTGTACCGCTACAAGGAGATCTGCAAGATCGGCTTCGA
CCTGTGGCAGGTCAAGTCTGGCACCATCTTCGACAACATCCTCATCACGGACGACGAAGAGTACGCCCGGGTGCACG
GCGAGGAGACCTGGGCCGCGCTCAANACGAGAGAAAAGATGANNNCAAGCAGGAAGAGAGAGAGCAAAGAGCAGA
AGAGATGACGCTAGACGAGGACGANN

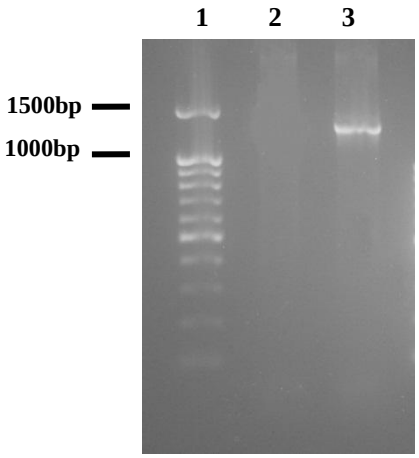
Figure 9 Sequence of the purified sample with the amplified fragment.

Table 3 Identity of *calreticulin* sequences from different ticks

	Sequence	*R. annulatus*	*R. microplus*	*R. sanguineus*	*D. variabilis*	*A. americanum*	*H. longicornis*	*I. ricinus*	*I. scapularis*
Sequence	100%	98%	97%	92%	90%	87%	87%	87%	85%
R. annulatus		100%	99%	93%	91%	88%	88%	84%	84%
R. microplus			100%	92%	89%	88%	88%	84%	83%
R. sanguineus				100%	89%	87%	87%	83%	84%
D. varibilis					100%	89%	89%	86%	85%
A. americanum						100%	89%	83%	83%
H. longicornis							100%	83%	83%
I. ricinus								100%	97%
I. scapularis									100%

4.2. Cloning and transformation

To express rCRT, pET101/D-TOPO® (Invitrogen Life Technologies, Carlsbad, California, USA) plasmids were used as vectors. Plasmids were incorporated into two different cell types: One Shot® TOP10 *E. coli* cells (Invitrogen Life Technologies, Carlsbad, California, USA), which allows a stable propagation and maintenance of recombinant plasmids, and BL21 Star™ (DE3) One Shot® *E. coli* cells (Invitrogen Life Technologies, Carlsbad, California, USA), that were used to produce the protein. Transformation procedures in both cell types were performed twice, always in the same conditions, until the PCR results showed a single band corresponding to CRT fragment (figure 10).



Lane 1. Ladder 100 bp
Lane 2. Negative PCR control
Lane 3. *E. coli* cells sample

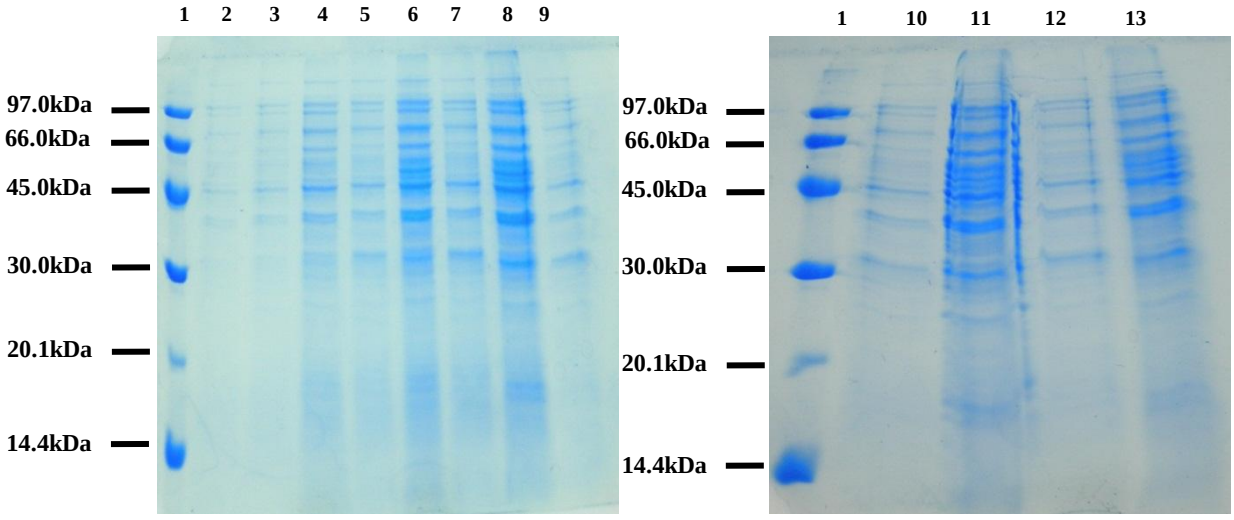
Figure 10 PCR results. Samples were electrophoresed in a 1% Agarose/CYBRsafe gel, 1x TAE.

Obtained fragments were sequenced to confirm the correct position of the fragment, since that is essential to produce the correct protein, as a different nucleotides sequence will correspond to different aminoacides and, consequently, to a different protein.

Sequence analysis of the fragment incorporated in the plasmid revealed a 500 bp length, instead of 1235 bp.

4.3. Recombinant calreticulin expression

After the cells transformation, several samples at different time points were picked from induced and non-induced cells cultures. The level of rCRT expression was first evaluated by SDS-PAGE, but, as shown in figure 11, no distinct rCRT band was obtained in induced fraction; instead there were several bands in the SDS-PAGE gel. So, a Western-Blot procedure was performed in order to confirm the expression of CRT and, as demonstrated by figure 12, a unique band of approximately 60 kDa was obtained in induced fraction of time points 1 and 2, which correspond to 1 and 2 hours of induction, respectively, and there were no bands in the other time points (T3 to T5).



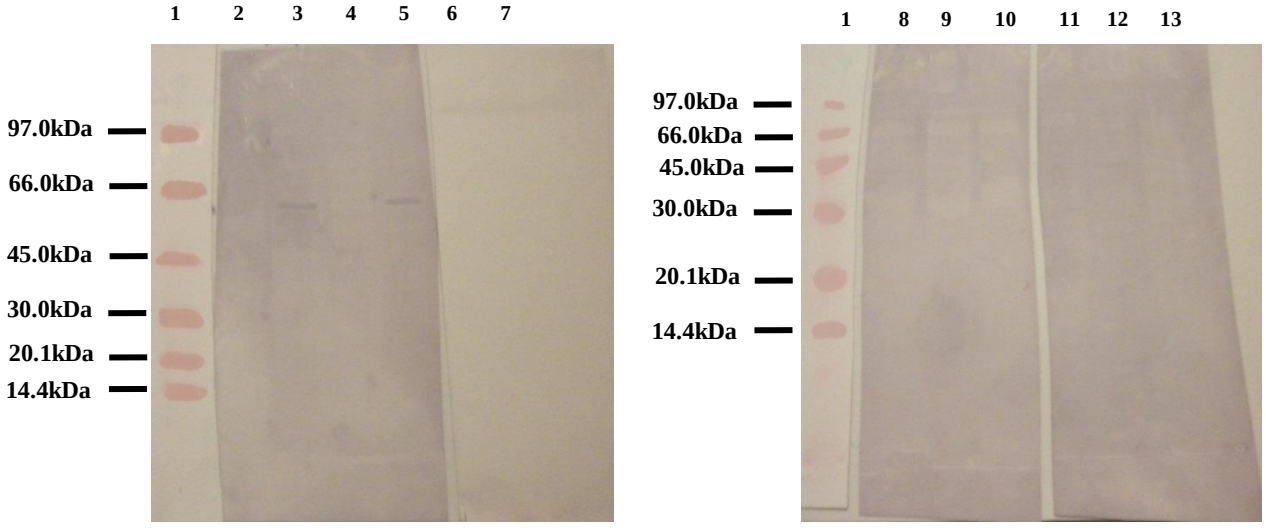
A

Lane 1. Ladder 100 bp
Lane 2. T0 - induced
Lane 3. T0 - non-induced
Lane 4. T1 - induced
Lane 5. T1 - non-induced
Lane 6. T2 - induced
Lane 7. T2 - non-induced

B

Lane 1. Ladder 100 bp
Lane 8. T3 - induced
Lane 9. T3 - non-induced
Lane 10. T4 - non-induced
Lane 11. T4 - induced
Lane 12. T5 - non-induced
Lane 13. T5 - induced

Figure 11 SDS-PAGE results.Protein bands from cell cultures induced by IPTG and non-induced at different time points (A) T0 to T3 and (B) T4 to T5.



A

Lane 1. Ladder 100 bp
Lane 2. T2 - non-induced
Lane 3. T2 - induced
Lane 4. T1 - non-induced
Lane 5. T1 - induced
Lane 6. T0 - non-induced
Lane 7. T0 - induced

B

Lane 1. Ladder 100 bp
Lane 8. T3 - non-induced
Lane 9. T3 - induced
Lane 10. T4 - non-induced
Lane 11. T4 - induced
Lane 12. T5 - non-induced
Lane 13. T5 - induced

Figure 12 Western-Blot results. Protein bands from cell cultures induced by IPTG and non-induced at different time points (A) T0 to T2 and (B) T3 to T5.

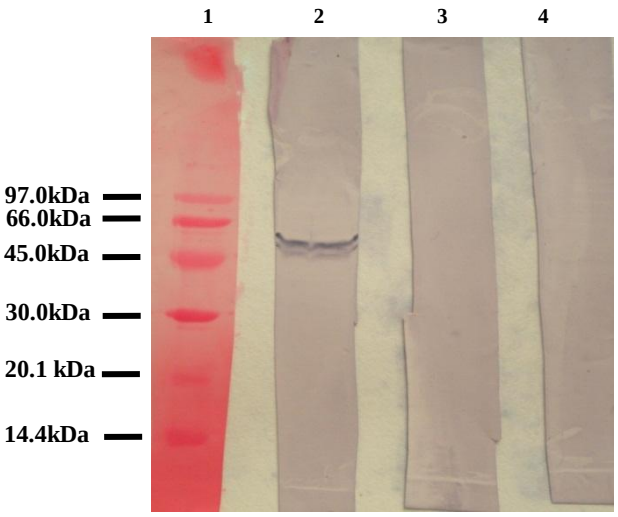
4.4. Purification of recombinant calreticulin

The first protocol used a binding buffer with 30 mM of imidazole, an elution buffer with 500 mM of imidazole, both with pH of 7.4, and one washing step. The results showed several contaminants in all samples (purified, non-purified and samples obtained from washes) and no distinct rCRT corresponding band. Thereafter, the protocol was slightly altered, using a different elution buffer with no imidazole and a pH of 4.5. However, the results contained even more contaminants than those obtained with the original protocol. Hence, as the pH reduction was worthless, it was decided to alter the original protocol by using different imidazole concentrations in the binding buffer (20 mM, 30 mM and 40 mM) and adding one more washing step. The results showed that samples obtained using the binding buffers with 20 mM and 40 mM of imidazole had almost no bands and those obtained using the binding buffer with 30 mM of imidazole had few contaminants, but also had the most visible bands in the purified fraction. Taking these results into consideration, the protocol using the binding buffer with 30 mM of imidazole and pH 7.4, two washing steps and the elution buffer with 500 mM was applied to the samples. After obtaining the purified sample, it was concentrated and, finally, results produced a single band with approximately 50 kDa, corresponding to rCRT (figure 16).

To determine the presence of rCRT in the soluble fraction, the first cells sample, suspended in PBS, were lysed by sonication. Figure 13 depicts the results of Western-Blot corresponding to the sonicated cells, showing that rCRT was only present in the insoluble fractions of the cellular lysate. Because CRT had been obtained in previous studies from the soluble fraction (Rachinsky et al., 2007; Gao et al., 2008; Parizi et al., 2009), the sonication protocol was altered and another method (freeze/thaw) was added to provoke cell lysis, it was admitted that sonication of cells in PBS was not enough to lyse them. Therefore, instead of using PBS, lysis buffer was used to suspend the cells, and, subsequently, sonication and freeze/thaw methods were applied. Once again, the SDS-PAGE results with those samples (figure 14) showed no distinct rCRT band. A Western-Blot approach was then performed, as that technique, in previous results (chapter 4.3.), revealed an rCRT corresponding band, where the SDS-PAGE procedure did not. The Western-Blot results confirmed the presence of rCRT, not only in the insoluble fraction, but also in the soluble phase, as two bands next to each other (doublet) were revealed by both methods results (figure 15).

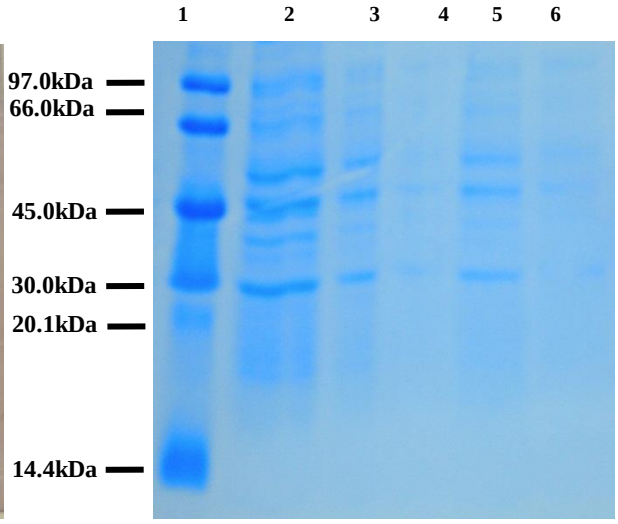
After rCRT purification in collumns, it was necessary to confirm the purity level of the obtained samples so a SDS-PAGE technique was used once more. Different purification protocols were apllied until a unique band corresponding to rCRT was obtained in the SDS-

PAGE results. Despite their differences, all the protocols applied 15 ml of distilled water, followed by an equilibration with 25 ml of binding buffer at a flow-rate of 5 ml/min, a washing step(s) with 75 ml of binding buffer and, finally, an elution step with 25 ml of elution buffer.



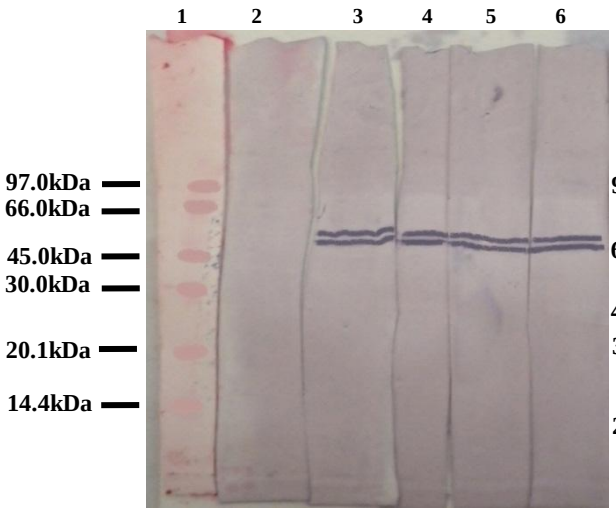
Lane 1. Ladder 100 bp
Lane 2. Insoluble fraction
Lane 3. Soluble fraction
Lane 4. T0 (non-induced)

Figure 13 Western-Blot results. Protein bands from soluble and insoluble fractions.



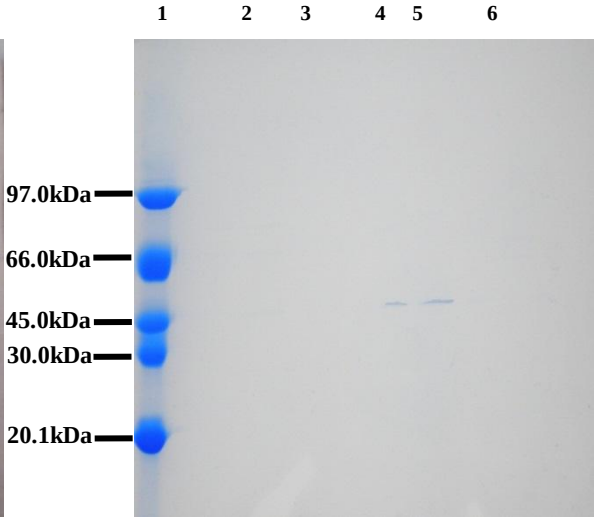
Lane 1. 1. Ladder 100 bp
Lane 2. T0 - non-induced
Lane 3. Insoluble fraction (cells sonication)
Lane 4. Soluble fraction (cells sonication)
Lane 5. Insoluble fraction (freeze/thaw)
Lane 6. Soluble fraction (freeze/thaw)

Figure 14 Western-Blot results. Protein bands from soluble and insoluble fractions.



Lane 1. Ladder 100 bp
Lane 2. T0 - non-induced
Lane 3. Insoluble fraction (cells sonication)
Lane 4. Soluble fraction (cells sonication)
Lane 5. Insoluble fraction (freeze/thaw)
Lane 6. Soluble fraction (freeze/thaw)

Figure 15 Western-Blot results. Protein bands from soluble and insoluble fractions.



Lane 1. Ladder 100 bp
Lane 2. Non induced sample
Lanes 3-6. Purified rCRT

Figure 16 SDS-PAGE results. CRT band from purified samples (2 to 6).

Chapter 5. Discussion

Babesiosis and other tick-borne diseases are relevant issues all over the world, especially in countries where cattle production plays a major role in the economy. Nowadays, vaccines against both tick-vector and parasites became an innovation and tend to be the most capable method to control ticks and their transmitted pathogens (de la Fuente et al., 2007).

This study reports the production of rCRT protein from *R. annulatus*, that is up-regulated in ticks infected with *B. bigemina* (Antunes et al., 2012), having in mind its further application in vaccination and immunolocalization tests.

The study is focused in the amplification of CRT gene by PCR technique. The results of the purified PCR product sequence showed 98% identity with native *R. annulatus*-CRT (table 3), though amplified fragment had only 1100 bp (instead of 1235 bp, as in the original one) (figure 9). It was considered that the fragment had been fully amplified within the PCR performance and the different length was due to a sequencing error. This hypothesis is corroborated by the high level of identity between original gene and the amplified fragment and also by the discrepancy found between bands length (in agarose gel and in the sequenced one), pointing out to the conclusion that the fragment probably had a length of 1235 bp, but only 1100 bp were amplified.

The following task involved rCRT cloning into a plasmid for its posterior use in bacteria transformation. Subsequent actions were facilitated due to the characteristics of the chosen plasmid, such as the C-terminal fusion tag for detection and purification of recombinant fusion proteins (V5 epitope and 6xHis, composed by 6 histidine residues), the antibiotic resistance marker for selection in *E. coli* and a T7 promoter that allows an IPTG-inducible expression of recombinant protein in *E. coli* strains (Invitrogen, 2010). The first characteristic is important, not only to protein detection by Western-Blot technique, but also to its purification, since rCRT has a histidine tail. The antibiotic resistance marker permits a "selector mechanism" that, after cell transformation, allow only those with an antibiotic resistance gene to grow in a selective medium. Finally, IPTG-inducible expression allows the growth of two groups of colonies, one induced and another uninduced, achieving the emergence of a negative control. Lastly, to clone the fragment into the plasmid, the forward primer had to have nucleotides CACC in 5' end, so that incorporation could take place

properly and in the correct sense. In the case of the primer reverse, it had to anneal to the last codon before the stop codon so that recombinant protein could have a histidine tail (Invitrogen, 2010).

Purified plasmids were sent to be sequenced, though the result showed only 500 bp instead of the expected 1235 bp. It was considered that this result was due to a sequencing error, corroborated by the fact the PCR previously performed using those purified plasmids produced a band with approximately 1200 bp. Moreover, previously purified PCR fragment sequence had also a different length with respect to the expected one (figure 9) and it was considered to be due to a sequencing error.

There are several recombinant systems that may be used to express recombinant proteins. In this study, an *E. coli* recombinant system was used, what is in line with other studies of recombinant proteins from ticks (Gao et al., 2008; Parizi et al., 2009; Tanaka et al., 2010; Almazán et al., 2012). As an alternative, a recombinant system with *Pichia pastoris* could have been used, because it is commonly chosen to express recombinant proteins from ticks and have been applied with success by several authors (Canales et al., 2008; Cunha, Andreotti & Leite, 2011; Ebrahimi, Dabaghian, Jazi, Mohammadi & Saberfar, 2012; Said et al., 2012)

There are numerous reported methods to include foreign DNA into *E. coli* cells (transformation), though chemical methods are the most commonly used, due to their accessibility and cost effectiveness (Singh, Yadav, Ma & Amoah, 2010). In this work, the transformation kit already provided chemical competent *E. coli* cells and transformation was performed through a brief incubation at 42ºC, followed by ice immersion, as it has already been demonstrated that these sequence improves transformation (Yoshida & Sato, 2009). The S.O.C. medium was added to the transformed cells, because it is a rich medium used in recovery step of *E. coli* cells transformation, maximizing its efficiency. Moreover, S.O.C. medium has glucose, which prevents induction of lac promoter by lactose, so preventing transcription of target gene (Sun et al., 2009).

To confirm transformation efficiency, colonies were screened by PCR and several did not generate the expected band in agarose gel. Supposedly, only cells with plasmid would be able to grow in LB medium with ampicillin, as it was previously explained. There are some possible explanations for this fact: ampicillin was not stable, primers did not anneal properly within PCR procedure or plasmid was unstable. Among all these possible causes, the most likely is the lack of ampicillin stability, since antibiotic solution was frozen and unfrozen several times impairing and corrupting its stability (Okerman, Van Hende & De Zutter, 2007). The other possible explanations are unlikely, since a previously performed PCR with purified plasmid, the same primers were used and a band with the expected length was obtained. In the

case of plasmid instability, although this phenomenon occurs during cellular growth period (Monbouquette & Ollis, 1986), Xu et al. (2006) showed that under resistance-selective pressure, cells with plasmid remain in a higher level than under nonselective pressure after induction, turning this hypothesis unlikely as well.

In rCRT expression, two cultures were used in order to provide induce and non-induce samples, what concurs to achieve more credible results, as negative control (non-induce sample) was available. The various time points adopted were decise to understand when rCRT production was at the highest level. This information was relevant to make decisions about subsequent cells cultures and the production and purification of rCRT. Therefore, having in hand the various time points samples, they were applied in a SDS-PAGE gel with the purpose to visualize which one had a band corresponding to rCRT (figure 11). In this procedure, proteins were denatured to achieve their primary conformation, so that the test would only be influenced by proteins molecular weight. In order to meet this goal, a SDS buffer was used, embodying a component that attaches to proteins and confers them a negative charge; β-mercaptoethanol was used to disrupt proteins disulphide bridges allowing molecules to adopt an extended monomeric form (EncorBio, 2012); glycerol was used to increase sample density, maintaining proteins on the wells bottom, overflow or uneven gel loading is restricted and bromophenol blue was used to visualize proteins migration, as it is a small molecule and migrates faster than all the other components (Grabski & Burgess, 2001). The heating process is important, because proteins heated with SDS are denatured and become negative charged (Hames, 1990). Concerning gel percentage, that was chosen according to proteins size and, since CRT would have, supposedly, 46 kDa (Fliegel et al., 1989) a percentage of 12.5 was selected.

Table 4 Gel percentage according to protein size.

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

Adapted from Abcam (2012).

However, with the SDS-PAGE gel, there was not a significant difference between induced and non-induced bands, so it was impossible to understand if rCRT had been produced or not. A Western-Blot was carried out because this technique favours the appearance of bands by

antibody-antigen binding, what is much more specific, and, as rCRT had a histidine tail, anti-histidine antibodies were used. The Western-Blot results showed a band corresponding to a protein with approximately 60 kDa, but only in time points 1 and 2 (figure 12). The predicted rCRT molecular weight was 46 kDa (Fliegel et al. 1989) plus 0.84 kDa, corresponding to hexa-histidine molecular weight (Terpe, 2003). The discrepancy between predicted and apparent rCRT molecular weight have already been described in CRT from other organisms, as ticks (Gao et al., 2008; Parizi et al., 2009), rats (Zhu, Zelinka, White & Tanzer, 1997; Rendón-Huerta, Mendoza-Hernández & Robles-Flores, 1999; Coling et al., 2007), humans (Coppolino & Dedhar, 1998; Hong et al., 2004), nematodes as *Heligmosomoides polygyrus* (Rzepecka et al., 2009) and *N. americanus* (Kasper et al., 2001; Winter et al., 2005) and insects as *Cotesia rubecula* (Zhang, Schmidt & Asgari, 2006), among others. The authors of these studies had two main explanations to molecular weight discrepancy: CRT glycosylation (Rendón-Huerta et al., 1999; Hong et al., 2004; Coling et al., 2007; Rzepecka et al., 2009), phenomenon that is common in proteins and the fact that CRT is highly negative charged with a pI of 4.7 (Coppolino & Dedhar, 1998; Rendón-Huerta et al., 1999; Gao et al., 2008; Rzepecka et al., 2009; Parizi et al., 2009). Besides those hypotheses, Zhu et al. (1997) described the putative existence of two isoforms of CRT, an endocalreticulin with 52 kDa and an exocalreticulin with 62 kDa. These results could also be due to the connection of another protein to collumns. Concerning that possibility, although it has been described the natural occurrence of histidine residues in *E. coli* proteins (Robichon, Luo, Causey, Benner & Samuelson, 2011), those molecules have non-consecutive histide residues and not a hexa-histidine molecule; moreover, those proteins were described as contaminants in IMAC technique and not in Western-Blot procedures. The exo/endocalreticulin hypothesis is also unlikely, since results showed only one band. Probably, the increased rCRT apparent molecular weight was due to either posttranslational modification that can increase proteins predicted molecular weight (Totten & Lory, 1990), as glycosylation, or to CRT negative charge. These hypothesis are more likely because CRT has several described binding sites to glycosylation (Schrag et al., 2001) and has already been defined as a negative charged protein (Michalak et al., 2009). About the fact that CRT was only detected in T1 and T2 time points, the result was unexpected, because the detectable rCRT at T1, supposedly should imply a higher protein level in the other time points. One explanation for these results may be related to technical errors during the manipulation of T3, T4 and T5 samples, namely incorrect sample loading, errors in proteins transference to blotting membrane or contamination of cell cultures. On the other hand, these explanations are not very likely, because cell cultures were always manipulated in a laminar flow chamber with all aseptic concerns; a transference error,

i.e., proteins not being transferred to the membrane or an incorrect sample loading are also unlikely because membrane coloring by means of Ponceau solution and SDS-PAGE gel, showed bands in all time points. Comparing with our cells cultures, their concentration was always increasing with time and, as IPTG was only added once at T0, there could be a lower impact of IPTG in distant time points, as described by Lewis, Taylor, Nienow & Hewitt (2004). Furthermore, substrate growth was always the same, so, as the experiment progressed in time, nutrients were degraded and excretion products accumulated, including acetate, which could inhibit cellular growth (Andersson, 1996) and recombinant protein production (Shimizu et al., 1998). Probably, from T2 on, there was a reduction of rCRT production due to lower impact of IPTG and to inhibition of recombinant protein production caused by either IPTG lower impact or acetate production, culminating in the absence of rCRT or such a low production level that would not be detectable at those time points samples.

Next, at the first attempt to produce soluble rCRT, cells were suspended in PBS and sonicated to provoke their lysis, as sonication was used in several studies of recombinant proteins from ticks (Liao et al., 2007; Gao et al., 2008; Almazán et al., 2012; Ebrahimi et al., 2012). As described in other studies, this method could have been associated to freeze/thaw technique (Parizi et al., 2009; Tian et al., 2011), a lysozyme addition (Miyoshi, Tsuji, Islam, Kamio & Fujisaki, 2004), cells could have been suspended in binding buffer instead of PBS and sonicated (Díaz-Martín, Manzano-Román, Siles-Lucas, Oleaga & Pérez-Sánchez, 2011); it could also have been used protein extraction with Triton X-100 in TBS (Almazán et al., 2005) or vortexing with glass beads (García-García et al., 2000). The sonicator has the ability to destroy cells membrane by powerful ultrasounds, which rise molecules distance exceeding the minimum molecular distance essential to maintain membrane intact, culminating with lysis (Santos, Lodeiro & Capelo-Martínez, 2009). The freeze/thaw method involved cells suspended in lysis buffer freezing at -80ºC and then thawing at 42ºC. This technique forms ice crystals in freezing phase, which contracts and breaks during thawing and, consequently, causes cells lysis (Thermo Scientific, 2009).

The Western-Blot results of the sonicated cells (figure 13) showed, in the insoluble fraction, two close bands (approximately 55 and 60 kDa). The lack of bands in the soluble phase may be explained by the presence of rCRT only in the other fraction or due to the fact that cells were not correctly lysed and, consequently, remained in the pellet (insoluble phase) and so was rCRT. The second explanation is most likely, because CRT from several ticks has already been produced as a soluble protein (Rachinsky et al., 2007; Gao et al., 2008; Parizi et al., 2009). Subsequently, sonication was used once more, but PBS was replaced by lysis buffer and another method (frozen and thaw samples suspended in lysis buffer) was adopted. When

the membrane was revealed (figure 15), there were bands in insoluble and soluble phases, probably due to an incomplete cells lysis and, consequently, part of rCRT was still in cells and so in pellet (insoluble phase). In each fraction, only one band corresponding to rCRT should appear; however, two close bands became visible (approximately 55 and 60 kDa), similar to the first attempt result. The two bands found in acrylamide gel are probably due to either protein denaturation, as the electrophoresis run in denaturing conditions (in the presence of SDS) or to protein degradation.

Concerning recombinant proteins purification, there are many described processes: IMAC (López et al., 2009), purification according to proteins sizes (Canales et al., 2008; Said et al., 2012) and excision from polyacrylamide gel (Ebrahimi et al., 2012) among others. In the process of rCRT purification, IMAC was the chosen method, because it is a quick and widely used method for histidine-tagged proteins purification (Kuo & Chase, 2011) and it has already been used successfully with recombinant proteins from ticks (Miyoshi et al., 2004; Almazán et al., 2005; Tanaka et al., 2010; Cunha et al., 2011; Díaz-Martín et al., 2011; Tian et al., 2011). There are other described purifying methods to recombinant proteins from ticks, such as purification according to proteins sizes (Canales et al., 2008; Said et al., 2012) and excision from polyacrylamide (SDS-PAGE) gel (Ebrahimi et al., 2012).

The columns used to purify rCRT have nickel ions. The imidazole concentration of binding buffer was low in order to minimize binding of host-cell proteins, though it may decrease binding of histidine-tagged proteins (GE Healthcare, 2005). The elution buffer had a higher imidazole quantity than the binding buffer, because these molecules have a greater affinity to the column than the protein, allowing the rCRT elution (GE Healthcare, 2005), though the pH diminishment would have the same effect (Du, Zhang, JieWang, Yao & Hu, 2008). Despite the facts described before, results in the first attempt showed that almost no rCRT was purified and that there was a huge contamination with other proteins, causing the appearance of several bands instead of just one band corresponding to rCRT. First of all, column protocol refers that an optimization should be performed, *i.e.*, different factors should be tested, such as imidazole concentration, in order to obtain the best balance of high purity and high yield, as well as the use of different metals, since protein-metal ion binding strength is affected by length, position and exposure of proteins affinity tag, ions and buffers pH. From the above mentioned aspects, the use of different metals is not of great importance, since nickel and cobalt are usually the chosen by several authors as the metal ions to bind to histidine-tagged proteins (Sun et al., 2005; Chong, Tan, Biak, Ling & Tey, 2009; Knecht, Ricklin, Eberle & Ernst, 2009). The use of pH reduction, instead of imidazole different concentrations, to purify rCRT revealed to be worthless, since there were almost no bands. On the other hand, lack of

optimization with different imidazole concentrations could be one of the main reasons for these results once, as explained above, imidazole concentration can decrease binding of histidine-tagged proteins. Next attempt, where three different binding buffer imidazole concentrations were tested, all samples had contaminants. However, the one washed with binding buffer with 30 mM of imidazole had the most visible bands, including rCRT corresponding band, being this imidazole concentration the one used in the first attempt. This result made clear that imidazole concentration was not influencing purification process. Low purification level could be related to some recombinant proteins having its histidine-tag partially hidden from protein surface, caused by intra or inter-molecular interactions (Mohanty & Wiener, 2004), leading to a lower liaison of rCRT to the columns and, consequently, to lower recombinant protein purification. There are many *E. coli* proteins described as contaminants when IMAC technique is used to purify histidine-tagged proteins. Most of them are probably due to bacteria response to stress conditions (nutrient starvation, heat shock or oxidative damage). Contaminant proteins production depends on culture conditions, media compositions and genetic background of expression strain (Bolanos-Garcia & Davies, 2006). Bolanos-Garcia & Davies (2006) described three contaminant proteins classes based on imidazole concentration required for elution: class I (≥80 mM), class II (55 to 80 mM) and class III (30 to 50 mM), placing our samples contaminants in class I, which includes contaminants protein ferric update regulator (Fur), cAMP regulatory protein (CRP), peptidoylproline cis-trans isomerase (SlyD), acetylornithinase (ArgE), Cu/Zn-superoxide dismutase (Cu/Zn-SODM) and metal-binding lipocalin (YodA). These proteins may bind to metal-chelating resins by possession of native metal-binding sites to Ni^{2+} or Co^{2+} (Fur, YodA, Cu/Zn SODM and ArgE) or due to the presence of histidine clusters (CRP and SlyD), both binding equally as histidine residues of a histidine-tagged protein, showing that contaminant proteins may not have histidine residues at all. Probably, the low concentration of rCRT and their histidine-tag, partially hidden from protein surface, consequently led to the liaison of contaminants to the column and their elution, instead of rCRT. In order to improve rCRT purification other methods were used, and only when two washes were applied, plus the concentration of purified samples, a single band correspondent to CRT appeared in the SDS-PAGE gel (figure 16). The IMAC technique might have been associated to a second purification step. Applying two different purification principles in sequence would improve the degree of purity, because each stage separates a certain amount of contaminants, increasing its total level of removal.

Chapter 6. Conclusion

The results of this project were optimistic and represent one more step to improve ticks control, as we showed in this study that CRT can be produced and purified without contaminants, though further vaccination and immunolocalization studies will be the key to understand CRT future use.

B. bigemina has a great importance worldwide, as it provokes a disease that affects not only animals, with a great impact in cattle production, but also humans, which turns it into a public health issue and a priority disease to be eliminated or, at least, to be controlled. The first attempt to control ticks and their transmitted pathogens was using molecules that would kill them in a quick and efficient way. However, all this pressure in the parasites survival leads to one of the biggest disasters of our existence: the appearance of resistant ticks against almost all acaricides. At this point, vaccines are the future of ticks and babesiosis control, especially vaccines that have action against more than one target, either several ticks or ticks and their transmitted pathogens. Possibly, due to be highly conserved among ticks, CRT may be a component of a large-spectrum anti-tick vaccine or in a vaccine against both vector and its transmitted pathogen, but only the future will confirm that.

Chapter 7. References

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Annex I - Material and Methods Appendix

1. Mediums

1.1. Luria-Bertani (LB)-agar:

6.25g of LB and 3.75g of agar in 250ml of distilled water

1.2. Luria-Bertani

6,25g of LB in 250ml of distilled water

1.3. S.O.C.

2% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose

2. Solutions

2.1. Lysis buffer

0.3ml KH_2PO_4, 4.7ml K_2HPO_4, 2.3g NaCl, 0.75g KCl, 10ml glycerol 100% (v/v), 0.5ml Triton X-100 and 0.068mg imidazole, obtain the final volume of 100ml with water and a final pH of 7.8

2.2. Coomassie Brilliant Blue dye

40% methanol, 10% acetic acid and 0.025% Coomassie Brilliant Blue R-250

2.3. Distaining solution

30% (v/v) of ethanol, 10% of acetic acid (v/v) and 60% of water

2.4. 1X SDS-PAGE sample buffer

1.25ml of 0.5M Tris-HCl at pH 6.8, 1.0ml of glycerol 100% (v/v), 0.2ml of β-mercaptoethanol, 0.01g of bromophenol blue and 0.2g SDS, obtain the final volume of 10ml with water

2.5. Running buffer (SDS 1%)

10g of SDS, 144.1g of glycine and 30.3g of Tris to a final pH of 8.3, obtain the final volume of 100ml with water

2.6. Transfer buffer

3.1g of Tris, 14.3g of glycine and 200ml of methanol, obtain the final volume of 1L with water

2.7. TTBS

18.18g Tris, 72.05g NaCl and 11.63g NaH2PO4 · H2O with a final pH 7.4, obtain the final volume of 1L with water, dilute 1:10 and add 0.05% of Tween 20

2.8. Blockage solution

0.3g of powder milk and 100ml of TTBS

2.9. Binding buffer

20mM Na_2PO_4, 500 mM NaCl and 30mM imidazole with a final pH 7.4

2.10. Elution buffer

20mM Na_2PO_4, 500 mM NaCl and 500mM imidazole with a final pH 7.4

3. Gels

3.1. Separating and Slacking gels

	Separating gel (12.5%)	**Slacking gel (2.5%)**
Bis acrylamide	3.125ml	0.625ml
Tris HCl 3M (pH=8,8)	0.938ml	
Tris-HCl 0,5M (pH= 6,8)		1.25ml
Sodium dodecyl sulfate **(**SDS) 10% (w/v)	75 µl	50µl
Ammonium sulfate (APS)	37.5µl	25µl
Tetramethylethylenediamine (TEMED)	3.75µl	3.75µl
H_2O miliQ	2.988ml	2.825ml