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Functional genomics of *Rhipicephalus bursa – Babesia ovis* interactions towards disease control

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Resumo

Estudo funcional de genes envolvidos nas interações *Rhipicephalus bursa* - *Babesia ovis* para o controlo da doença.

Fábio David Ferreira Rodrigues

Palavras-chave: *Babesia*, Carraça, Vacina, RNA de interferência, *Lachesina*, *Vitelogenina*.

As carraças são ectoparasitas hematófagos obrigatórios de animais selvagens e domésticos, sendo o Homem um hospedeiro acidental. Enquanto em saúde humana são consideradas o segundo vetor mais importante de doenças, a seguir aos mosquitos, em saúde animal são os principais vetores. Os protozoários do género *Babesia*, responsáveis pela doença denominada babesiose, são agentes patogénicos que afetam uma ampla variedade de animais, incluindo o Homem. Apesar de a babesiose humana ser uma zoonose emergente, é na área animal que a babesiose se destaca, pois tem um grande impacto na produção animal. Nomeadamente a espécie *Babesia ovis*, transmitida principalmente por *Rhipicephalus bursa*, é altamente patogénica, particularmente em ovinos, podendo provocar febre, anemia, aborto, hemoglubinúria e até levar à morte. Atualmente, o principal método de controlo de carraças e agentes a estas associados baseia-se no uso de acaricidas, mas a vacinação é um método alternativo. O desenvolvimento de vacinas inicia-se com a identificação e caracterização de antigénios com papel essencial no desenvolvimento da carraça. Assim, o objetivo do presente estudo foi clarificar a função de genes de carraças diferenciadamente expressos em resposta à alimentação e infeção por *B. ovis*, visando o desenvolvimento de vacinas anti-carraças. Com base em estudos prévios foram identificados genes potencialmente envolvidos no processo de alimentação e infeção. Quatro destes foram selecionados para caracterização funcional utilizando a metodologia de RNA de interferência. A redução dos níveis de mRNA alvo nas carraças mostrou que a *lachesina* poderá estar envolvida no processo de infeção, uma vez que reduziu significativamente os níveis de infeção de *B. ovis*. Além disso, foi observado um efeito na fixação da carraça ao hospedeiro e aumento da mortalidade. O silenciamento de *vitelogenina-3* e do gene codificante para uma proteína de cimento demonstrou que ambos poderão estar associados ao processo de alimentação. Vários estudos têm caracterizado a interface carraça-patogénio a nível molecular. Porém, este é o primeiro estudo de genómica funcional em *R. bursa* em resposta à infeção por *B. ovis*. Os resultados obtidos permitem avaliar o interesse destes genes como potenciais candidatos a vacinas.

Abstract

Functional genomics of *Rhipicephalus bursa* - *Babesia ovis* towards disease control.

Fábio David Ferreira Rodrigues

Keywords: *Babesia*, Tick, Vaccine, RNA interference, *Lachesin*, *Vitellogenin*.

Ticks are obligate hematophagous ectoparasites of wild and domestic animals, whereas is Man an accidental host. While in human health are considered the second most important vector of diseases, after mosquitoes, in animal health are the main vectors. Protozoans of the genus *Babesia*, responsible for the disease called babesiosis, are pathogens that affect a wide variety of animals, including Man. Although human babesiosis is an emerging zoonosis, this disease has its greatest impact on animal production. Namely *Babesia ovis*, mainly transmitted by *Rhipicephalus bursa*, is highly pathogenic, particularly in sheep, and can cause fever, anemia, abortion, hemoglobinuria and even lead to death. Currently, the principal TTBD control method is based on the use of acaricides, nevertheless vaccination is an alternative method. Vaccine development begins with the identification and characterization of antigens that have an essential role in tick development. Therefore, the goal of this study was to clarify the function of genes differentially expressed in response to blood-feeding and infection by *B. ovis*, aiming the development of anti-tick vaccines. Based on previous studies, genes potentially involved in tick feeding and infection processes were identified. Four of these were selected for functional characterization using the RNA interference methodology. Reduction of target mRNA levels showed that *lachesin* may be involved in the infection process, since it significantly reduced the infection levels of *B. ovis*. Furthermore, it was observed an effect on tick attachment to the host and increased mortality. Silencing of *vitellogenin-3* and the gene coding for a cement protein demonstrated that both may be associated to tick feeding. Several studies have characterized the tick-pathogen interface at the molecular level. However, this is the first functional genomics study in *R. bursa* in response to infection by *B. ovis*. The results obtained allow assessing the interest of these genes as potential vaccine candidates.

Abbreviations

µL	Microliter
µM	Micro molar
ºC	Degree Celsius
64TRP	64 tick recombinant protein
AGE	Agarose Gel Electrophoresis
AN	Avogadro's number
ATP	Adenosine triphosphate
Ave	Average
bp	Base pair
cDNA	Complementary DNA
Cq	Quantification cycle
CRT	Calreticulin
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
e.g.	*exempli gratia*
ELF	Elongation factor
ELI	Expression library immunization
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
g	gram
g	G-force
gDNA	Genomic DNA
GRP	Glycine-rich protein
ICT	Immunochromatographic test
IFAT	Immunoflorescence antibody test
kDa	Kilo Dalton
Kg	Kilo gram

Lac	Lachesin
Mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
Mw	Molecular weight
nl	Nanolitre
LAMP	Loop-Mediated Isothermal PCR
OTEs	Off-target effects
OVs	Ovaries
PBS	Phosphate buffered saline
PCR	Polymorphism chain reaction
qPCR	Quantitative PCR
rCRT	Recombinant calreticulin
rDNA	Ribosomal DNA
RISC	RNA-induced silencing complex
RLB	Reverse line blot
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA sequencing
RT-PCR	Reverse transcriptase polymerase chain reaction
RTT	Replication-Transcription-Translation
s	Second
S.D.	*Standard* deviation
SGs	Salivary glands
siRNAs	Small interfering RNAs
SNPs	Single nucleotide polymorphisms

spp.	Species (Plural)
s.s.	*sensu stricto*
SSH	Suppression-Subtractive Hybridization
TBD	Tick borne diseases
TBE	Tris/Borate/EDTA
TBEV	Tick-borne encephalitis virus
TNF-α	Tumor necrosis factor α
Tris-HCl	Tris hydrochloride
TROSPA	Tick receptor for outer surface protein A
TTBD	Tick and tick borne diseases
UV	Ultraviolet
V	Volt
Vg	Vitellogenin
VgR	Vitellogenin receptor
Vn	Vitellin
w/v	Weight per volume

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1 Introduction

1.1 Ovine babesiosis

1.1.1 Background

Tick-borne protozoan parasites of the phylum Apicomplexa such as *Babesia* spp. are important pathogenic agents with a major impact in animal health leading to substantial financial losses especially regarding livestock (Domingos et al., 2013; Guerrero et al., 2012a; Erster et al., 2015b). In addition, some of these organisms can infect humans increasing the risks posed by ticks and tick-borne diseases (TTBD). The first documented case of human babesiosis occurred in 1956 near Zagreb, Croatia, when a splenectomized farmer was diagnosed with a *B. divergens* infection (Skrabalo & Deanovic, 1957) ever since, babesiosis came into view as a potentially life threatening zoonotic infection in humans (Herwaldt et al., 2003; Homer et al., 2000; Hunfeld et al., 2008). Notably, *Babesia* spp. can infect three host groups: domestic animals, humans and, most recently acknowledged, some wildlife species make of this pathogen one of the most important protozoan transmitted by ticks (Schnittger et al., 2012).

B. ovis is responsible for ovine babesiosis which as the name suggests is mainly associated with small ruminants. This protozoan species represents the principal ethiological agent of the disease, although other species like *B. motasi, B. crassa, B. taylori, B. foliata,* among others have been described (Guan et al., 2008; Erster et al., 2015a; Ranjbar-Bahadori et al., 2012). Ovine babesiosis is widespread in Eastern Asia, Iran, the Mediterranean basin and North Africa (Erster et al., 2015a; Ranjbar-Bahadori et al., 2012; Rjeibi et al., 2014; Sevinc et al., 2013; Uilenberg, 2006), being considered of great economic importance to the livestock industry, due to animal mortality, yield losses and costs of treatment (Sevinc et al., 2015; Ranjbar-Bahadori et al., 2012). The principal vector of *B. ovis* is the tick species *Rhipicephalus bursa*, whose distribution ranges from Asia to Africa, across the Mediterranean region (Erster et al., 2015a; Ranjbar-Bahadori et al., 2012). *R. bursa* is a two-host tick, feeding on a varied range of hosts, comprising hares, dogs and humans, as well as several species of ungulates (Erster et al., 2015; Yeruham et al., 1996). Like other pathogens, *B. ovis* displays transstadial and transovarial transmission, which means that the infection sustains tick moulting and is successfully passed to progeny (Razmi & Nouroozi, 2010). Outbreaks of ovine babesiosis were described mostly from April to July, corresponding with the seasonality of the adult *R. bursa* activity (Erster et al., 2015a; Yeruham et al., 2000, 2001).

1.1.2 Historical overview

It was at the end of the 19th century that Babes discovered microorganisms in erythrocytes of cattle in Rumania and associated them with bovine hemoglobinuria or red water fever (Babes, 1888). Afterwards, the same author found similar organisms in sheep red blood cells. This seems to have been the first report of the transmission of a protozoan parasite by an arthropod. In 1893, Starcovici named these parasites as *Babesia bovis, Babesia ovis* and *Babesia bigemina* (Starcovici, 1893).

1.1.3 Hosts

As other protozoa pathogens, also *Babesia* spp. needs to interact with two different hosts to complete its life cycle: a vertebrate host and an arthropod, namely a tick. Initially, *Babesia* spp. was considered to be specific to a given vertebrate host but with the subsequent development of molecular tools, some *Babesia* species have been shown to have a wider vertebrate host range than thought before. *B. bovis* and *B. bigemina,* primarily described as pathogens of cattle in tropical and sub-tropical areas, were both identified by specific serology and PCR in white-tailed deer (*Odocoileus virginianus*) in northern Mexico (Cantu et al., 2007; Chauvin et al., 2009; Mosqueda et al., 2012). *B. divergens* represents another parasite of cattle in temperate climates. Yet, it is capable to infect humans with a special impact in immunocompromised individuals (Cantu et al., 2007), primates (chimpanzees and rhesus monkeys) (Garnham & Bray, 1959), ungulates (roe deer, fallow deer, red deer, mouflon and sheep) (Penzhorn, 2006), and rodents (rat) (Ben Musa & Phillips, 1991) as well as reindeer (Zintl et al., 2011), sheep (Malandrin et al., 2009) and gerbils (Lewis & Williams, 1979).

1.1.4 Vectors

The main experimental and biological vector of babesiosis in sheep is the tick *Rhipicephalus bursa* Canestrini and Fanzago, 1877 (Erster et al., 2015a; Erster et al., 2015b; Ferrolho et al., 2016).

R. bursa is a common ectoparasite of sheep and goat, although has also been documented in equines, cattle, dogs, gazelles and hares (Yeruham et al., 2000) and is widespread in the north hemisphere, being particularly frequent in Mediterranean basin and central-western Asia (Erster et al., 2015a; Ferrolho et al., 2016; Rjeibi et al., 2014; Sevinc et al., 2013; Uilenberg, 2006). This tick is also known to act as vector of *B. bigemina* and *B. bovis,* the agents of bovine babesiosis, *Theileria ovis, T. equi* and *T. annulata*, etiological agents of theileriosis, *Anaplasma marginale* and *A. ovis*, agents of anaplasmosis, and *Ehrlichia canis*, responsible for canine monocytic ehrlichiosis (de la Fuente et al., 2008; Uilenberg, 2006; Dahmani et al., 2016; Ferrolho et al., 2016; Masala et al., 2012). Other diseases have been associated with this tick, for example, Crimean Congo haemorrhagic fever virus (Gargili et al., 2011; Papadopoulos & Koptopoulos, 1980).

R. bursa is a two-host tick, though immature stages are commonly found in the same host as adults (Yeruham et al., 1996). As described previously, *R. bursa* can act as vector of many pathogens,

but is mostly associated to the transmission of *B. ovis.* Like in all *Babesia*l infections, once infected with *B. ovis, R. bursa* ticks remain infected during the course of their life cycle and transmit the parasite to the progeny, resulting in the emergence of infected larvae, nymphs and adults capable of infect susceptible hosts during feeding. Besides this transstadial transmission, *Babesia* spp. also presents transovarial transmission (Erster et al., 2015a; Razmi & Nouroozi, 2010). Transovarial transmission is considered a *Babesia* spp. adaptation for long-lasting persistence by the fact that some ticks remain infected and infective for many generations without needing to feed on infected animals again, thus increasing transmission efficiency (Chauvin et al., 2009). *Babesia* spp. are usually divided into large and small forms such as *B. ovis* and *B. microti*, respectively. The large *Babesia*, also named as *Babesia* sensu stricto (*s.s.*), differ from small *Babesia* by their susceptibility to anti-*Babesia* drugs (Gray & Pudney, 1999) and by their life cycles, principally the occurrence of transovarial transmission (Hunfeld et al., 2008; Uilenberg, 2006).

Using light microscopy, Weber and Friedhoff (1971) showed the development of *B. ovis* in *R. bursa* and could characterize the differentiated merozoites in the salivary glands (SGs) of female ticks (Weber & Friedhoff, 1971). Later, a study by Moltmann et al. (1982) using electron microscopy has determined the development of *B. ovis* in the SGs of *R. bursa*. *R. sanguineus* and *R. turanicus* were reported that they could act as a vector of *B. ovis* as well (Moltmann et al., 1982; Razmi et al., 2002; Shayan et al., 2007). A study in Iran demonstrates that *B. ovis* DNA was found not just in *R. bursa* ticks but also in *R. sanguineus* and *R. turanicus* (Shayan et al., 2007). *Hyalomma marginatum* is also known to act like a vector of *B. ovis* in cattle (Razmi & Nouroozi, 2010; Razmi et al., 2002; Taylor, 2015).

1.1.5 *Babesia* spp. life cycle

As referred previously, *Babesia* spp. life cycle takes place in two hosts, vector and mammalian host, and sexual and asexual reproduction proceeds through three stages, in which gamogony – sexual development with formation and fusion of gametes inside the tick gut, sporogony – asexual reproduction in tick SGs, and merogony – asexual reproduction in the vertebrate host (Mehlhorn & Piekarski, 2002).

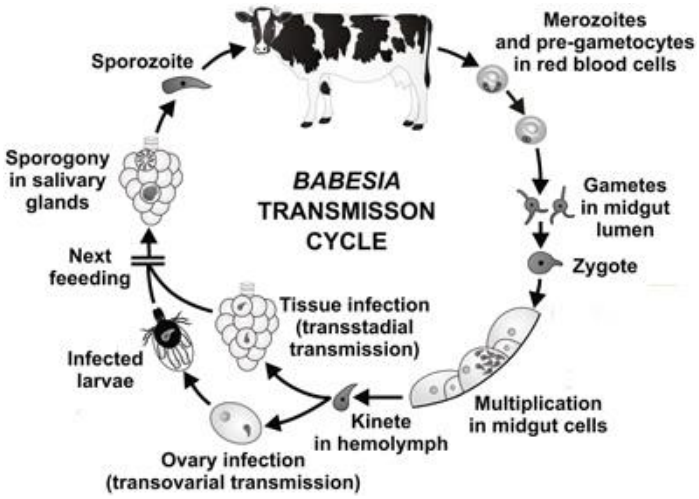

Figure 1.1: The transmission cycle of *Babesia* spp. in cattle (adapted from Hajdušek et al., 2013).

1.1.5.1 *Babesia* spp. development in the vertebrate host

Babesia spp. that belong to sensu stricto groups invade the host by injection of sporozoites with the saliva of the infected tick larvae, nymph or adult (Mehlhorn & Piekarski, 2002). Once in circulation sporozoite penetrate erythrocyte's cell membranes with the aid of a specialized apical complex forming a parasitophorous vacuole (Moltmann et al., 1982; Suarez & Noh, 2011; Yokoyama et al., 2006). The parasite is left with the defining piroplasm feature of a single membrane by the gradual disintegration of the vacuole membrane, contrasting to *Plasmodium* species that invade by a similar mode but retain the host membrane in addition to its own (Homer et al., 2000). Sporozoite transforms into trophozoite by binary fission, from which two merozoites develop by merogony. These last structures lyse the cell and continue to infect more erythrocytes. Rapid reproduction destroys the host cell and results in hemoglobinuria in the host. Different divisional stages can be observed in the bloodstream at the same time due to the asynchronous multiplication of the parasite (Chauvin et al., 2009). Some trophozoites develop into a diploid ovoid type of merozoite, named gamont precursor, which do not develop further until they are taken up by the tick in the blood meal later on, when in the tick gut, even before to leaving the erythrocytes, these precursors develop into gametocytes (Chauvin et al., 2009; Hildebrandt et al., 2013; Homer et al., 2000; Hunfeld et al., 2008; Mackenstedt et al., 1995).

1.1.5.2 *Babesia* spp. development in the vector

Once in the arthropod vector, most of the parasites degenerate and are destroyed when *Babesia*-infected erythrocytes are ingested by ticks. Nevertheless, in the passage from host blood to the midgut of the tick there are environmental changes that stimulate the development of "pre-gametocytes" into elongated bodies, with arrowhead-shaped ray appearance, believed to be gamonts, the ray bodies ("strahlenkorper") (Chauvin et al., 2009; Hildebrandt et al., 2013; Mackenstedt et al.,

1995; Schnittger et al., 2012). The ray bodies go through further multiplication inside the infected erythrocyte, leading to the formation of large aggregations of multinucleated ray bodies. Once gametogenesis is accomplished and after digestion of the consumed erythrocyte, single-nucleated and haploid gametes emerge from the aggregates (Mackenstedt et al., 1995). The elongated zygote is formed by the fusion of gametes in the lumen of tick's digestive tract. When the zygote reaches the midgut cell membrane invaginates at the point of contact, apparently due to the action of enzymes released by the invading parasite and no parasitophorous membrane is formed (Chauvin et al., 2009). At some moment, the zygote undergoes one-step meiosis to form a haploid zygote (Mackenstedt et al., 1995) and motile kinetes are formed (primary schizogony). These organisms escape the midgut epithelium into the haemolymph and infect a variety of cell types and tissues, including ovaries (OVs) where successive cycles of secondary schizogony occur (Chauvin et al., 2009; Homer et al., 2000; Mackenstedt et al., 1995). Hence, transovarial transmission succeeds with further development taking place in tick larvae. Sporogony occurs at each tick stage and the *Babesia* spp. infection acquired during one life stage is passed on to the next (transtadial transmission). Kinetes are transformed into multinucleated stages in the SGs and break up to form sporozoites (Mackenstedt et al., 1995). A recent study showed an inefficient transmission of the parasite by immature tick stages that indicates that the transmission of *B. ovis* by *R. bursa* occurs mainly by the adult stage (Erster et al., 2015a), which is in agreement with previous reports on the seasonality of ovine babesiosis, describing that the outbreak of the disease corresponds to the emergence of adult *R. bursa* (Erster et al., 2015a; Yeruham et al., 1998a).

1.1.6 Pathogenesis and clinical signs

After *Babesia* spp. transmission to the vertebrate host, the pathogenesis of babesiosis consists of an incubation period of between 1 week and 6 weeks followed sequentially by acute, subclinical, and in some cases chronic phases (Conrad et al., 1991; Figueroa et al., 1992; Homer et al., 2000).

A few studies suggest that the pathogenesis is possibly related to an excessive immunological reaction of the vertebrate host to the *Babesia* agent (Hemmer et al., 2000; Hunfeld et al., 2008; Telford III & Maguire, 2006). Studies in a mouse model of *B. microti* infection demonstrated that T-cell receptor-deficient mice are easily infected in comparing with B-cell receptor-deficient mice (Hunfeld et al., 2008; Telford III & Maguire, 2006). Immunological studies on mice also reveal an important role of CD4+ T cells in controlling parasitemia (Hemmer et al., 2000; Hunfeld et al., 2008). These data are in agreement with the known difficulties of depressed cellular immunity individuals to control persistent parasitemia (Telford III & Maguire, 2006; Haselbarth et al., 2007; Hunfeld et al., 2008; Hildebrandt et al., 2008). In the same way, reduction of host macrophages and natural killer cells increases susceptibility to infection (Hunfeld et al., 2008). A devastating production of pro-inflammatory cytokines such as TNF-α and interferon-Y in animal studies with *B. duncani* propose that the pathobiology principally results from the host response and not from the parasite itself. In

human cases, symptoms occur at parasitemias of less than 1% and experiments on several *Babesia* spp. suggest that an excessive host immune response is an important pathogenetic cofactor for severe babesiosis (Hunfeld et al., 2008; Gray & Weiss, 2008). The acute phase generally runs a course of one week, in which mild and non-specific signs are described, such as fever, loss of appetite, tachycardia, dyspnea, icterus, hemoglobinuria and hemolytic anemia, with lymphadenopathy, splenomegaly and hemorrhagic tendencies in worst cases, which eventually might lead to death (Conrad et al., 1991; Yeruham et al., 1998a; Yeruham et al., 1998b). Mortality rates in susceptible hosts range from 30% to 50% after field infections with *B. ovis* (Aktas et al., 2005; Hashemi-Fesharki, 1997). Whereas *B. ovis* infections of young animals are not usually followed by clinical signs, primary exposure of adult sheep and goats to this parasite may lead to clinical symptoms of the disease (Yeruham et al., 1998b; Carletti et al., 2015; Suarez & Noh, 2011). The pathogenicity of *B. ovis* strains are directly related to erythrocyte destruction. In the case of strains of *B. bovis*, the ethiological agent of bovine babesiosis, hemolysis involves the release of many pharmacologically active agents like proteolytic enzymes, which affect microcirculation by vasodilatation and increased permeability, leading to hypotension and edema, and affect blood viscosity, coagulation and cytoadherence, resulting in ischemia. Central nervous system complications due to adhesion of parasitized erythrocytes in brain capillaries can occur with *B. bovis* infections (Mosqueda et al., 2012; Seifert, 1996). Afterwards, babesial infections may continue after spontaneous clinical recovery or ineffective treatment, and such animals may enter the subclinical phase of babesiosis with no clinical signs. Consequently, clinically healthy sheep in the subclinical phase of babesiosis are carriers of the parasite for years without developing clinical disease, during which time tick vectors could still acquire and spread the pathogen to other hosts (Buling et al., 2007; Conrad et al., 1991; Homer et al., 2000). For an unknown reason, certain animals will progress to the chronic phase of babesiosis, which can be absent of clinical signs for years due to complete cure or more often associated with the persistence of small numbers of parasites, being consequently considered natural reservoirs of *Babesia* spp. (Homer et al., 2000). Chronically infected animals maintain elevated antibody titers, and some can develop signs of other chronic diseases, such as liver disease (Conrad et al., 1991; Homer et al., 2000).

Babesiosis is a multisystemic disease and several factors may contribute to its severity, such as pathogenicity of different strains, host age, immunocompetence and co-infections with other pathogenic agents (Homer et al., 2000; Marathe et al., 2005).

1.1.7 Diagnosis

The diagnosis of babesiosis should begin with a descriptive history, which might include clinical manifestations, history of travel to an area where it is endemic, tick bite, or exposure to a tick-infested area, recent blood transfusion and splenectomy (Homer et al., 2000).

Clinical cases of babesiosis can be detected by microscopy, immunological assays or using molecular detection methods (Mosqueda et al., 2012). Relatively to the first group, blood smears can

be dyed by staining with Giemsa or acridine orange. Thin blood films are prepared from capillary blood, since blood of general circulation may contain fewer parasites due to sequestration of infected erythrocytes in capillaries of brain or other organs (Böse et al., 1995). For low levels of parasitemia, diagnosis is carried on by thick smears of infected blood stained with Giemsa (Mosqueda et al., 2012). The advantage of the thick smear consists in a large amount of erythrocytes analyzed in a reduced space. Hence, the probability of finding infected cells is higher than in a thin smear. Such methods are inexpensive and portable, nevertheless, accuracy of diagnosis depend on the skills of the microscopist (Mosqueda et al., 2012).

Some immunological tests have been described for *Babesia* spp. detection, as the indirect immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) and the immunochromatographic test (ICT), being all based on the recognition of parasite antigens by serum antibodies in the blood of the tested animal. ELISA includes the use of recombinant antigens and monoclonal antibodies, increasing specificity and decreasing unspecific binding and signal (Goff et al., 2008; Mosqueda et al., 2012). The ICT is a quick diagnostic device that detects antibodies against a specific antigen in a small amount of serum by means of specific antibody and a recombinant antigen both imbued on a nitrocellulose membrane-based test strip (Weigl et al., 2008). Since it is very easy to perform and read, does not require a trained technician, can be implemented in the field and is inexpensive (Mosqueda et al., 2012). The main disadvantage of the immunological tests consists in the relying on the presence of specific antibodies against parasites and that may take days or weeks to develop in an infected animal (Mosqueda et al., 2012).

The molecular diagnosis methods can distinguish active infections by detection and amplification of pathogen DNA (PCR based assays). Since the improvement of the sensitivity of PCR based techniques, many methods for the detection and differentiation of babesiosis infections have been described, among them nested PCR (Figueroa et al., 1993), reverse line blot (RLB) hybridization (Schouls et al., 1999), LAMP (Loop-Mediated Isothermal PCR) (Iseki et al., 2007) and real time PCR (Buling et al., 2007; Criado-Fornelio et al., 2009). Due to factors like costs, contaminations and validation, none of these methods is globally used, in spite of the advantages of these techniques concerning to sensitivity. There are some studies that used PCR to diagnose *B. ovis* and this technique demonstrates to be specific and sensitive in detecting the pathogen (Aktaş et al., 2005; Shahzad et al., 2013).

1.1.8 Babesiosis treatment

Due to its implications in animal production and in public health, babesiosis control is crucial (Bock et al., 2004). Currently, due to the introduction of exotic breeds, which typically do not display natural immunity against *Babesia* spp., babesiosis control is even more important (Graf et al., 2004). Disease control can be assured either by tick management, immunization, anti-*Babesia* drugs administration or by a combination of these approaches (Suarez & Noh, 2011).

Chemotherapy is usually effective against ovine babesiosis and several chemical compounds have been reported to be active against *Babesia* parasites (Vial & Gorenflot, 2006). An early diagnosis and the rapid administration of drugs are factors that contribute to a successful treatment. Present treatments afford protection from disease but normally permit an appropriate level of infection (low level parasitemias) in order to develop immunity which is important in babesiosis endemic areas. Only a few *Babesia*cides are available commercially, being diminazene aceturate and imidocarb dipropionate the most used:

Diminazene aceturate – In Pakistan, chemotherapy against babesiosis was studied (Rashid et al., 2010). Diminazene® was administered to a group of sheep at the dose of 3.5 mg/kg body weight and showed 80% efficacy at day 10 post-medication. These results agree with the study of Baby and his colleagues, who treated simultaneous babesiosis and anaplasmosis in goat with diminazene (Baby et al., 2001). Equivalent results were also found by Cordoves & Polanco (1983), Simitch et al. (1956), Aliu & Odegaard (1985), Mohamed & Yagoub (1990) and Manget (1983), who obtained an acceptable effect of diminazene against babesiosis.

Imidocarb dipropianate – used subcutaneously at a dose of 1.2 mg/kg for treatment or at a dose of 3 mg/kg for chemoprophylactic use will prevent babesiosis (Vial & Gorenflot, 2006). Several studies have presented that imidocarb is retained in comestible tissues of ruminants for long periods after treatment (McHardy et al., 1986; Mosqueda et al., 2012; Suarez & Noh, 2011). High doses of this drug completely eliminate parasites, leaving the animals susceptible to reinfection and for this motive reduced drug levels are sometimes designated (Bock et al., 2004; Vial & Gorenflot, 2006), particularly in endemic areas where the development of protective immunity is desired. In other hand, the use of reduced drug doses increases the risk of resistance acquisition against the drug by the extensive use (Rodriguez & Trees, 1996). Rashid et al. (2010) also studied the effect of imidocarb dipropianate in treatment of babesiosis in sheep. Imizol® was administered to a group of sheep at the dose rate of 2 mg/kg body weight. The efficacy was 60% at day 3, 90% at day 7 and 100% at day 10 post-medication. Ramin (2000) and McHardy et al. (1986) have found similar results, by recording 97.28% and 100% imidocarb efficacy.

1.1.9 Zoonotic risk

Although recognized as an animal disease, more attention is being given to babesiosis as a worldwide emerging zoonosis due to the increase of reports of human cases. The rodent parasite *B. microti* and the cattle parasite *B. divergens* are the most commonly implicated species in North America and Europe, respectively. Cases reported in splenectomized or otherwise immunocompromised individuals are often fatal (Herwaldt et al., 2003, 2004).

The first human case of babesiosis was identified in 1957 near Zagreb, Croatia (Skrabalo & Deanovic, 1957). A young farmer had been grazing cattle on tick-infested pastures and presented with fever, anemia and hemoglobinuria. He was asplenic and died of renal insufficiency during the second

week of illness. Firstly, described as *B. bovis*, the agent most likely was *B. divergens*. In 1968, *B. divergens* was confirmed as the etiologic agent in a splenectomized person infected while vacationing in the Irish countryside (Fitzpatrick et al., 1968; Vannier et al., 2008). Primarily detected in Europe and North America, human babesiosis is now described worldwide.

Over the past 50 years, the epidemiology of the human babesiosis has changed from a few isolated cases to the establishment of endemic areas in southern New England, New York, and the north central Midwest. Human babesiosis due to *B. microti* has been reported in Connecticut, Massachusetts, Minnesota, New Jersey, New York, Rhode Island, and Wisconsin (Esernio-Jenssen et al., 1987; Meldrum et al., 1992; Spielman, 1988; Spielman et al., 1981; Spielman et al., 1979; Steketee et al., 1985; Western et al., 1970; Spielman et al., 1985; Eskow et al., 1999; Herwaldt et al., 2002; Krause et al., 1991). Moderately severe illness caused by *B. duncani* occurred in Washington state and California (Conrad et al., 2006; Persing et al., 1995). Cases of *B. divergens*-like infection have been reported from Missouri (Herwaldt et al., 1996), Kentucky (Beattie et al., 2002), and Washington state (Herwaldt et al., 2004). In Europe, *B. divergens, B. microti,* and *B.* EU1, an etiological agent of babesiosis found in ticks from Slovenia (Duh et al., 2005), have been reported to cause babesiosis in humans and are thought to be transmitted by *Ixodes ricinus* (Herwaldt et al., 2003; Hildebrandt et al., 2007). In Asia, babesiosis has been reported in Japan (*B. microti*-like) (Wei et al., 2001), Korea (KO1) (Kim et al., 2007), Taiwan (TW1) (Shih et al., 1997), and India (Marathe et al., 2005). Human babesiosis also has been reported in Africa (Bush et al., 1990) and South America (Ríos et al., 2003).

The disease manifestations are similar to the other types of babesiosis (Benach & Habicht, 1981; Persing et al., 1995). The cases due to *B. divergens* infections seen in Europe are usually more severe than those caused by *B. microti*. Onset of disease symptoms usually occurs within 1 to 3 weeks of the infecting tick bite (Homer et al., 2000; Hunfeld et al., 2008; Leiby, 2006). In splenectomized patients, illness appears suddenly, with hemoglobinuria followed by jaundice due to severe hemolysis. In the most severe cases, patients show renal failure and pulmonary edema (Homer et al., 2000; Vial & Gorenflot, 2006).

1.2 Tick vector

1.2.1 Classification

Ticks belong to phylum Arthropoda, subphylum Chelicerata, class Arachnida, subclass Acari, superorder Parasitiformes, order Ixodida and superfamily Ixodoidea. There are three families of ticks, in which Argasidae or soft ticks with 193 species, Ixodidae or hard ticks with 702 species and, with only one species, Nuttalliellidae (Brites-Neto et al., 2015). The most remarkable difference between the two most representative tick families is the presence of a hard sclerotized shield or scutum on the anterior dorsal surface of hard ticks, which is absent in soft ticks. There are other dissimilarities, like the aspect of the outer body wall or integument that is rough on soft ticks while smooth with fine

grooves in hard ticks, and the position of mouthparts, which are located ventrally in soft ticks and anterior in hard ticks, making them visible from a dorsal view. Nuttalliellidae family is considered the most ancestral lineage of ticks, sharing features characteristic of both Argasidae and Ixodidae (Klompen et al., 2007; Mans et al., 2011). The largest family of ticks can be divided in Prostriata, which is considered as the most basal line and can copulate either on or off the host, aggregating only the *Ixodes* genus, in contrast with Metastriata that can mate only on the host (Barker & Murrell, 2008).

Figure 1.2: Tick examples. (A) dorsal (left) and ventral (right) view of an *Rhipicephalus annulatus* female, representative of a hard tick species (*original and authorized from Sandra Antunes)*. (B) dorsal (left) and ventral (right) view of an *Ornithodoros savignyi* with eggs, representative of a soft tick species (*original and authorized from Ard Nijhof)*.

1.2.2 Life cycle

Ticks go through four stages, specifically egg, larvae, nymph and adult (Oliver, 1989; Sonenshine & Roe, 2014). Hard ticks only have one nymph instar, differing to the several nymphal instars of soft ticks (Oliver, 1989). Ixodid ticks require some days to feed and after the female is engorged falls from the host to lay thousands of eggs and then dies. Argasid ticks may feed for several times and intermittently in their lifetime and lay few hundreds of eggs in batches on different hosts because these parasites don't remain attached to the hosts. These last have a huge longevity living for many years and may tolerate long periods of starvation (Sonenshine & Roe, 2014). Relatively to Ixodid ticks, larval, nymphal and adult feeding normally requires 3-7, 4-8 and 7-9 days, respectively. Through this time, occurs the growth of gut and cuticle in order to accommodate the blood meal, mostly acquired in the last 24 hours of engorgement. Male hard ticks feed intermittently, since small quantities of blood are enough to mature reproductive organs. As soon as genus *Ixodes* male ticks moult from the nymphal stage, they have already active reproductive organs and do not need to feed. Resulting of many factors of nature such as photoperiods, temperature, humidity and availability of appropriate hosts, the length of life cycles is variable. In colder regions, ticks can take until three years to complete their life cycle, being one generation a year the usual pattern for most ticks in warmer regions (Oliver, 1989; Sonenshine & Roe, 2014).

1.2.3 Tick-host interactions

Ixodid ticks can be three-, two-, or one-host arthropods. Regarding the two-host ticks, larvae attach to the host and when full of blood they hatch and nymphs reattach feeding again until repletion. Nymphs drop from the host and, after some days, adults hatch and search for a new host to complete the life cycle. Under certain conditions, ticks can use one or two hosts or use two instead of three (Oliver, 1989), demonstrating some flexibility in feeding behavior. There are ticks that accept an

extensive variety of host species, other might be more selective and other attach to only one host species.

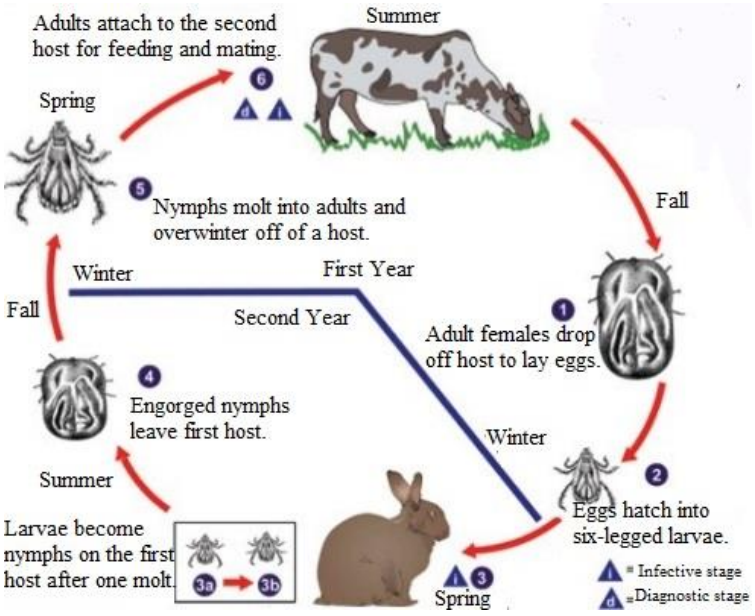

Figure 1.3: Characteristic Ixodid two-host life cycle. (Adapted from: http://www.cdc.gov/ accessed in 20 July 2016).

1.2.4 Tick anatomy and physiology

Ticks body is externally divided in two main parts: the anterior capitulum or gnathosoma containing the head and mouthparts and the posterior idiosoma that contains the legs, digestive tract and reproductive organs. Whole tick body is covered by cuticle that works as an exoskeleton, like in other arthropods. The exterior part of cuticle, termed procuticle, is sclerotized in certain parts and forms sclerites. The biggest sclerite, scutum, covers the anterior part of the body and protects the dorsal side of it. Cuticle major components are proteins and chitin, whereas lipids represent a minor part (Sonenshine & Roe, 2014).

Within ticks body there are different organs surrounded by hemolymph, including the midgut, SGs and the ovary, which are organs that can be easily detected upon dissection of engorged female ticks.

The midgut is the most notable organ in the tick body and is divided into an anterior and a post-ventricular region, lined by a simple pseudo-statified epithelium composed of cells with diverse classifications and functions (Coons & Alberti, 1999). Throughout feeding, almost all body cavity of the tick is occupied by it and his branches are for storage. Unlike in insects, the digestion in ticks is an intracellular process, except the intraluminal digestion of erythrocytes (Coons & Alberti, 1999; Sonenshine & Roe, 2014). At a structural level, midgut's cells of Ixodidae ticks are complex by having different organelles and many cytoplasmic inclusions and that reflects the multifunctional activity of the midgut (Caperucci et al., 2010).

SGs accomplish a range of complex functions that are essential to tick survival as well as for

the development and transmission of tick-borne pathogens, designed transstadial transmission (Sonenshine & Roe, 2014). The pair of SGs is located in the lateral regions of the body cavity in both Argasid and Ixodid ticks, and was described as grape-like (alveolar structures) clusters composed of the granular and agranular acini. The saliva is drained by a system of small secondary ducts to the main duct towards the opening in the mouthpart (Sonenshine & Roe, 2014). Previously to feeding, SGs are crucial in water balance regulation, during attachment and feeding are responsible for cement proteins secretion as well as other molecules transported by saliva (Sonenshine & Roe, 2014). During feeding SGs expand several times and once females fully engorge suffer degeneration and transformation processes that are under hormonal regulation (L'Amoreaux et al., 2003).

Female reproductive system consists of a single U-shaped ovary, which is found in the posterior region of the body and is responsible for transovarial transmission in some pathogens. In the unfed females the ovary is thin and small otherwise in fed females it's a big organ with a tube-like structure of luminal epithelium and developing oocytes connected with an epithelium by a short hollow stalk called funiculus (Sonenshine & Roe, 2014).

The sequential life cycle stages of *Babesia* spp. occur in different sections of ixodid ticks. So, these pathogens have to cross barriers like midgut and salivary gland epithelium, and, in ticks that transmit these parasites transovarially, also need to cross ovary epithelium (Florin-Christensen & Schnittger, 2009).

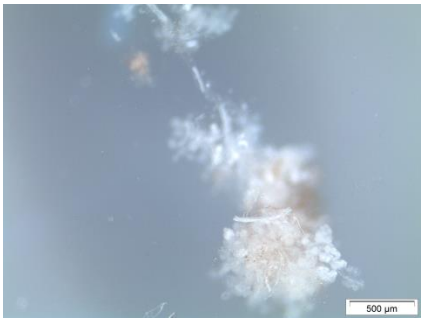

Figure 1.4: Dissected SGs from *Rhipicephalus annulatus*. (*original and authorized from Sandra Antunes).*

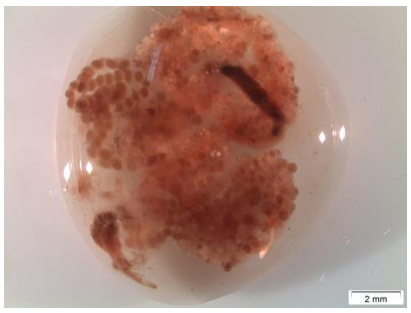

Figure 1.5: Dissected ovaries from *Rhipicephalus annulatus*. *(original and authorized from Sandra Antunes).*

Ticks represents one of the most important groups of arthropod vectors of pathogens worldwide and are considered obligate, bloodsucking, nonpermanent ectoparasitic arthropods that feed on all animals except fish (Schwan, 2011).

Although ticks are considered zoophilic, several species can be related to the transmission of agents to humans, making these last accidental hosts (Silva et al., 2006). In order to qualify as a vector, a tick must feed on infectious vertebrates, acquire the pathogen during the blood meal, keep the pathogen through one or more stages of life-cycle and has to be able to transmit the pathogen to other unexposed hosts while feeding again (Estrada-Peña et al., 2013; Jongejan & Uilenberg, 2004).

1.3 Tick control

Tick control is fundamental to reduce impact on livestock productivity and also to contract tick-borne diseases occurrence, including control measures predominantly based on the application of acaricides, however, other methods such as vaccination has been applied (Willadsen, 2006).

1.3.1 Chemical tick control – acaricides

Up to now, the use of acaricides has been a main factor of an integrate tick control measures. There are several 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 (George et al., 2004; Latif & Walker, 2004). Owing to limitations like contamination problems, ineffective issues or resistance arising, some acaricides were withdrawn from the market unless others are still accessible. Acaricides can lead to residual effects in milk and meat products, as well as in the environment. Acaricides application strategies are frequently hard to preserve and consequently, tend to be improperly used, being responsible for acaricide-resistant ticks increasing (George et al., 2004; Graf et al., 2004). This resistance is associated with mutations in genes related to drug susceptibility, like detoxificating enzymes, like esterases, glutathione-S-transferases and mono-oxidases, and due to genetic drift (Guerrero et al., 2012a). Combinations of acaricides have been used globally, which products combine active components, in order to exploit a diverse number of mechanisms of action, aiming the reduction of insecticide resistance (Veiga et al., 2012).

The public awareness of the damaging effects of pesticides on the environment and increasing concerns about resistance of insecticides, demands the need of discovering new methodologies in tick control. Besides, the introduction in the market of a new acaricide is time-consuming and has a massive economic burden (Graf et al., 2004).

1.3.2 Alternative methods in tick control - Vaccines

Ticks have relatively few natural enemies, although the use of predators, parasites and pathogens has been studied aiming tick control (Miranda-Miranda et al., 2011). Tick control strategies could be based on interference with tick bacteria endosymbionts, which are essential to arthropod survival (Ghosh et al., 2007). Other approach is the application of entomopathogenic fungi that have been reported to attack and kill ticks. These organisms have been applied in field trials with moderate success and commercial products have been developed (Samish et al., 2004; Stafford & Allan, 2010).

Other measure in controlling the tick vector is the genetic control, in which consists in the release of sterilized ticks into the environment, identical to sterile insect techniques developed for the control of pests. Ticks can be sterilized through hybridization (Hilburn et al., 1991), treatment with chemicals (Hayes & Oliver, 1981) or by RNA interference (RNAi) (de la Fuente et al., 2006b; Merino et al., 2011a).

1.3.3 Immunological tick control

Alternatives to acaricide treatments have been developed and anti-tick vaccines are among the most significant developments. The identification of antigens capable of induce animal protection to ticks is critical and during the decade of 1980´s several midgut protein combinations were tested until an antigen termed Bm86, a membrane-bound glycoprotein in the cell surface, was discovered, which conferred significant protection of cattle against *R. microplus* infestations (Willadsen et al., 1989). This protein of unknown function in tick biology is localized on the microvilli of the midgut digest cells, and tick ingestion of antigen specific antibodies leads to lysis of these cells, resulting in mortality and a deleterious effect on the reproductive performance of tick (de la Fuente et al., 1998c; Willadsen, 2004). The discovery of this protective antigen was a revolutionary moment in the development of anti-tick vaccines. From this breakthrough, two commercial vaccines containing the Bm86 recombinant protein emerged in the early 1990's, Gavac in Cuba and TickGARD in Australia (Willadsen, 2004; Ghosh et al., 2007). In spite of the effectiveness of these commercial Bm86-based vaccines for cattle tick infestations control, they show strain-to-strain variation in efficacy being predominantly effective against *Rhipicephalus* tick species (de la Fuente & Kocan, 2003; Guerrero et al., 2012b; Willadsen, 2006).

Since the commercialization of these vaccines no other has become available but research focusing this alternative tick control method has ascended. In the last decade new molecular tools such as next generation sequencing, proteomics or RNA interference (RNAi) have brought to light some potential vaccine candidates allowing a rapid, systematic and comprehensive approach to tick vaccine discovery (de la Fuente & Kocan, 2006c; Domingos et al., 2013). Nevertheless, the availability of these techniques, the identification and characterization of effective antigens remains a noteworthy challenge.

This approach is based on recombinant protein as antigens to immunize animals and demonstrate to be a good-looking alternative for the control of tick plagues, since they exhibit several advantages, such as prevention or reduction of pathogens transmission (Almazán et al., 2005; de la Fuente et al., 1998c; de la Fuente et al., 2007a, 2011; Merino et al., 2011b), environmental safety, low cost production (Kiss et al., 2012), avoidance of drug-resistant selection (Parizi et al., 2012) and inclusion of multiple antigens that are able to target many tick species (de la Fuente et al., 2000; de la Fuente & Kocan, 2006c; Parizi et al., 2012; Willadsen, 2008; Willadsen, 2004).

A study from Rodriguez-Mallon focused on *Rhipicephalus* ribosomal proteins (Rodríguez-Mallon et al., 2012) identified a unique immunogenic region of protein P0. This protein seems to be important in the assembly of 60S ribosomal subunit (Rodríguez-Mallon et al., 2012). Silencing effects of tick protective antigens 4D8 and Rs86, homologues of Bm86, were evaluated in *R. sanguineus* (de la Fuente et al., 2006a). Silencing of *4D8* alone had effect on tick feeding, attachment and oviposition and silencing of *Rs86* had an effect on tick weight and oviposition. Silencing of expression of both genes had substantial effect on *R. sanguineus* survival, attachment, feeding, weight and oviposition (de la Fuente et al., 2006a). The authors of this study suggested the development of multi-antigenic vaccines, in order to prevent infestation from *R. sanguineus* (de la Fuente et al., 2006a).

1.4 Tick and tick-borne diseases control

The aim of anti-arthropod vaccines is not only the control of vector infestations but also the agents harbored by them. The effect of such vaccines could be achieved by a) reducing vector populations and therefore the exposure of hosts to vector-borne pathogens, b) reducing the arthropod vector capacity for pathogen transmission, and, ideally, c) a combination of these factors (Merino et al., 2013). As it is more and more clear that disease transmission can implicate complex interactions between host, vector and disease organism, it is accepted that by disturbing the tick the vaccine will also have impact on the disease (Willadsen, 2004).

1.4.1 Tick antigens

Current molecular techniques are supporting in the identification of potencial tick-protective antigens. Bioinformatic tools and high throughput DNA sequencing technologies development enable undertake of pr*ovis*ional function to expressed sequence tags (ESTs). ESTs are fragments of mRNA sequences of approximately 200-800 base pairs (bp) derived from single sequencing reactions performed on randomly selected cDNA clones and show to be really useful for gene identification and verification of gene predictions, since they represent the expressed portion of a genome and offer a low-cost alternative to full genome sequencing, particularly for eukaryote organisms, whose genomes tend to be larger and less gene-dense than prokaryotes (Parkinson & Blaxter, 2009). The first study that reports the use of ESTs was in 1983 (Putney et al., 1983). An alternative approach for identification of potential vaccine antigens is expression library immunization (ELI), a high-

throughput technology that uses the immune system to screen the entire genome of a pathogen, in combination with sequence analysis of EST's, resulting in the expressed genes without prior knowledge of the antigens encoded by the cDNAs (Almazán et al., 2003; Barry et al., 2004; Ghosh et al., 2007). Also, suppression subtractive hybridization (SSH) is a broadly used technique for separating DNA molecules that discriminate two closely related DNA samples of either cDNA or genomic DNA (Diatchenko et al., 1999). Both these methods assure antigen identification without introducing prior criteria to manage the selection of candidate genes and thereby may result in the finding of new and unexpected antigens.

Several studies successfully identified antigens related to tick feeding and pathogen infection using SSH technique (Antunes et al., 2012; Heekin et al., 2012, 2013; McNally et al., 2012). A study performed by Antunes et al. (2012) applied this technique to identify *R. microplus* and *R. annulatus* genes induced by infection with *B. bigemina* (*e.g.* TROSPA, calreticulin, serum amyloid A, subolesin). Posterior studies perfomed *in vitro* and *in vivo* supported the inclusion of TROSPA in the development of new anti-TTBD vaccine (Antunes et al., 2014, 2015; Merino et al., 2013).

Direct RNA sequencing (RNA-Seq) offers the possibility to obtain both sequence and frequency of RNA molecules that are present at any particular time in a particular cell type, tissue or organ. Briefly, a population of RNA is converted to a library of cDNA fragments with adaptors attached to one or both ends. After this, fragments are sequenced in a high-throughput manner to obtain short sequences, typically 30–400 bp. After sequencing, the resulting reads are either aligned to a reference genome or reference transcripts, or assembled *de novo* without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene (Wang et al., 2009).

Although RNA-Seq is a technology under development, it presents several advantages over existing technologies. First, contrasting with hybridization-based approaches like microarrays, based on the use of probes to simultaneously analyze the expression of thousands of genes in a certain point in time, this method is not limited to detecting transcripts that correspond to existing genomic sequence (Nagalakshmi et al., 2008; Wang et al., 2009). For example, 454-based RNA-Seq has been used to sequence the transcriptome of the Glanville fritillary butterfly (*Melitaea cinxia*) (Vera et al., 2008). For non-model organisms, whose genomic sequences that are yet to be determined, this technique shows to be particularly attractive. RNA-Seq has very low, if any, background signal because DNA sequences can been unambiguously mapped to unique regions of the genome, unlike microarrays that have high background signal and cannot distinguish two closely related sequences due to cross-hybridization of probes (Nagalakshmi et al., 2008; Wang et al., 2009). This technique can also reveal sequence variations, as single nucleotide polymorphisms (SNPs), in the transcribed regions (Cloonan et al., 2008). The results of RNA-Seq also demonstrate high levels of reproducibility for both technical and biological replicates, as well as high accuracy in quantifying expression levels, as determined using quantitative PCR (qPCR) and spike-in RNA controls of known concentration

(Cloonan et al., 2008; Mortazavi et al., 2008). Finally, because there are no cloning steps, RNA-Seq requires less RNA sample. These factors make RNA-Seq useful for studying complex transcriptomes.

There are reports that use RNA-Seq in order to get new insights into the sialotranscriptome of *A. americanum* tick (Karim & Ribeiro, 2015), as well for *A. parvum, A. cajennense* and *A. triste* (Garcia et al., 2014), aiming the identification of antigens that might confer anti-tick immunity.

1.4.2 Functional genomics in ticks

RNAi or post transcriptional gene silencing is a conserved and natural process that cells use to turn down or silence specific genes (Fire, 1999; Montgomery et al., 1998). Though RNAi mechanism in ticks has not been fully elucidated, it has been well studied in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* (Hannon, 2002; Mello & Conte, 2004). RNAi begins with the uptake of dsRNA by the cell, followed by its cleavage, which is assessed by an RNAse III called Dicer, producing small interfering RNAs (siRNAs). The siRNAs are then incorporated into RNA-induced silencing complex (RISC), which results in a huge sequence-specific degradation of cytoplasmic mRNAs containing the same sequence as dsRNA trigger, leading to gene silencing (de la Fuente et al., 2007b; Mello & Conte, 2004). This silencing signal may spread among the cells and different tissues, generating a systemic gene silencing in the organism (Whangbo & Hunter, 2008). A study from Kurscheid et al. (Kurscheid et al., 2009) shown that some components of RNAi machinery in other invertebrates are also present in the ticks.

The key challenge is to find easy and trustworthy methods for delivering dsRNA. There are three main strategies to delivery dsRNA, being microinjection the most commonly used method for dsRNA delivery to arthropods and insects (Antunes et al., 2012). Ingestion of dsRNA through oral feeding of diet mixed with dsRNA or transgenic plants expressing dsRNA could be performed (Baum et al., 2007). In some cases, dsRNA could be delivered by soak organisms in dsRNA solution (Galay et al., 2016; Whyard et al., 2009) but is mainly used for cell culture work. Prior experiments proposed that dsRNAs could be designed as specific pesticides due to its high specificity (Baum et al., 2007; Whyard et al., 2009).

Since the first report of RNAi application in *Amblyomma americanum* (Aljamali et al., 2002), in which ticks injected with histamine binding protein (HBP) dsRNA presented a decrease in feeding compared to control ticks, several studies have been used RNAi in ticks to evaluate tick gene function in response to pathogen infections, aiming the development of vaccines against tick specific antigens (Antunes et al., 2012; Galay et al., 2013, 2016; Hajdušek et al., 2016; Lu et al., 2016). *TROSPA* and *serum amyloid A* knockdowns using RNAi reduced *B. bigemina* infection in *R. annulatus* whereas in *R. microplus*, knockdown of *TROSPA*, *serum amyloid A* and *calreticulin* reduced pathogen infection as well, comparing with controls (Antunes et al., 2012). Recently, knockdown of *subolesin*, a transcription factor that regulates gene expression (Naranjo et al., 2013), by RNAi decreased the engorgement, attachment, oviposition and body weight in *R. microplus* ticks (Lu et al., 2016). In other

recent study using RNAi, silencing of gene encoding for an intracellular ferritin-1, an iron-binding protein, in *Haemaphysalis longicornis* ticks led to lower weight after feeding when compared with control group, as well as high mortality and low oviposition (Galay et al., 2016).

In the past years, RNAi revealed to be a valuable tool to carry gene functional studies in ticks, the characterization of the tick–pathogen interface and the screening and characterization of tick-protective antigens (de la Fuente & Kocan, 2006c; Merino et al., 2013).

1.5 Aims of this Master project

Due to its capacity of transmit a wide variety of infectious agents to different vertebrate hosts, ticks represent great medical and veterinary importance (de la Fuente et al., 2008). Particularly, babesiosis continues having a great economic impact in livestock industry due to the lack of effective control methods. Alternatively to acaricides, anti-tick vaccines have been developed to control tick infestations (de la Fuente et al., 1998; Willadsen et al., 1989; Willadsen, 2004). Still, identification and evaluation of new candidate antigens implicate laborious and often expensive work, since it is necessary to define the immunological mechanisms of these antigens and develop appropriate methods for their production, as well as optimization studies on the host immune system response, field trials to test the vaccine and product registration are needed (Willadsen, 2004). Presupposing that improved vaccine formulations and the discovery of new tick-protective antigens related to infection and feeding will improve control of TTBD, as well as increase our understanding on tick-pathogen interface, this Master project was designed with one main objective, that consists in validation of the influence of antigens, which were identified in previous studies as differentially expressed in response to tick feeding and *B. ovis* infection. Functional analyses using RNA interference was applied in order to assess about the role of specific gene expression disruption in pathogen transmission and tick feeding. Parameters such as tick weight, attachment to the host and mortality were evaluated as well as the *B. ovis* infection levels in tick´s SGs and OVs.

These assays are expectable to contribute for the characterization of the tick-pathogen interface, as well as provide new targets for the development of alternative TTBD control methods, such as vaccination.

2 Materials and Methods

2.1 Lamb infection with *Babesia ovis*

A six month old lamb bred and maintained at the INIAV (Instituto Nacional de Investigação Agrária e Veterinária) animal facility was used in the gene silencing assays. To ensure infection establishment, the lamb was splenectomised and, 45 days after, intravenously inoculated with 1 ml of cryopreserved *B. ovis* culture with 9% parasitemia. The infection in the lamb was monitored daily by collecting and screening blood for *B. ovis* using the protocol described in the section below. Animal ethics approval was given for this work. The lamb used in the present work was cared for in accordance with *standard*s specified in the Guide for Care and Use of Laboratory Animals.

2.2 Genomic DNA extraction from lamb blood

DNA from the lamb blood was extracted using the NZY Blood gDNA Isolation Kit (NZY Tech, Genes and Enzymes) according to the protocol provided by the manufacturer. The DNA samples were maintained at -20ºC. Following DNA extraction, quantity and purity was assessed in a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific™) using the elution buffer as a blank.

2.3 Detection of *Babesia ovis* in blood by PCR

To amplify a 549 bp fragment of *B. ovis 18S* ribosomal DNA (*18S* rRNA), primers described by Aktaş et al. (2005) were used. Primers were synthetized by StabVida (Lisbon, Portugal) and reconstituted in nuclease free water (Sigma-Aldrich, St. Louis, MO, USA) to a 100 mM stock. PCR were performed in 25 µl reactions with Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 1 µM primers and 5 µl of template DNA. There was also prepared a negative control, without DNA, and a positive control, which contained DNA extracted from "in house" *B. ovis* (Israel strain) culture. The PCR was carried out with a thermal cycling profile of 95ºC for 2 min, and 35 cycles of 95ºC for 30 s, 62ºC for 45 s and 72ºC for 45 s, followed by a 72ºC extension for 5 min and a 4ºC hold (T-100® Thermal Cycler, Bio-Rad, Hercules, CA, USA). The PCR products were visualized by agarose gel electrophoresis.

2.4 Agarose gel electrophoresis

PCR products were visualized by AGE as follows. To prepare 1.2% agarose gel as required, 0.36 g of agarose (Sigma-Aldrich, St. Louis, MO, USA) was added to 30 ml of 0.5X Tris-Borate-EDTA (TBE) buffer (Sigma-Aldrich, St. Louis, MO, USA) and heated in a microwave oven for 1 min, in order to dissolve the agarose powder. 2 µl SYBR® Safe (Thermo Fisher Scientific™) was added. 10 µl of each sample was loaded directly into the wells. 5 µl NZY DNA Ladder I (NZY Tech, Genes and Enzymes) was loaded in the lane M. Gels were run at 90 V for 20 min. The products were visualized using a UV transilluminator.

2.5 Identification of differentially expressed genes in *R. bursa* ticks in response to *B. ovis* infection

This work is a continuation of a research project that started earlier entitled "Functional genomics of *Rhipicephalus bursa* and *Babesia ovis* interactions towards disease control" PTDC/CVT-EPI/4339/2012, thus part of the project plan was already concluded, which made ground for the selection of genes to be characterized under this MSc project.

The identification of *R. bursa* genes potentially involved in response to *Babesia* infection was previously performed by the IHMT´s team based on RNA sequencing results. In summary, *R. bursa* differentially expressed genes in response to both infection and blood feeding were identified by sequencing RNA from SGs of tick populations' correspondent to each of the conditions: *B. ovis* infected and fed females, non-infected and fed females and finally non-infected and unfed females. The annotation of each transcript was done based on the BLAST similarity results comparing the transcript to a database of reference proteins. Two comparisons were made (infected *vs* non-infected and fed *vs* non-fed) and, subsequently, two catalogues of genes differentially expressed were obtained. The obtained data was analyzed and the selection of genes to further characterize was made according to their potential role on each of the conditions (feeding and infection) studied and fold change of expression.

2.5.1 Selection of genes

The genes *cf1* (Uniprot ID: A0A034WWF8), *cf2* (Uniprot ID: L7M018), *st1* (Uniprot ID: A0A034WWS7) and *mt5* (Uniprot ID: L7M1K6_9ACAR) were selected for functional analysis.

The *cf1* and *cf2* transcripts were found to be upregulated in fed ticks showing a fold change above 15 and belong to cell function class. The *cf1* encodes for the vitellogenin-3 protein (Vg-3), a precursor of the major yolk protein vitellin (Vn), both considered heme-binding storage proteins (Horigane et al., 2010). Vg has already been identified in other tick suggesting that their role may be linked to lipid transport and its production is one of the most important events for egg development since, once synthesized by the fat body or less commonly by the midgut (Coons et al., 1982), Vg are secreted in the haemolymph and incorporated into oocytes by a specific Vg receptor on the surface (Boldbaatar et al., 2008, 2010; Horigane et al., 2010; Khalil et al., 2011; Smith & Kaufman, 2013, 2014; Taheri et al., 2014; Mitchell et al., 2007). The *cf2* encodes for a putative lachesin which is a cell surface protein that is thought to be involved in the morphogenesis of the tracheal system of *Drosophila* and in other arthropods, known to be involved in neurogenesis (Karlstrom et al., 1993; Llimargas et al., 2004). Its role in ticks has not yet been described. Regarding the *st1* and *mt5* transcripts identified on the catalogue of differentially expressed genes in response to infection, both were found to be upregulated in infected ticks with again fold changes above 15. The *st1* transcript encodes for a putative glycine-rich secreted cement protein with unknown function belonging to the

structural class while the *mt5* transcript encodes for a protein that belongs to the metabolism class with unknown function on ticks. Performing functional analysis using RNA interference permits to unravel possible function of these proteins during infection.

2.6 *in vivo* gene silencing in ticks by RNA interference

2.6.1 dsRNA synthesis

Gene-specific double-stranded RNAs (dsRNA) were synthesized based on identified *Rhipicephalus* spp. sequences prior to the beginning of the present work and used to knockdown the expression of selected genes in *R. bursa* ticks, since the role of genes carried in this study were not previously described in *R. bursa*. Briefly, specific primers containing T7 promoter sequences (5´-TAATACGACTCACTATAGGGTACT-3´) at the 5´- end were manually designed and synthesized by StabVida (Lisbon, Portugal) (Table 2.2). *R. bursa* cDNA was synthetized using the iScript cDNA synthesis (Biorad, CA, USA) following the manufacturer instructions and further used as template to amplify fragments of interest by PCR. Amplification of target DNA was achieved using the iProof High Fidelity PCR kit (Biorad, CA, USA). 50 µl of final volume was used including 200mM each primer. Cycling conditions were for 40 cycles: 30 s at 94 ºC, 30 s at specific annealing temperature and 30 s at 72 ºC with a final extension step of 7 min at 72º C. All PCR assays were performed in a T100 thermal cycler (Biorad, CA, USA). Amplification results were analyzed on a 0.5X TBE, 1.2 % (w/v) agarose gel. Amplicons were purified using the NZYGelpure kit (NZYtech, Lisbon, Portugal) and sent for Sanger sequencing at StabVida (Lisbon, Portugal). The obtained sequences were aligned, compared to reference sequences and sequences deposited in the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast). After confirmation of the amplified sequences the MEGAscript RNAi Kit (Ambion, Austin, TX, USA) was used to synthesize dsRNA according to manufacturer's instructions. The resulting dsRNA was purified, quantified by spectrometry and checked on a 0.5X TBE, 1.2 % (w/v) agarose gel. Table 2.1 shows the determination of the number of molecules per µl of each dsRNA used in silencing experiments.

Table 2.1: Number of molecules per µL of each dsRNA used to knockdown *R. bursa* genes in the present study.

Group	Mw (g/mol)	[ ] (g/µL)	n (moles)	Molecules per µL
Vitellogenin (*cf1*)	132311,4	1,11E-06	8,38E-12	5,05E+12
Lachesin (*cf2*)	141611	1,53E-06	1,08E-11	6,51E+12
Metabolism protein (*mt5*)	140110,2	1,32E-06	9,43E-12	5,68E+12
Glycine-rich secreted cement protein (*st1*)	132832,2	7,01E-07	5,28E-12	3,18E+12
Double-knockdown (*cf1+cf2*)	136969,2	8,20E-07	5,99E-12	3,61E+12

*The number of molecules per µL of each dsRNA was calculated using the formula nº of molecules= n×AN, in which AN represents Avogadro's number, AN=6.022×10^{23}. Molecular weight (Mw) was calculated based in the number of each nitrogenous base (adenine, timine, cytosine and guanine) present in the dsRNA sequence, using the following formula Mw=(A*329.2)+(T*306.2)+(C*305.2)+(G*345.2)+159.*

2.6.2 Rhipicephalus bursa colony

A laboratory *R. bursa* colony was established in Centro de Estudos de Vectores e Doenças Infecciosas (CEVDI) from Instituto Nacional de Saúde Dr. Ricardo Jorge. To establish a *R. bursa* colony, ticks were originally collected from the field in Sétubal, Portugal, region. Briefly, after oviposition, each female and a sample of eggs were tested by PCR for pathogens detection according to Aktas et al (Aktaş et al., 2005), during two generations. *R. bursa* life cycle was maintained in Hyla white rabbits under the appropriate conditions. The ticks that were used in the present study were at least the 10th generation of the laboratory colony.

2.6.3 dsRNA injection in ticks

R. bursa adult female ticks were detached from a Hyla white rabbit ear, using fine forceps, observed, cleaned and placed ventral side up on double sticky tape affixed to a plane wood table. The ticks were closely positioned together in groups of 10 ticks leaving the body exposed. After, ticks were injected with 69 nl of dsRNA (1×10^{11} to 1×10^{12} molecules) in the trochanter articulation. Thirty female ticks per group were injected using the nano-injector (Nanoject, Drummond Scientific, Broomall, PA, USA). Control ticks were injected with buffer (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone (negative control). According to the described above, six groups were formed, *cf1, cf2, cf1+cf2, st1, mt5* and control. The group *cf1+cf2* corresponds to a double knockdown performed by injection of an equal mixture of both dsRNA. After dsRNA injection, female ticks were held in a humidity chamber for 4 hours after which they were allowed to feed on splenectomized sheep infected with *B. ovis* together with 30 male ticks per tick feeding cell. Tick-feeding cells (450mmX400mm) (cotton fabric) were glued to shaved skin using Pattex® contact glue (Henkel Nederland, Nieuwegein, The Netherlands) on the day before infestation. Ticks were allowed to feed in the infected lamb and 8 days after attached ticks were removed. Ticks were monitored daily and mortality was evaluated.

Figure 2.1: Injection of *Rhipicephalus bursa* female tick in the trochanter articulation. (*original from the author*).

2.6.4 Analysis of tick survival after RNAi

Tick survival after feeding was evaluated by determining the number of ticks that survived and tick weight. Tick mortality was evaluated as the ratio of dead ticks to the total number of fed ticks on the lamb. To analyze tick mortality, the Chi-square test (P=0.05) was used with the null hypothesis that tick mortality was not dependent on gene knockdown. The number of attached ticks and the number of ticks that started to feed, in each group, were as well analyzed using the Chi-square test (P=0.05). Tick weight was determined in individual female ticks collected after feeding and further compared between ticks injected with test genes dsRNA and control dsRNA by Student's t-test with unequal variance (P = 0.05). Ticks SGs and OVs were posteriorly dissected and used for DNA and total RNA extraction for further evaluation of silencing efficiency determination and influence on infection acquisition.

2.7 Tick dissection

Ticks were twice rinsed individually in distilled water. To carry out tick dissection, each tick was covered with a drop of PBS in a Petri dish, to prevent desiccation of the tissues. All tissues were washed in PBS giving special attention to guts samples from which the luminal contents were carefully removed, and remaining tissue was gently washed from the host blood excess in the same buffer. SGs and OVs were removed and stored in 100 µl of RNA later (Ambion®, Austin, TX, USA) at 4ºC for further total RNA and DNA extraction.

2.8 DNA and total RNA extraction from tick SGs and OVs

gDNA and RNA from tick tissues were extracted using a TRI Reagent® Protocol (Sigma-Aldrich, St. Louis, MO, USA). The manufacturer's protocol was followed.

Subsequently to DNA and RNA extraction from OVs and SGs, the concentration as well as

the optical density of each sample was measured spectrophotometrically with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific™) using water as blank for RNA samples and 8 mM NaOH for DNA samples.

Table 2.2: Sequences of primers used for dsRNA synthesis and amplification conditions.

Uniprot ID	Upstream/downstream primer sequence 5´-3´*	PCR annealing conditions
L7M1K6_9ACAR (*mt5*)	ACGTGTTCGGCCTATCTCAC TGGCGATTCAAGTACACCAG	64ºC/30s
A0A034WWS7_RHIMP (*st1*)	CGGTGGATATGGTGCTCTTT GGGAAACCTCCGTATGATCC	55ºC/30s
A0A034WWF8 (*cf1*)	CCGCCAAGGTTCTGTTGTAT GCATCTTCGCTCCTCTGTTC	65ºC/30s
L7M018 (*cf2*)	GCGCTGGTGTCTTTAGGTTC GTGGCATAGCACTCCAAGGT	65ºC/30s

**All primers contained T7 promoter sequences (5´-TAATACGACTCACTATAGGGTACT-3´) at the 5´end.*

2.9 Gene knockdown assessment by qPCR

2.9.1 Determination of tick mRNA levels by qPCR

The efficiency of gene specific silencing in different tick tissues was evaluated by qPCR, using the minimum information for publication of qPCR experiments (Bustin et al., 2009). The efficiency of *cf1, cf2, st1* and *mt5* knockdown was assessed in the SGs and OVs. Several potential reference genes were tested and based on the geNorm algorithm included in the CFX Manager™ Software (Bio-Rad) and on the expression stability value M of each gene (Bustin et al., 2009); the ideal reference genes were then selected to normalize the silenced gene levels in tick SGs and OVs. For each gene used in qPCR, primer sets were designed and can be found in Table 2.3 as well as PCR conditions. RNA extracted from individual tick SGs was used to synthetize cDNA using the iScript™ cDNA Synthesis Kit (Biorad) and the iQ™ SYBR® Green Supermix (Biorad) was used for amplifications. The qPCR was carried out in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Negative controls were prepared with water. *Standard* curves were constructed with 5-fold serial dilutions of cDNA from *R. bursa*. The CFX Manager™ Software was used to analyse the qPCR data. Gene expression was normalized to the total amount of RNA used to generate the cDNA, as previously described (Bustin et al., 2009). The absence of PCR product in control reactions has shown

the reaction specificity. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently in the same temperature range for every sample. The mRNA levels in ticks after feeding were compared between dsRNA and saline-injected control ticks by Student's t test (P=0.05).

Table 2.3: Sequences of primers used for qPCR.

Gene	Upstream/downstream primer sequence 5´-3´	PCR annealing conditions
L7M1K6_9ACAR (*mt5*)	GTGCGCTTCAATGTGTTTGT AAGAATGGCCTTTGTGTTGG	56ºC/45s
A0A034WWS7_RHIMP (*st1*)	CGGTGGATATGGTGCTCTTT ACGCCACCAAGTCCTGAGTA	54.5ºC/45s
A0A034WWF8 (*cf1*)	AGACCTTCGACAACGTCACC GCATCTTCGCTCCTCTGTTC	54.5ºC/45s
L7M018 (*cf2*)	GCGCTGGTGTCTTTAGGTTC GATCTGGTACGATGGCCTTG	62ºC/45s
β-actin	GACATCAAGGAGAAGCT(TC)TGC CGTTGCCGATGGTGAT(GC)	62ºC/45s
16S rRNA	GACAAGAAGACCCTA ATCCAACATCGAGGT	56ºC/45s
ELF (elongation factor)	CGTCTACAAGATTGGTGGCATT CTCAGTGGTCAGGTTGGCAG	62ºC/45s
β-tubulin	AACATGGTGCCCTTCCCACG GCAGCCATCATGTTCTTTGC	62ºC/45s

2.9.2 Determination of *Babesia ovis* infection by qPCR

B. ovis DNA levels were evaluated by qPCR normalizing against tick *16S* rDNA as described previously for *B. bigemina* (Antunes et al., 2012). The primers used for quantitative detection of *B. ovis* were the same used previously for traditional PCR firstly described by Aktas et al (Aktaş et al., 2005). Normalized Cq values were compared between ticks injected with test A and control dsRNA by Student's t-test with unequal variance (P = 0.05).

3 Results

3.1 Monitorization of lamb infection

The infection in the lamb was monitored daily by collecting and screening blood for *B. ovis*. Fig 3.1 shows the evolution of parasitemia from day 0 to day 17. The lamb was considered infected on day 6.

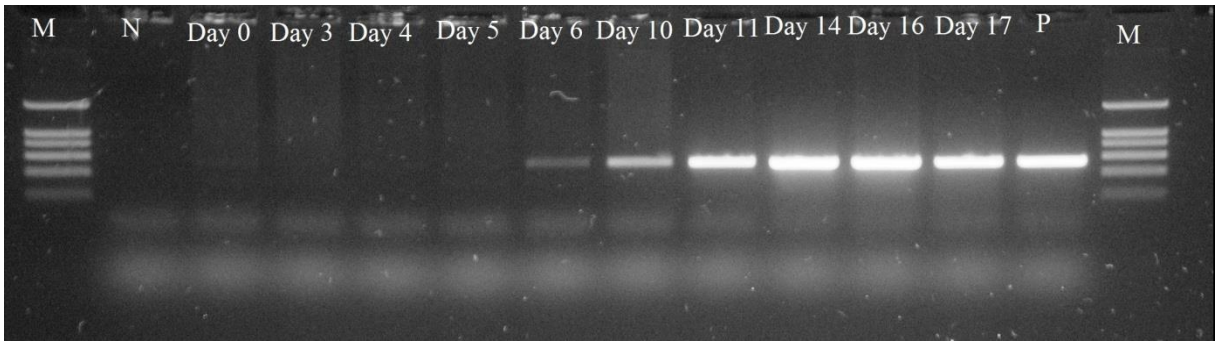

Figure 3.1: Detection of *B. ovis* in the lamb blood by AGE of PCR products. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The first and last lane corresponds to the ladder (M), the second lane corresponds to the negative control (N) and the 13th lane corresponds to the positive control (P).

3.2 Sequence analysis of tick genes differentially expressed in response to *B. ovis* infection

Additional sequence analysis was conducted on different *Rhipicephalus* spp. ESTs differentially expressed in response to *Babesia* spp. infection and tick feeding, since sequences from genes analyzed in this study are not available in databases for *R. bursa*. *R. microplus* (UniProt Accession number A0A034WWF8) obtained sequence analysis showed that *cf1* which encodes for vitellogenin-3 is a conserved gene in ticks, with 93.61% homology between *R. appendiculatus* (Figure 3.2, Table 6.1). Regarding *cf2* which encodes for a putative lachesin, *R. pulchellus* (L7LSG7) showed a 94.91% identity to *R. appendiculatus* (A0A131YVX3) (Table 6.2). Relatively to *st1* that encodes for a putative glycine-rich secreted cement protein, *R. pulchellus* (L7MBM8) showed the highest homology (45.85%) to *A. americanum* (A0A0C9SFJ8) and *I. scapularis* (A0A023FLK9) share with *A. cajennense* (A0A023GE61) 61.60% of identity with the obtained sequence (Table 6.3).

Figure 3.2: Analysis of *vitellogenin* orthologue sequences. Unrooted phylogram inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

3.3 Reference genes selection

In order to normalize gene mRNA and DNA levels in tick SGs and OVs, the best reference genes were selected for each tissue based on the geNorm algorithm included in the CFX Manager™ Software (Bio-Rad) and on the expression stability value M of each gene (Bustin et al., 2009). Relatively to OVs, after test several potential reference genes, *β-actin*

and *β-tubulin* were chosen and were used in data analysis with a mean M value of 0,5578 and a coefficient of variation (C.V.) of 0,1933. Regarding the SGs, *ELF* and *β-tubulin* were used with a mean M value 0,9585 and a C.V. of 0,3325. These values are within the recommended stability values for heterogeneous samples.

3.4 Efficiency of gene silencing in *R. bursa* SGs and OVs

Under the conditions undertaken in this study gene knockdown after dsRNA-mediated RNAi was demonstrated in *R. bursa* SGs for all genes, except for *mt5*, whose silencing values were approximately 92%, 51% and 65% for *cf1*, *cf2* and *st1*, respectively. In the case of the double knockdown performed, only two ticks were recovered, thus, it was not possible to determine gene silencing efficiency. Concerning to OVs the silencing of the four genes was not demonstrated.

3.5 Functional analysis of tick genes differentially expressed in response to *B. ovis* infection

3.5.1 Analysis of tick phenotype after RNAi

The knockdown effect of selected genes on tick weight and mortality was determined and statistically analyzed. Gene silencing assays results showed first that tick mortality was significantly affected in dsRNA-injected ticks, particularly in *cf1* and *cf2* gene knockdown, when compared to controls (P=0.001), suggesting that Vg-3 and putative lachesin had a role in tick survival. After allowed to feed, it was possible to determine that not all the ticks engorged or even attached to the host: group *cf1*, 4/7 ticks attached, *cf2* 8/9 ticks, *cf1*+*cf2* 3/5 ticks, from the *mt5* group 11/19, *st1* group 13/24 ticks and finally from the control group 22 of the 25 ticks recovered were attached. By comparing injected test groups with control group using a Chi-square test (P=0.05) there is evidence that *st1* (P=0.008774) knockdown reduced significantly tick attachment. *Vitellogenin-3* (*cf1*) and gene encoding for a putative glycine-rich secreted cement protein (*st1*) knockdowns resulted in a reduction of female tick weight in *R. bursa* (Table 3.1). The low number of fed females in *cf1* did not allow any statistical analysis but these results are indicative of impact of the silenced gene in the process of feeding. When comparing ticks injected with specific dsRNA to control group using the Student's *t*-test (P= 0.05) results demonstrate that *st1* knockdown significantly reduced female tick weight (P=0.02). Figure 3.3 shows the recovered ticks after gene silencing assays. Regarding *mt5*, since gene silencing was not demonstrated, no inferences can be assumed.

Table 3.1: Female tick weight after gene knockdown by RNA interference in *Rhipicephalus bursa* ticks.

Group	*R. bursa* weight Ave ± S.D. (mg)
Vitellogenin-3 (*cf1*)	40 ± 19[b]
Lachesin (*cf2*)	149 ± 108
Metabolism protein (*mt5*)	73 ± 72[b]
Glycine-rich secreted cement protein (*st1*)	52 ± 46 [a]
Double-knockdown (*cf1*+*cf2*)	19 ± 1[b]
Control	136 ± 163

Thirty female ticks per group were injected with double stranded RNA or saline control. Ticks were allowed to feed in six separated patches on a lamb experimentally infected with B. ovis. All attached ticks were removed after 7 days of feeding, weighed and held in a humidity chamber for 4 days to allow ticks to digest the blood meal. Tick mortality was evaluated as the ratio of dead ticks to the total number of placed ticks on the lamb. Female tick weight after feeding was compared between dsRNA and saline-injected control ticks by a Student's t-test ([a]P < 0.05; [b] no statistical study was made).

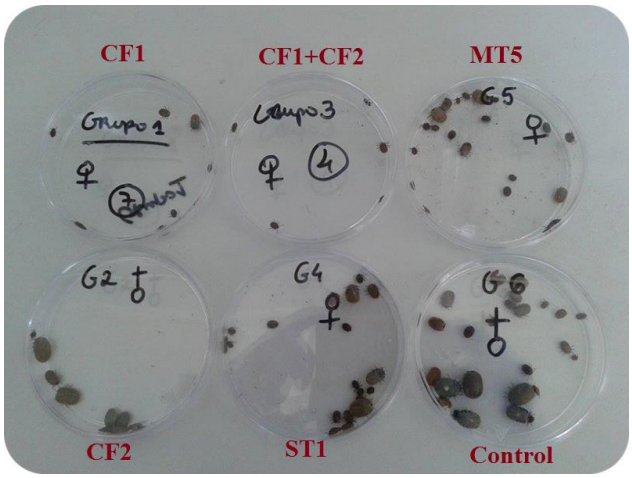

Figure 3.3: Silenced and control female ticks recovered after feeding in *B. ovis* infected lamb.

3.5.2 Analysis of *Babesia ovis* infection levels after RNAi

Knockdown of *lachesin* significantly reduced *B. ovis* infection levels by 70% in *R. bursa* SGs compared with control ticks (Table 3.2). The *vitellogenin-3*, gene encoding for a metabolism protein and gene encoding for a glycine-rich secreted cement protein knockdowns did not affect *B. ovis* infection levels in SGs of *R. bursa* ticks (Table 3.2). Considering the OVs, *B. ovis* infection levels were not analyzed since gene silencing was not confirmed in none of the four genes studied.

Table 3.2: *Babesia ovis* infection levels after gene knockdown by RNA interference in *Rhipicephalus bursa* ticks SGs.

Group	N	Gene silencing (% Ave ± S.D.)	*B. ovis* infection levels (Ave ± S.D.)	Test/Control (Ave ± S.D.)
Vitellogenin-3 (*cf1*)	4	92 ± 2	$4{,}67e^{-04} \pm 3{,}05e\text{-}^{04}$	314 ± 255.17^{a}
Lachesin (*cf2*)	8	51 ± 9	$4{,}48e^{-07} \pm 1{,}20e^{-07}$	0.30 ± 0.09^{a}
Glycine-rich secreted cement protein (*st1*)	13	65 ± 11	$2{,}97e^{-06} \pm 2{,}68e^{-06}$	1.99 ± 2.18
Metabolism protein (*mt5*)	11	ND	ND	ND
Control	22	---	$1{,}49e^{-06} \pm 1{,}09e^{-06}$	---

Thirty female ticks per group were injected with double stranded (ds)RNA or saline control. Ticks were allowed to feed in six separated patches on a lamb experimentally infected with B. ovis. All attached ticks (n = 4–22) were removed after 7 days of feeding and held in a humidity chamber for 4 days to allow ticks to digest the blood meal. Gene knockdown was analyzed by real-time reverse transcription (RT)-PCR by comparing mRNA levels between dsRNA-injected and control ticks. The B. ovis infection levels were determined by quantitative PCR of the 18S rRNA gene and normalized against tick 16S rRNA using the ddCT method ($2^{-CT_{target}-CT_{\beta\text{-}actin}}$). The mRNA levels and B. ovis infection in ticks were compared between dsRNA and saline-injected control ticks by a Student's t-test ($^{a}P < 0.05$). ND means not determined, since gene knockdown was not demonstrated.

4 Discussion and Conclusions

Understanding the paradigm host-pathogen-tick remains a complex issue, mostly because, in fact, there´s three interactions present: Pathogen-Vector, Pathogen-Host and Host-Vector. In this way, basic research focusing on each one of these interplays is necessary that can be after integrated to realize a broader image of this triangle. One of the models that can be used is the *Babesia ovis* - *Rhipicephalus bursa* system. Focusing on the molecular alterations that *Babesia* induces on the tick vector offers insight on the possible molecular strategies that this pathogen uses to surpass mechanic and immune barriers. Moreover, these perceptions can be used in the discovery of tick key molecules with a role in vector infestation or vector capacity. Recent studies performed with different methodological approaches, have reported that the expression of *Rhipicephalus* tick factors can be modulated in response to tick feeding and *Babesia* infection (Domingos et al., 2015; de la Fuente et al., 2011; Merino et al., 2011a,b; Heekin et al., 2012; Lu et al., 2016; Taheri et al., 2014; Antunes et al., 2012, 2014, 2015; Erster et al., 2015a; Merino et al., 2013). In this work, we characterized *R. bursa* genes differentially expressed in response to feeding and *B. ovis* infection using a RNAi approach to analyze their role in tick biological parameters and also pathogen acquisition.

Under the study conditions, babesiosis was detected 6 days post-inoculation in the lamb blood and clinical signs were observed 11 days after experimental infection, which is concomitant with a recent study related to transmission of *B. ovis* in *R. bursa* (Erster et al., 2015a). Gene knockdown was carried out by inoculation of dsRNA on the trochanter of *R. bursa* females. This technique allowed to diminish the damage provoked to the specimens in comparison to the traditional inoculation site, the abdominal region. Ticks were placed in the lamb at day 12 post-inoculation, when the lamb presented high parasitemia and were allowed to feed until drop-off or until day 8 post-infestation. After these assays, gene knockdown evaluation was performed.

Silencing efficiency: In *R. bursa* SGs but not in OVs, gene knockdown was assessed for all of the genes tested, except for the unknown metabolism related protein (*mt5*). These results suggest that for some genes and for different tissues, the amount of injected dsRNA necessary to knockdown a gene is different. Moreover, as referred, these genes were selected based on transcriptomic data relative to SGs, thus we are not able to determine the basal expressions levels of such genes in OVs. The cement related protein, denominated here as *st1*, probably does not exist in OVs, since these type of proteins are exclusive to SGs. Vitellogenins are commonly found and highly expressed in OVs, thus the amount of dsRNA necessary for an efficient knockdown should be higher in comparison to other tissues. Regarding the gene, denominated here as *mt5,* not much is known about its function in either tissue, so, we might suggest that further studies should be performed to confirm that the gene is also found on OVs and, if so, the expression profile should be assessed in order to determine the amount of dsRNA needed to silence this gene. Finally, the *lachesin* gene, here *cf2*, is not described as present in OVs of invertebrate organisms, consequently, as in *mt5*, more analyses should be carried to explain the result obtained in the present work. A noteworthy element, that cannot be excluded in our gene

knockdown assays, is the RNAi off-target effects (OTEs) (Scacheri et al., 2004) previously described in *R. microplus* (Lew-Tabor et al., 2011). The absence of full tick genomic data and the lack of a confirmed tick RNAi pathway can under estimate the OTEs in current tick RNAi experiments (Lew-Tabor et al., 2011). Notwithstanding, the use of long dsRNAs in RNAi treatments in ticks has been accepted as a routine method for validation of tick gene function (Galay et al., 2016; Lu et al., 2016; de la Fuente et al., 2007c; Merino et al., 2011b). The following part of the discussion will be centered in each of the genes studied.

Vitellogenin (Vg), a large phosphoglycolipoprotein (200-700 kDa), is a lipid transfer protein (Avarre et al., 2007) that constitutes the precursor of major yolk protein, such as vitellin (Vn). The two major storage proteins found in ticks are the hemelipoglyco-carrier protein (CP), which seems to be expressed throughout most of tick development, and the female-specific yolk proteins, denominated vitellogenin (Vg), only found in female ticks at the point of egg development. Curiously, both these proteins also demonstrate to have a common evolutionary origin (Donohue et al., 2008, 2009; Sonenshine & Roe, 2014) and, due to its similarities, differentiation between these proteins can be difficult. In ticks, vitellogenesis takes place in midgut, fat body and ovary (Coons et al., 1989; Thompson et al., 2007; Boldbaatar et al., 2010; Horigane et al., 2010; Khalil et al., 2011), being induced by a blood meal and regulated by ecdysteroids (Seixas et al., 2008; Thompson et al., 2005), while tissues where CP is more abundant is in SGs and fat body, being suggested to be a component of saliva and cement cone (Donohue et al., 2008; Guddera et al., 2002). The first complete tick Vg cDNA sequence was reported from *Dermacentor variabilis* (Thompson et al., 2007). Since then, complete Vg sequences have been described from *Ornithodoros moubata* (Horigane et al., 2010) and *H. longicornis* (Boldbaatar et al., 2010) with multiple *Vg* genes described from both *D. variabilis* (Khalil et al., 2011) and *H. longicornis* (Boldbaatar et al., 2010). In *H. longicornis,* there were identified three different *Vg* mRNA sequences, described as *HlVg-1*, *HlVg-2* and *HlVg-3*, and further molecular structure studies aiming these proteins revealed a great similarity of these to CPs (Sonenshine & Roe, 2014). Vitellogenin uptake by growing oocytes in oviparous animals is carried by a receptor-mediated endocytosis pathway, including in invertebrates, such as the nematode *Caenorhabditis elegans* (Grant & Hirsh, 1999), arthropods, like, *Drosophila melanogaster* (Meigen, 1830) (Schonbaum et al., 1995) and *Aedes aegypti* (L., 1762) (Sappington et al., 1995), *D. variabilis* (Mitchell et al., 2007) and vertebrates, such as chicken (Bujo et al., 1994) and fish (Prat et al., 1998). A study reports that knockdown of *Vg* in *H. longicornis* led to lower tick body weight and egg weight in *Vg* dsRNA-injected ticks owing to insufficient uptake of Vg for oocyte development (Boldbaatar et al., 2010). In other study, Vg receptor (*VgR*) knockdown using RNAi in *H. longicornis* resulted in decreased tick ability for oviposition due to failure of Vg uptake by developing oocytes (Boldbaatar et al., 2008). In *A. hebraeum, VgR* knockdown resulted in decreased oocyte length, delayed ovarian development, and a longer latency to oviposition (Smith & Kaufman, 2013). These results suggest that VgR is crucial for

binding and transporting Vg via receptor-mediated endocytosis into oocytes in the tick ovary (Boldbaatar et al., 2008; Smith & Kaufman, 2013). *Vg-3* (*cf1*) was found to be upregulated in SGs of fed ticks when comparing with non-fed ticks (Antunes et al., in prep.). *Vg-3* knockdown was successfully assessed in SGs, presenting 92% of gene silencing. Under the conditions of this study, *Vg-3* knockdown did not affect pathogen infection, thus suggesting that this molecule is not essential to control *B. ovis* infection in *R. bursa* ticks. However, *Vg-3* knockdown resulted in increased tick mortality. Based on the principal functions associated to this type of molecule, we can suggest that a decrease of the expression of *Vg-3* reduces the transport of lipids and normal production of energy (ATP) provided by the disruption of lipids, leading to death. Whereas Vgs are mainly present in midgut and OVs, *Vg-3* was found in SGs of fed ticks. As referred, CPs and Vgs share some molecular features, being CPs mostly present in SGs, composing the cement cone, and were suggested to have a role in tick attachment and feeding (Donohue et al., 2008; Gudderra et al., 2002). This result stimulates future research with this molecule in tick life cycle to further develop vaccines against tick infestations.

Lachesin (Lac), a cell surface protein of the immunoglobulin superfamily (Karlstrom et al., 1993; Llimargas et al., 2004), was found to regulate organ size by influencing cell length and cell detachments, suggesting a role for Lac in cell adhesion (Llimargas et al., 2004). Lac was first identified in a grasshopper embryo as a membrane protein specifically expressed in neural cells (Karlstrom et al., 1993). Further, a *Lac* homologue in *D. melanogaster* was identified (Karlstrom et al., 1993) and is expressed in a dynamic pattern including in the trachea development (Llimargas et al., 2004). Strong expression of this protein is detected in specific tissues such as the trachea, hindgut, foregut and nervous system (Llimargas et al., 2004). Using bead aggregation assays, it was shown that Lac works as a homophilic cell adhesion molecule necessary to afford epithelial integrity to the tracheal tubes and to control tubular epithelium length (Llimargas et al., 2004). Also, it was reported that Lac accumulates at the Septate Junctions (SJs), specific invertebrate cell junctions located in the apical part of the lateral membrane of ectoderm-derived cells, whose role is the formation of a trans-epithelial diffusion barrier, establishing and/or maintaining cell polarity, cell adhesion and cell-cell interactions (Tepass & Hartenstein, 1994; Tepass et al., 2001). Recently, *lachesin* has been identified in the *R. appendiculatus* sialotranscriptome upon blood feeding (de Castro et al., 2016). Also, this gene has firstly been identified in the genome of *Ixodes scapularis* (Caler et al., 2008), but until date there is no studies focusing this molecule in ticks. *Lac* (*cf2*) was found to be upregulated in fed ticks when comparing with non-fed ticks (Antunes et al., in prep.). *Lac* knockdown was demonstrated in SGs, presenting 51% of gene silencing. Gene knockdown under the conditions undertaken here led to a significantly lower pathogen infection of about 70% in SGs. In addition, *Lac* dsRNA-injected ticks presented increased tick mortality when compared to PBS-injected ticks, as well as it was observed for *Vg-3* (*cf1*). No effect was evidenced in tick weight after feeding. These results suggested the

possibility that *Lac* may play a role in tick survival, as in *B. ovis* infection, in *R. bursa* ticks. Based on the putative role of this molecule in the cell adhesion and cell-cell interactions might influence pathogen invasion. Plus, as mentioned, it has been described as a molecule necessary to confer epithelial integrity and consequently involved in the development of specific organs therefore manipulating the expression of such gene can induce abnormal cell growth.

Putative secreted glycine-rich cement protein, *st1,* is a component of the cement cone, which consists of several glycine-rich proteins (GRPs) (Bishop et al., 2002; Trimnell et al., 2005), found to be expressed in SGs of several hard tick species (Bishop et al., 2002; Mulenga et al., 1999a,b; Trimnell et al., 2005; Untalan et al., 2005). For feeding, ticks attach to their hosts with the help of specialized mouthparts and remain attached by the secretion of adhesive (cement) GRPs that glue the mouthparts into the host's skin, enabling ixodid ticks to remain attached to the host during the prolonged feeding period and prevent host immune response molecules from coming into contact with the tick proboscis (Sonenshine & Roe, 2014; Bishop et al., 2002; Trimnell et al., 2005). SGs are not only responsible for the attachment cement secretion that allows the parasite to remain attached to the host for several days, but also enable pathogen transmission from the vector to the host. When on-host, tick avoids the host's immune system through SGs secretions (L'Amoreaux et al., 2003). Therefore, these structures are of great interest for on-host activities. GRP was isolated from *H. longicornis*, and was proposed to be a component of cement to help anchor the tick's mouthparts to the host during tick feeding (Mulenga et al., 1999a,b). Few years later, a study has identified genes encoding cement-like antigens in *H. longicornis*, which were upregulated upon feeding (Harnnoi et al., 2006). Cement cone proteins are similar to those of epidermis/dermis, which reflects their flexibility and need to avoid host rejection, representing good-looking candidates for inclusion in vaccines against ticks and pathogen transmission, since formation of the cone is essential for the tick to attach and feed (Bishop et al., 2002). 64P, a 15 kDa protein, was identified as a putative cement protein involved in attachment and feeding of *R. appendiculatus* ticks (Trimnell et al., 2002), found to be expressed in SGs (Havlíková et al., 2009). In vaccination experiments with recombinant versions from *R. appendiculatus* 64P protein (64TRPs), a dual-action vaccine was proposed, since it was demonstrated that it could act both as "exposed" and "concealed" antigen (Almazán et al., 2005a,b; Havlíková et al., 2009; Trimnell et al., 2002, 2005). Moreover, a posterior study showed that immunization with 64TPR protected mice from tick-borne encephalitis virus (TBEV) transmission by *Ixodes ricinus* (Labuda et al., 2006). Gene encoding for a putative secreted glycine-rich cement protein (*st1*) was found to be upregulated in SGs infected ticks when comparing with non-infected ticks (Antunes et al., in prep.). Subsequent gene knockdown was successfully assessed in SGs, showing 65% of gene silencing. Under the conditions of this study, *st1* knockdown did not affect pathogen infection, thus suggesting that this molecule is not essential to control *B. ovis* infection in *R. bursa* ticks. Though, *st1* knockdown resulted in decreased tick weight after feeding and tick attachment to the host. Previous studies concerning cement cone

proteins showed that immunization with these proteins affected significantly ixodid tick attachment to the host (Trimnell et al., 2005), as well as reducing pathogen transmission (Labuda et al., 2006). As in the 64P case, using a cement cone protein, such as *st1*, for the development of new anti-tick vaccines, represent a promising approach for the control of tick infestations since these molecules are considered "exposed" antigens by targeting the tick-feeding site, which results in impaired feeding and attachment. "Exposed" antigens have the advantage of not needing further vaccination boosts, since tick feeding promote continuous exposition of such molecules to the immune system of the vertebrate host (Ghosh et al., 2007; Trimnell et al., 2005, 2002).

***Mt5*,** an unknown metabolism related protein, was found to be upregulated in SGs infected ticks when comparing with non-infected ticks (Antunes et al., in prep.). Until date, there is no study that focuses on this protein, consequently, not much is known about its function in either tissue, and so, we might suggest that further studies should be performed. Since *mt5* gene knockdown was not assessed in SGs or OVs, functional analysis after RNAi were not carried.

The main objective of the present Master project consisted in the validation of the influence of selected genes in tick development and infection acquisition towards TTBD. New genes involved in the tick feeding and in *B. ovis* infection were identified using RNA-Seq. Using this technique, catalogues of genes upregulated in an infected tick population and in a fed tick population were obtained, improving our understanding of the molecular mechanisms involved in tick–pathogen and tick-host interactions (Antunes et al., in prep.). *Vitellogenin-3* (*cf1*) and putative *lachesin* (*cf2*) were selected from the catalogue of fed tick population, and gene encoding for a putative secreted glycine-rich cement protein (*st1*) and gene encoding for an unknown metabolism protein (*mt5*) were selected from the catalogue of infected tick population, based on fold change expression. RNA interference assays were employed, allowing functional analysis of genes by disrupting their expression. Our results showed that gene disruption was achieved in *cf1*, *cf2* and *st1* groups, in SGs. The gene *Lac* presented a significant influence on *B. ovis* infection in *R. bursa* ticks. Additionally, *Vg-3* (*cf1*) knockdown resulted in increased tick mortality. Finally, *st1* gene disruption resulted in decreased tick attachment and decreased tick weight after feeding. The results reported here increased our understanding of the role of tick genes in *Babesia* infection/multiplication and tick feeding, which is fundamental for the development of novel tick control measures. Some of the *R. bursa* genes discovered in the present study such as *Vg-3*, *Lac* and the gene encoding for a putative secreted glycine-rich cement protein could contribute to the development of novel vaccines designed to reduce tick infestations and prevent or minimize pathogen infection in ticks and transmission to vertebrate hosts.

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6 Appendix

Appendix I

Table 6.1: Sequence identity between different available *vitellogenin* nucleotide sequences and the obtained *vitellogenin* sequences.

	R. microplus Vg-3 A0A034WTS6	*H. longicornis Vg-3* E1CAY0	*R. microplus Vg-1* A0A034WTV5	*R. microplus Vg-2* A0A034WWF8	*R. appendiculatus Vg-3* A0A131YWP6
R. microplus Vg-3 A0A034WTS6	100	39.06	41.02	42.67	41.75
H. longicornis Vg-3 E1CAY0		100	42.67	43.48	42.84
R. microplus Vg-1 A0A034WTV5			100	49.06	48.49
R. microplus Vg-2 A0A034WWF8				100	93.61
R. appendiculatus Vg-3 A0A131YWP6					100

Table 6.2: Sequence identity between different available genes encoding for secreted glycine-rich cement proteins nucleotide sequences and the obtained sequence.

	I. scapularis A0A023FLK9	*A. cajennense* A0A023GE61	*A. triste* EEC18366	*A. parvum* A0A023FYT7	*R. pulchellus* L7MBM8	*A. americanum* A0A0C9SFJ8	*R. microplus* A0A034WZ12
I. scapularis A0A023FLK9	100	61.60	37.99	35.92	41.51	39.65	36.33
A. cajennense A0A023GE61		100	36.00	35.92	40.97	37.80	36.13
A. triste EEC18366			100	41.71	43.26	35.79	34.46
A. parvum A0A023FYT7				100	45.34	37.71	37.24
R. pulchellus L7MBM8					100	45.85	44.04
A. americanum A0A0C9SFJ8						100	49.45
R. microplus A0A034WZ12							100

Table 6.3: Sequence identity between different available *lachesin* nucleotide sequences and the obtained *lachesin* sequence.

	Ixodes scapularis B7QM16	*R. pulchellus* L7LSG7	*R. appendiculatus* A0A131YVX3
Ixodes scapularis B7QM16	100	46.01	46.43
R. pulchellus L7LSG7		100	94.91
R. appendiculatus A0A131YVX3			100

Appendix II

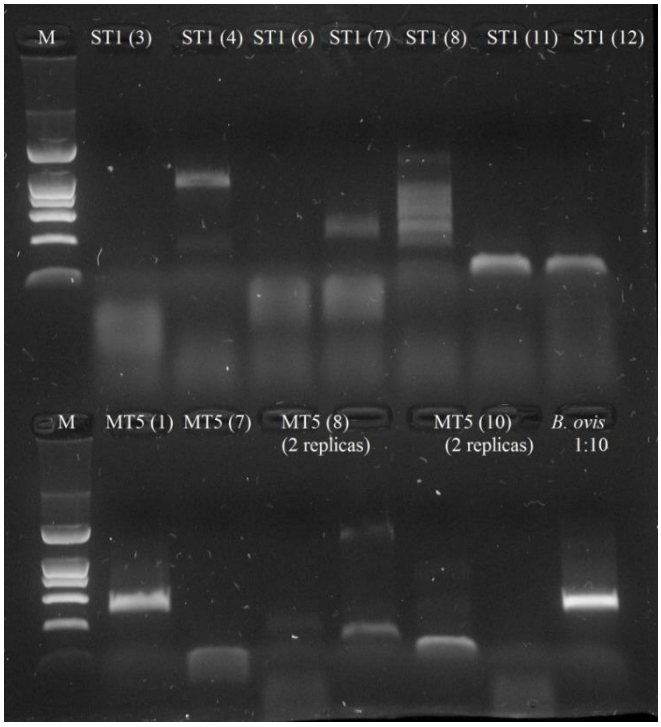

Figure 6.1: Detection of *B. ovis* in the tick salivary glands by AGE of qPCR products in silenced groups *st1* and *mt5*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder. The last lane corresponds to a *standard* of *B. ovis* (positive control).

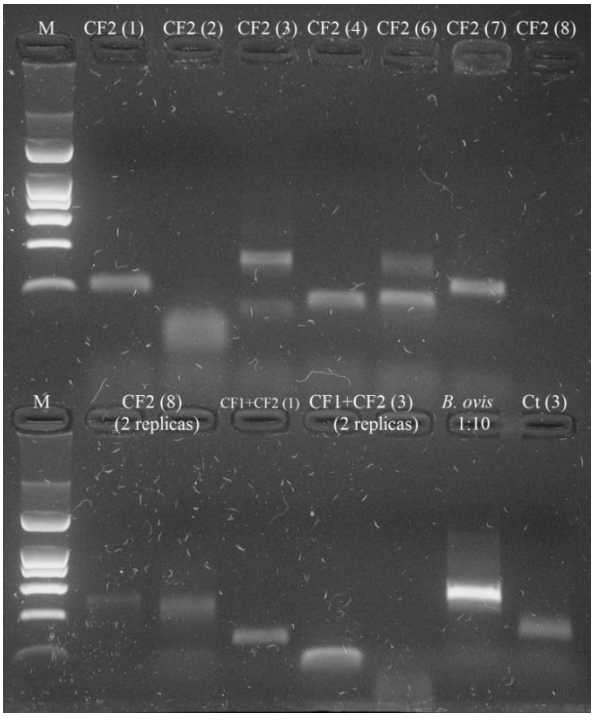

Figure 6.2: Detection of *B. ovis* in the tick salivary glands by AGE of qPCR products in the group Control and in the silenced groups *cf2* and *cf1+cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder and a 1:10 *standard* sample of *B. ovis* was run (positive control).

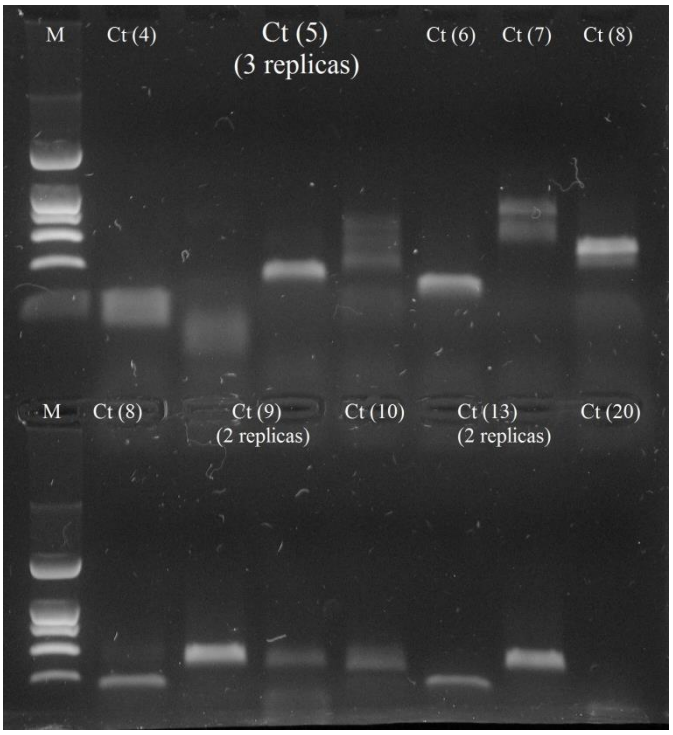

Figure 6.3: Detection of *B. ovis* in the tick salivary glands by AGE of qPCR products in the group Control. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

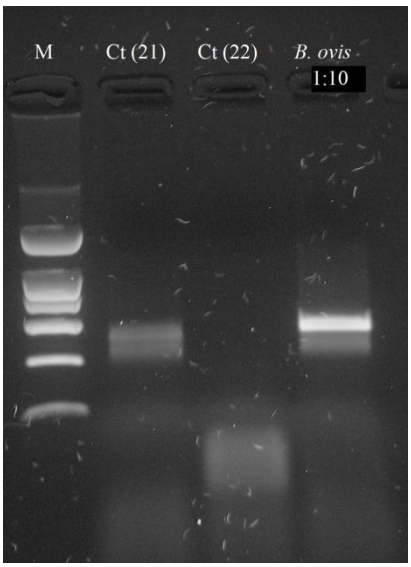

Figure 6.4: Detection of *B. ovis* in the tick salivary glands by AGE of qPCR products in the group Control. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The first lane (M) corresponds to the ladder and the last lane corresponds to a 1:10 *standard* sample of *B. ovis* (positive control).

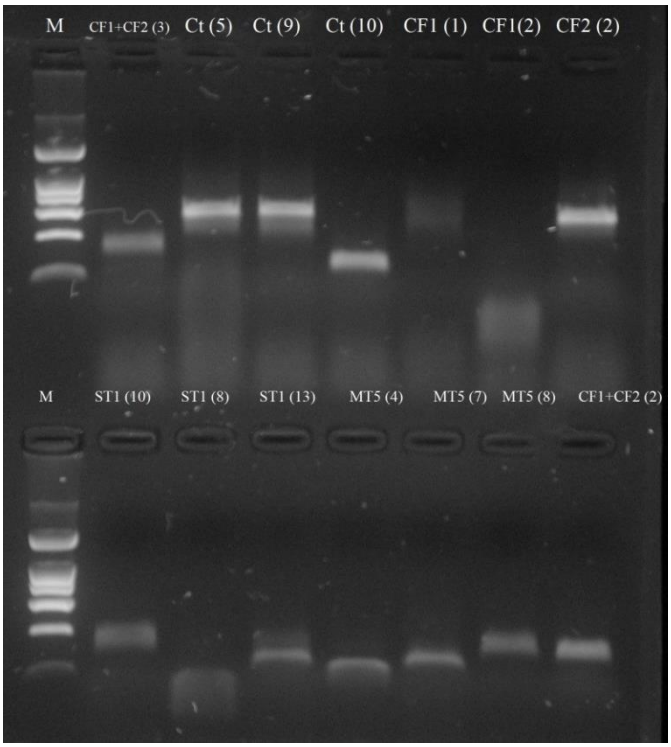

Figure 6.5: Detection of *B. ovis* in the tick ovaries by AGE of qPCR products in the group Control and in the silenced groups *cf1*, *cf2*, *st1*, *mt5* and *cf1+cf2*. The protocol followed is described in (Aktas et al 2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

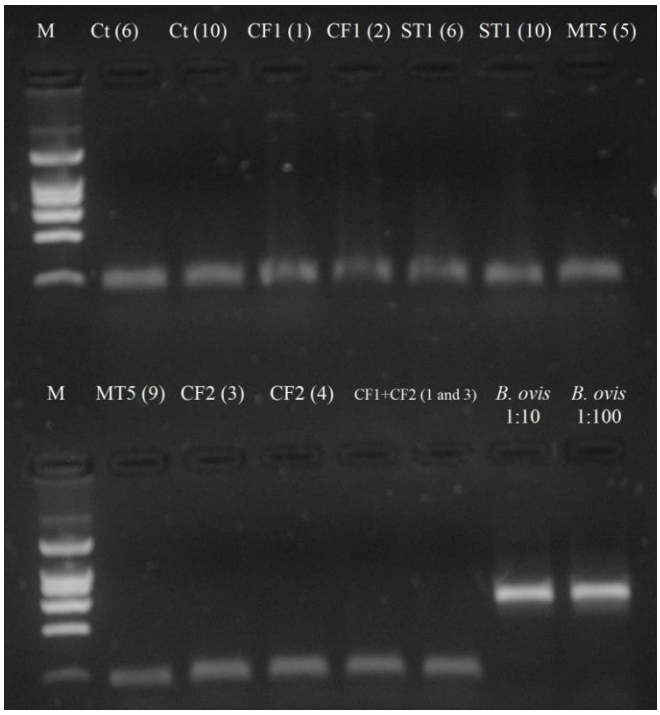

Figure 6.6: Detection of *16S* tick gene in the tick ovaries by AGE of qPCR products in the group Control and in the silenced groups *cf1*, *st1*, *mt5*, *cf2* and *cf1+cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder. Two *B. ovis standard* samples with different dilutions (1:10 and 1:100) were run.

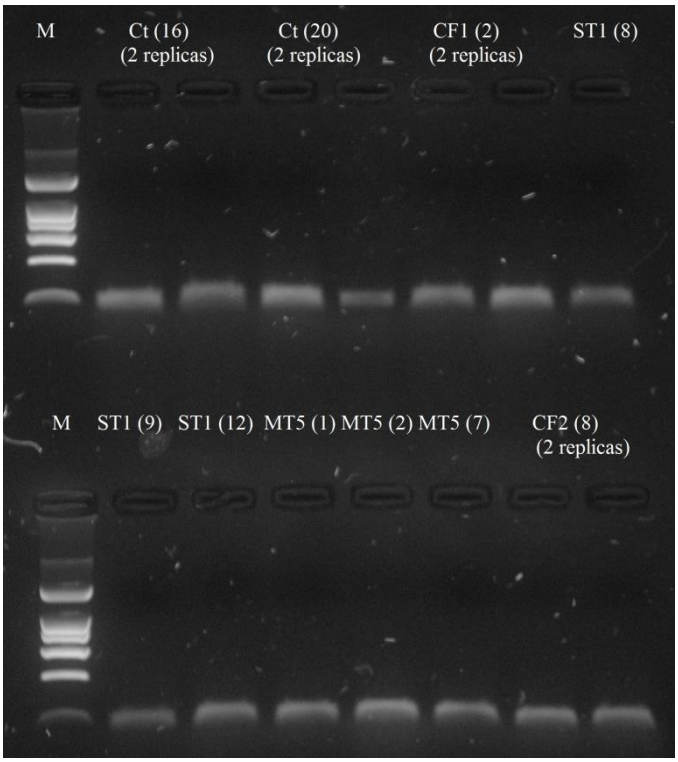

Figure 6.7: Detection of *16S* tick gene in the tick salivary glands by AGE of qPCR products in the group Control and in the silenced groups *cf1*, *st1*, *cf2* and *mt5*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

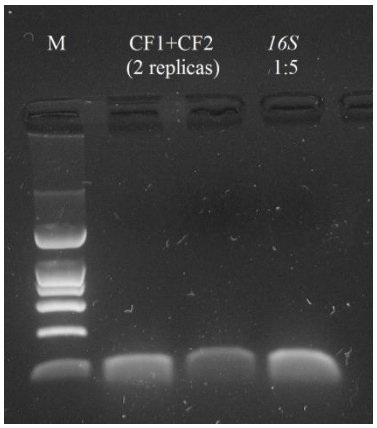

Figure 6.8: Detection of *16S* tick gene in the tick salivary glands by AGE of qPCR products in the silenced group *cf1*+*cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The first lane (M) corresponds to the ladder and the last lane corresponds to a 1:5 *standard* sample of *16S* (positive control).

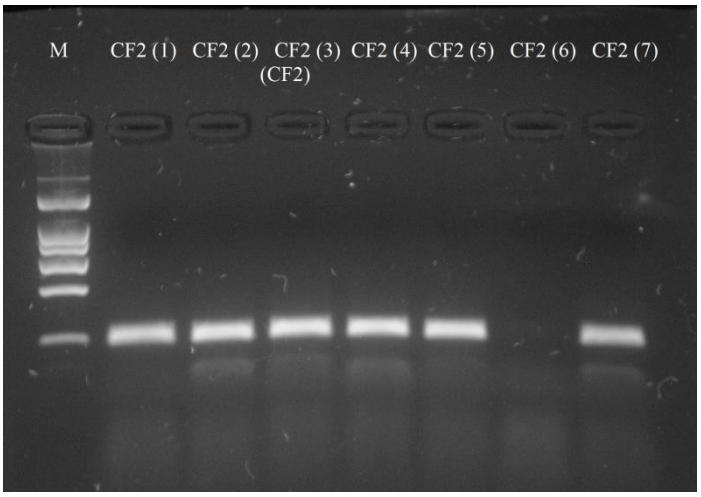

Figure 6.9: Detection of *cf2* tick gene in the tick ovaries by AGE of qPCR products in the silenced group *cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The first lane corresponds to the ladder (M).

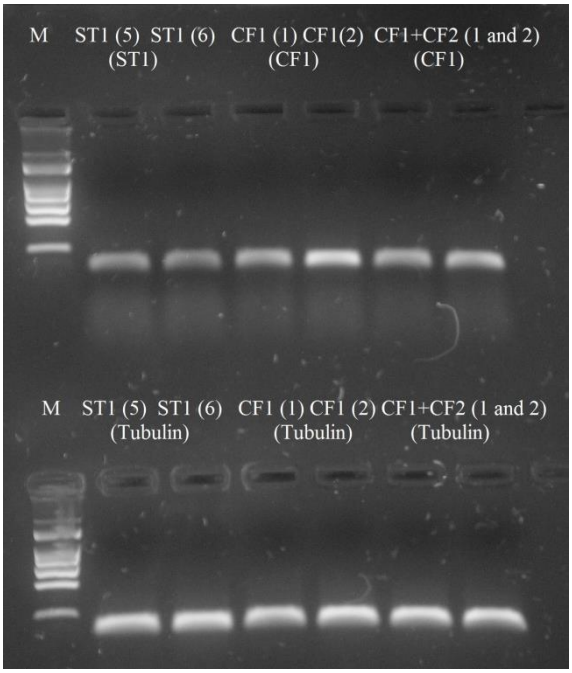

Figure 6.10: Detection of *st1*, *cf1* and *β-tubulin* tick genes in the tick ovaries by AGE of qPCR products in the silenced groups *st1*, *cf1* and *cf1+cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

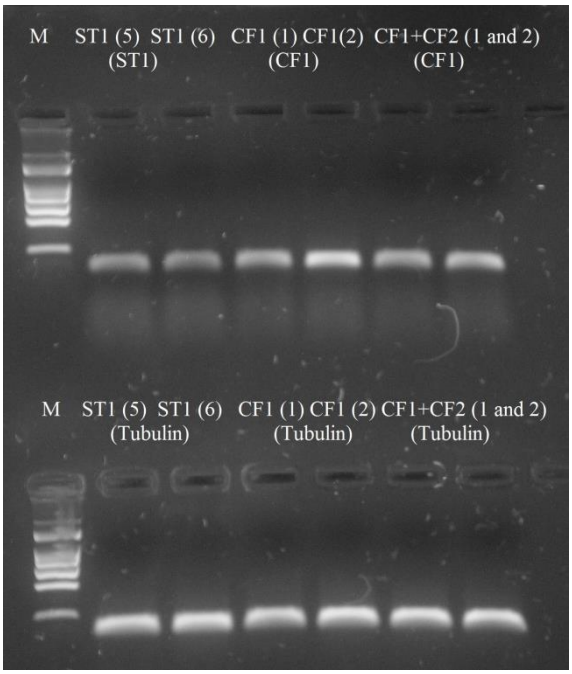

Figure 6.11: Detection of *st1*, *cf1* and *ß-tubulin* tick genes in the tick ovaries by AGE of qPCR products in the silenced groups *st1*, *cf1* and *cf1+cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

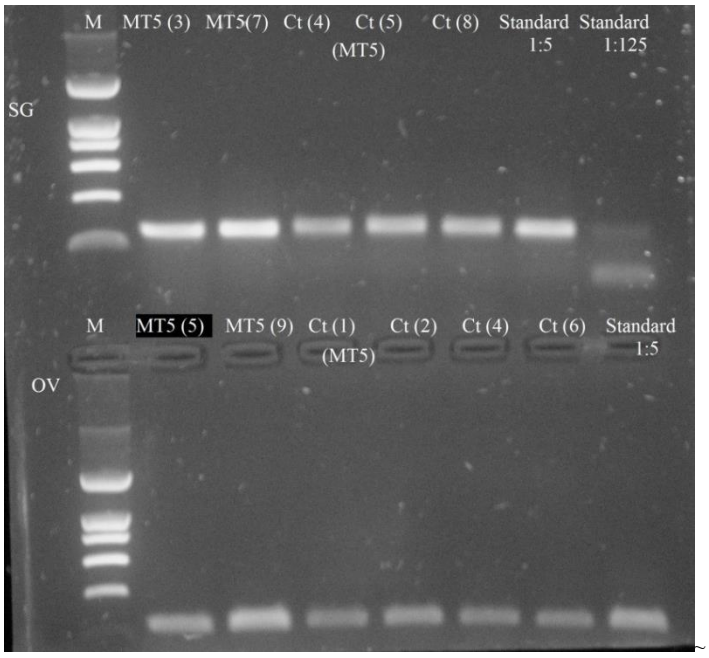

Figure 6.12: Detection of *mt5* tick gene in the tick salivary glands and ovaries by AGE of qPCR products in the group Control and in the silenced group *mt5*. The protocol followed is described in Aktas et al (2005). The lanes M correspond to the ladder and *standard* samples (1:5 and 1:125) were run (positive controls).

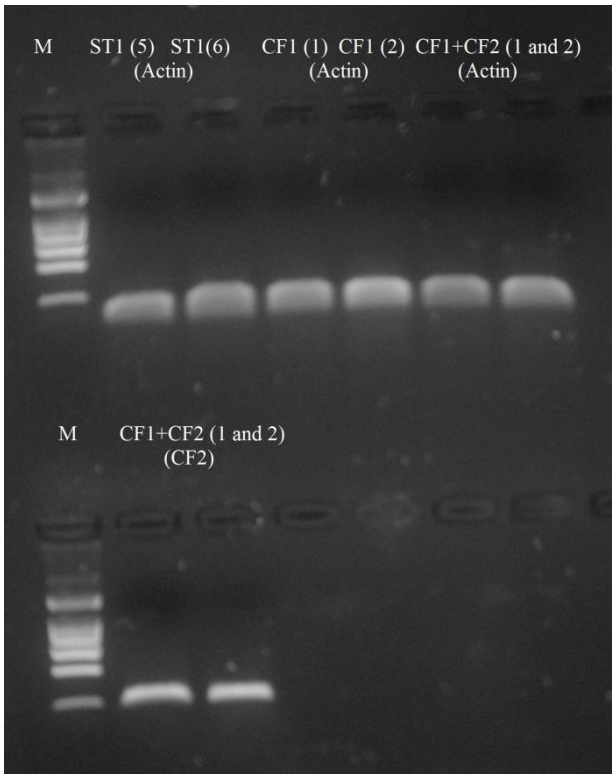

Figure 6.13: Detection of *β-actin* and *cf2* tick genes in the tick ovaries by AGE of qPCR products in the silenced groups *st1*, *cf1* and *cf1+cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

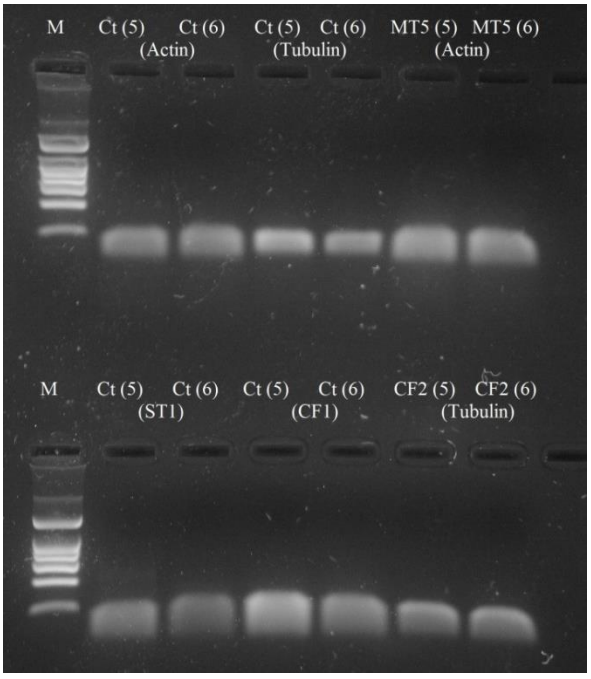

Figure 6.14: Detection of *β-actin*, *β-tubulin*, *st1* and *cf1* tick genes in the tick ovaries by AGE of qPCR products in the group Control and in the silenced groups *mt5* and *cf2*. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The lanes M correspond to the ladder.

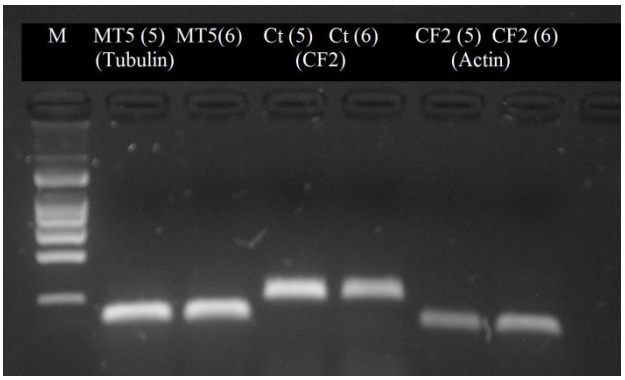

Figure 6.15: Detection of *β-tubulin*, *cf2* and *β-actin* tick genes in the tick ovaries by AGE of qPCR products in the silenced group *mt5*, group Control and in the silenced group *cf2*, respectively. The protocol followed is described in Aktas et al (2005). Samples were electrophoresed on a 1.2% Agarose/SYBR® Safe gel, 0,5X TBE. The first lane (M) corresponds to the ladder.